GRAS Notice for Synthetic Sodium Aluminosilicate for Use in Feed as an Aid to Maintain Calcium Balance in Periparturient Dairy Cows

Protekta, Inc., a partner of Vilofoss® U.S.
2680 E. Main Street, Suite 205
Plainfield, IN 46168

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PART 1. §570.225. SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §570 Subpart E consisting of §570.203 to 280, Protekta, Inc. (hereafter referred to as "Protekta") herby informs the U.S. Food and Drug Administration (FDA) that they are submitting a Generally Recognized As Safe (GRAS) notice for synthetic sodium aluminosilicate.

1.1 NAME AND ADDRESS OF ORGANIZATION

Protekta, Inc.
2680 E. Main Street, Suite 205
Plainfield, IN 46168

1.2 NAME OF THE NOTIFIED SUBSTANCE

The notified substance is synthetic sodium aluminosilicate.

1.3 INTENDED CONDITIONS OF USE

Synthetic sodium aluminosilicate is intended for use in the feed of periparturient dairy cows for a period of at least 14 days and no more than 28 days, pre-calving. The ingredient will be incorporated into dairy feed as a formulation on a wheat carrier and marketed under the trade name "X-Zelit®". The cows will be provided with 500 g X-Zelit®/head/day equivalent to 400 g synthetic sodium aluminosilicate/head/day as top-dressing or part of the total mixed ration (TMR). The use of synthetic sodium aluminosilicate will be discontinued at calving.

1.4 STATUTORY BASIS FOR THE CONCLUSION OF GRAS STATUS

Pursuant to 21 CFR §570.30(a) and (b), synthetic sodium aluminosilicate manufactured by Protekta, has been concluded to have GRAS status for use as an aid to maintain calcium balance in feed for periparturient dairy cows under the conditions described in Part 1.3, on the basis of scientific procedures.

1.5 PREMARKET EXCEPTION STATUS

Protekta herby informs the U.S. FDA of the view that synthetic sodium aluminosilicate is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FFDCA) based on Protekta's conclusion that the notified substance is GRAS under the conditions of intended use as described in Part 1.3 above.

1.6 AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS notification will be made available to the U.S. FDA for review and copying upon request during customary business hours at the offices of:

Protekta, Inc.
2680 E. Main Street, Suite 205

Plainfield, IN 46168

In addition, upon request, Protekta will supply the U.S. FDA with a complete copy of the data and information either in an electronic format that is accessible for the Agency's evaluation or on paper.

1.7 FREEDOM OF INFORMATION ACT, 5 U.S.C. 552

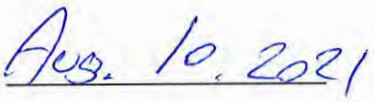
In Protekta's view, all data and information presented in Parts 2 through 7 of this notice do not contain any trade secret, commercial or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempt from the Freedom of Information Act, 5 U.S.C. Section 552. The exceptions are Appendices 01, 02A to E, 03A to D, 04A, 04D, 05A to D, 06A to C, 07A and B, and 08A to C which contain proprietary information and are considered confidential.

1.8 CERTIFICATION

Morten Jakobsen hereby certifies that to the best of his knowledge, all data and information presented in this notice constitutes a complete, representative and balanced submission, which includes all unfavorable as well as favorable information known to Protekta and pertinent to the evaluation of the safety and GRAS status of synthetic sodium aluminosilicate.

Signed,

Morten Jakobsen, Founder & CEO



Date

PART 2. §570.230. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

2.1 IDENTITY

2.1.1 Common or Usual Names

The common or usual name of the ingredient is synthetic sodium aluminosilicate. Other names for the ingredient are Zeolite A and sodium aluminosilicate.

2.1.2 Description

Synthetic sodium aluminosilicate refers to a range of amorphous and crystalline hydrated sodium aluminosilicates with varying proportions of sodium oxide (Na_2O), aluminum oxide (Al_2O) and silicon oxide (SiO_2) manufactured by reacting aluminum sulfate and sodium silicate followed by precipitation. The subject of the GRAS determination is synthetic sodium aluminosilicate with the chemical formula $Na_{12}Al_{12}Si_{12}O_{48}{\cdot}27H_2O$ and which is known as Zeolite A. The additive is a white crystalline powder with a typical particle size between 2 and 5 µm.

2.1.3 Identity

The identity of synthetic sodium aluminosilicate is detailed in Table 2.1.

Table 2.1: Identity of Synthetic Sodium Aluminosilicate	
Common names	Synthetic sodium aluminosilicate; Zeolite A
Chemical name	Sodium aluminum silicate
Synonyms	Sodium silicoaluminate (sodium silico aluminate); aluminum sodium silicate; silicic acid, aluminum sodium salt; aluminosilicic acid, sodium salt; Linde type A zeolite
Chemical formula	$Na_{12}Al_{12}Si_{12}O_{48}{\cdot}27H_2O$
CAS number	1344-00-9
INS number	554

Abbreviations: CAS = Chemistry Abstracts Service; INS = International Numbering System.

2.2 METHOD OF MANUFACTURE

The manufacturing information is described in Appendix 01 (CONFIDENTIAL).

2.2.1 Raw Materials and Processing Aids for the Manufacture of Synthetic Sodium Aluminosilicate

The raw materials and processing aids used in the production of synthetic sodium aluminosilicate are listed in Table 2.2. All of the raw materials and processing aids are considered safe and suitable for the manufacture of a feed ingredient. Silicon dioxide and sodium hydroxide are recognized technical additives for direct addition to feed, and although there is no listing in the CFR or Association of American Feed Control Officials (AAFCO) Official Publication (OP) for alumina, other aluminum salts such as aluminum sulfate and aluminum calcium silicate are permitted for use.

Table 2.2: Raw Materials used in the Manufacture of Synthetic Sodium Aluminosilicate		
Raw Material	**Function**	**Regulatory Status**
Silicon dioxide (silica sand)	Source of silicon dioxide	Permitted for use as a food additive in specific feed components as (a) an anti-caking agent and/or grinding aid; use levels in the feed components range from 0.8% in piperazine, piperazine salts to 3% in vitamins; (b) a carrier at levels of 50% in flavors and 95% in selenomethionine hydroxy analogue (21 CFR §573.940; U.S. FDA, 2020) [Appendix 02A; CONFIDENTIAL]
Sodium hydroxide	Source of sodium	GRAS for use as a general purpose food additive in accordance with good manufacturing and feeding practice (21 CFR §582.1763; U.S. FDA, 2020) [Appendix 02B; CONFIDENTIAL]
Aluminum oxide (alumina)	Source of aluminum oxide	Aluminum sulfate, aluminum ammonium sulfate, aluminum potassium sulfate, aluminum sodium sulfate, sodium aluminum phosphate and aluminum calcium silicate are GRAS for use as general purpose food additives in accordance with good manufacturing and feeding practice (21 CFR §582.1125, §582.1127, §582.1129, §582.1131, §582.1781, §582.2122; U.S. FDA, 2020) [Appendix 02C; CONFIDENTIAL] Note: alumina (aluminum hydroxide) is permitted for use as a color additive for coloring drugs in amounts consistent with good manufacturing practice to color drugs generally (21 CFR§73.1010; U.S. FDA, 2020)
Demineralized water	Processing aid	Not applicable

Abbreviations: CFR = Code of Federal Regulations;

2.2.2 Raw Materials and Processing Aids for Formulation of X-Zelit®

As mentioned in the introduction, synthetic sodium aluminosilicate is marketed for the intended use for periparturient dairy cattle as a formulation under the trade name X-Zelit®. The formulation comprises (b) (4) sodium aluminosilicate, (b) (4) (carrier) and (b) (4) rapeseed oil (anti-dusting agent). The formulation components are listed in Table 2.3. All components are considered feed-grade, and safe and suitable for the intended use.

Table 2.3: Raw Materials used in the Formulation of X-Zelit®		
Raw Material	**Function**	**Regulatory Status**
Sodium aluminosilicate	Aid to maintain calcium balance	GRAS for use as an anti-caking agent in feed at levels not to exceed 2% in the diet (21 CFR §582.2727; U.S. FDA, 2020)
Wheat (*Triticum aestivum* L., *Triticum durum* Desf. and other wheat cultivars)	Carrier	No official definition but considered a common food and listed within the collective ingredient definition for Grain Products in the AAFCO OP (Collective Term 22.3; AAFCO, 2021) [Appendix 02D; CONFIDENTIAL]
Rapeseed (*Brassica napus*) oil	Anti-dust agent	Vegetable fat listed in the AAFCO OP (Ingredient definition 33.2; AAFCO, 2021) [Appendix 02E; CONFIDENTIAL]
Potable water	Processing aid	Not applicable

Abbreviations: AAFCO = Association of American Feed Control Officials; CFR = Code of Federal Regulations; OP = Official Publication.

2.2.3 Manufacturing Process to Synthetic Sodium Aluminosilicate

Synthetic sodium aluminosilicate is manufactured by (b) (4)

Synthetic sodium aluminosilicate is manufactured in accordance with cGMP and a Hazard Analysis Critical Control Point (HACCP) system is in place. The manufacturer will comply with the requirements for importing feed into the U.S. as laid down by the Food Safety Modernization Act (FSMA) including the foreign supplier verification program (FSVP) and Bioterrorism Act (2002).

2.2.4 Formulation of the Feed Product (X-Zelit®)

(b) (4)

The final product is extruded to yield the market formulation, X-Zelit®.

X-Zelit® is manufactured in accordance with cGMP and a HACCP plan is in place. The manufacturer will comply with the requirements for importing feed into the U.S. as laid down by the Food Safety Modernization Act (FSMA) including the foreign supplier verification program (FSVP) and Bioterrorism Act (2002).

2.3 PRODUCT SPECIFICATIONS AND ANALYTICAL DATA

2.3.1 Product Specifications for Synthetic Sodium Aluminosilicate

Appropriate feed-grade specifications have been established for synthetic sodium aluminosilicate and are presented in Table 2.4. Synthetic sodium aluminosilicate complying with these specifications is also currently used as an anti-caking agent in the EU and U.S.

The relative amounts of the nominal components comprising synthetic sodium aluminosilicate, that is sodium oxide (Na_2O), alumina (Al_2O_3) and silicon dioxide (SiO_2), reflect the stoichiometry and manufacturing conditions and are specific to the form known as Zeolite A. The theoretical composition of synthetic sodium aluminosilicate based on its chemical formula is presented in Appendix 04A (CONFIDENTIAL).

Heavy metal specifications are set for synthetic sodium aluminosilicate which reflect the maximum limits specified by the European Commission (EC) under Directive 2002/32/EC on undesirables in animal nutrition (EC, 2002 – as amended) for lead and cadmium in feed additives belonging to the functional groups of binders and anti-caking elements of 30 and 2 mg/kg, respectively. The maximum limits for arsenic and mercury are based on the levels considered acceptable for complementary mineral feeds under the same legislation of 12 and 0.2 mg/kg, respectively.

Additionally, the National Research Council (NRC, 2005) has established maximum tolerable limits for lead, cadmium, arsenic and mercury on a DM basis in cattle feed of 100, 10, 30 and 2 mg/kg, respectively. These levels equate to a maximum exposure to lead, cadmium, arsenic and mercury of 1,000, 100, 300 and 20 mg/head/day by a periparturient cow consuming around 10 kg DM/head/day. By comparison, a periparturient cow consuming 400 g/head/day of synthetic sodium aluminosilicate will be exposed to no more than 12, 0.8, 4.8 and 0.08 mg/head/day of lead, cadmium, arsenic and mercury, respectively based on the maximum limits set by the product specifications. These levels fall well below

the maximum tolerated levels on a per head basis and therefore, are not anticipated to pose any safety concerns under the conditions of intended use of synthetic sodium aluminosilicate as an aid to maintain calcium balance for at least 14 days, and no more than 28 days, pre-calving.

Similarly, specifications are set for dioxins, the sum of dioxins and dioxin-like PCBs, and non-dioxin-like PCBs are set which reflect the maximum limits specified by the European Commission in feed materials of mineral origin under Directive 2002/32/EC (EC, 2002 – as amended) of 0.75 ng WHO-PCDD/F-TEQ kg, 1.0 ng WHO-PCDD/F-PCB-TEQ/kg and 10 µg/kg, respectively.

All methods of analysis follow internationally recognized standards or are validated in-house. Copies of the methods of analysis for the determination of heavy metals, and dioxins and PCBs are provided in Appendices 04B and C, respectively.

Table 2.4: Proposed Product Specifications for Synthetic Sodium Aluminosilicate

Parameter	Specification	Method of Analysis
Composition		
Na_2O	Min. 17%	XRD (Internal method)
Al_2O_3	Min. 28%	XRD (Internal method)
SiO_2	Min. 30%	XRD (Internal method)
Physical Properties		
Color	White	Visual inspection (Internal method)
Form	Powder	Visual inspection (Internal method)
Heavy Metals		
Arsenic	Max. 12 mg/kg	EN 13805m:2014; EN ISO 17294m:2016 (ICP-MS)
Cadmium	Max. 2 mg/kg	EN 13805m:2014; EN ISO 17294m:2016 (ICP-MS)
Lead	Max. 30 mg/kg	EN 13805m:2014; EN ISO 17294m:2016 (ICP-MS)
Mercury	Max. 0.2 mg/kg	EN 13805m:2014; EN ISO 17294m:2016 (ICP-MS)
Dioxins and PCBs[1]		
Dioxins	Max. 0.75 ng WHO-PCDD/F-TEQ/kg	Commission Regulation (EC) No 152/2009 (GC-MS/MS)
Sum of dioxins and dioxin-like PCBs	Max. 1.0 ng WHO-PCDD/F-PCB-TEQ /kg	Commission Regulation (EC) No 152/2009 (GC-MS/MS)
Non-dioxin-like PCBs (ICES-6)	Max. 10 µg/kg	Commission Regulation (EC) No 152/2009 (GC-MS/MS)

Abbreviations: EN = European Standards; GC = gas chromatography; ICES = sum of PCBs 28, 52, 101, 138, 153 and 180; ICP = inductively coupled plasma; ISO = International Organization for Standardization; MS = mass spectrometry; PCB = polychlorinated biphenyl; PCDD = polychlorinated dibenzo-p-dioxins; PCDF = polychlorinated dibenzofurans; TEQ = toxic equivalency; WHO = World Health Organization; XRD = X-ray diffraction.
[1]Reported as upper-bound concentrations (assumption that all values of the different cogeners below the limit of quantification are equal to the limit of quantification).

2.3.2 Analytical Data on Synthetic Sodium Aluminosilicate

Analytical data for 3 independently produced commercial batches of synthetic sodium aluminosilicate which confirm compliance with the compositional specifications are provided in Table 2.5. The

Certificate of Analysis is provided in Appendix 05A (CONFIDENTIAL). Across the 3 batches, the amounts of each of the nominal components are consistent, varying by no more than 1%. The batches were manufactured in 2017 and 2018 but the process is well-established, relatively simple and has not been altered for many years. On this basis, the batches were considered representative of the material currently marketed by the manufacturer as an anti-caking agent in the EU and U.S.

Heavy metals, and dioxins and PCBs are not routinely measured on every batch of sodium aluminosilicate and the results for 2 of the commercial batches are provided in Table 2.5. The Certificates of Analysis are provided in Appendices 05B and C (CONFIDENTIAL). These data confirm that the levels of heavy metals, and dioxins and PCBs generally fall well below the limits set by the product specifications and by Directive 2002/32/EC on undesirables in animal nutrition (EC, 2002 – as amended) for feed materials of mineral origin.

The only detectable heavy metal was arsenic, with levels of (b) (4) reported for the 2 batches of sodium aluminosilicate, respectively. As mentioned in Section 2.3.1, the NRC has established a maximum tolerable limit on a DM basis in cattle feed of 30 mg/kg (NRC, 2005), equating to 300 mg/head/day for a periparturient cow consuming 10 kg DM/head/day. Synthetic sodium aluminosilicate containing 4.2 mg/kg arsenic will lead to an exposure of 1.7 mg arsenic/head/day when incorporated into feed at the intended use level of 400 g/head/day. The contribution by synthetic sodium aluminosilicate to the arsenic intakes by dairy cows is therefore, anticipated to be negligible (<1%) compared to the maximum tolerable limit set by the NRC.

Table 2.5: Analytical Data on 3 Representative Commercial Batches of Synthetic Sodium Aluminosilicate					
Parameter	**Units**	**Specifications**	**Analytical Data**		
			Batch 18008001	**Batch 17100001**	**Batch 17229001**
Composition					
Na_2O	%	Min. 17.0	(b) (4)		
Al_2O_3	%	Min. 28.0			
SiO_2	%	Min. 30.0			
Physical Properties					
Color	-	White			
Form	-	Powder			
Heavy Metals					
Arsenic	mg/kg	Max. 12			
Cadmium	mg/kg	Max. 2			
Lead	mg/kg	Max. 30			
Mercury	mg/kg	Max. 0.2			
Dioxins and Dioxin-Like PCBs[1]					
Dioxins	ng WHO-PCDD/F-TEQ/kg	Max. 0.75			
Sum of dioxins and dioxin-like PCBs	ng WHO-PCDD/F-PCB-TEQ /kg	Max. 1.0			
Non-dioxin-like PCBs (ICES-6)	µg/kg	Max. 10			

Abbreviations: "-" = not measured; ICES = sum of PCBs 28, 52, 101, 138, 153 and 180; PCB = polychlorinated biphenyl; PCDD = polychlorinated dibenzo-p-dioxins; PCDF = polychlorinated dibenzofurans; TEQ = toxic equivalency; WHO = World Health Organization.

2.3.3 Additional Analytical Data

The chromium and aluminum levels in 2 commercial batches of synthetic sodium aluminosilicate are presented in Table 2.6. The Certificates of Analysis are provided in Appendices 05C and D (CONFIDENTIAL). No chromium was detected in either of the batches tested. Based on the chemical formula of synthetic sodium aluminosilicate, the theoretical aluminum level is 148,000 mg/kg[1] and the analytical results of (b) (4) respectively for the 2 batches are considered to fall within acceptable ranges of this value.

The NRC has set a maximum tolerable limit for aluminum in cattle feed on a DM basis of 1,000 mg/kg (NRC, 2005) which equates to 10,000 mg/head/day for a periparturient cow consuming 10 kg DM/day. By comparison, synthetic sodium aluminosilicate containing 150,000 mg/kg of aluminum provides 60,000 mg/head/day of aluminum. A safety evaluation of synthetic sodium aluminosilicate including the aluminum component for target animals is provided in Section 6.

[1] Theoretical Al_2O_3 content of 27.9%, equates to an aluminum content of 14.8%.

Table 2.6: Chromium and Aluminum Analysis of 2 Representative Commercial Batches of Synthetic Sodium Aluminosilicate			
Parameter	**Units**	**Analytical Data**	
		Batch 17100001	**Batch 17229001**
Chromium	mg/kg	(b) (4)	
Aluminum	mg/kg		

2.3.4 Product Specifications for X-Zelit®

Synthetic sodium aluminosilicate (b) (4) is combined with wheat as a carrier (b) (4) and rapeseed oil as an anti-dust agent (b) (4) to yield the market formulation, X-Zelit®. Appropriate feed-grade specifications have been established for X-Zelit® on the basis that this is the market formulation, and are presented in Table 2.7. The formulated product is a gray granular product without a distinctive odor.

The relative amounts of the nominal components comprising synthetic sodium aluminosilicate, that is sodium oxide (Na_2O), alumina (Al_2O_3) and silicon dioxide (SiO_2), reflect the stoichiometry of the Zeolite A form of synthetic sodium aluminosilicate, taking into account dilution on the wheat carrier. The theoretical amount of each component in the formulation is described in Appendix 04D (CONFIDENTIAL).

Heavy metal specifications are set for X-Zelit® which reflect the maximum limits set by European Commission (EC) under Directive 2002/32/EC (EC, 2002 – as amended) for feed supplements (referred to as complementary feeds or compound feeds) of mineral origin of 12 mg/kg for arsenic, 5 mg/kg for cadmium[2], 0.2 mg/kg for mercury and 15 mg/kg for lead.

As mentioned previously, the NRC (2005) has established maximum tolerable limits for arsenic, cadmium, mercury and lead on a DM basis in cattle feed of 30, 10, 2 and 100 mg/kg, respectively. These levels equate to a maximum exposure to arsenic, cadmium, mercury and lead of 300, 100, 20 and 1,000 mg/head/day by a periparturient cow consuming around 10 kg DM/head/day. By comparison, a periparturient cow consuming 500 g/head/day of X-Zelit® will be exposed to 6, 2.5, 0.1 and 7.5 mg/head/day of arsenic, cadmium, mercury and lead, respectively based on the maximum limits set by the product specifications. These levels fall well below the maximum tolerated levels on a per head basis and therefore, are not anticipated to pose any safety concerns under the conditions of intended use of synthetic sodium aluminosilicate as an aid to maintain calcium balance for at least 14 days, and no more than 28 days, pre-calving.

Similarly, specifications are set for dioxins, the sum of dioxins and dioxin-like PCBs, and non-dioxin-like PCBs are set which reflect the maximum limits specified by the European Commission in feed materials of mineral origin under Directive 2002/32/EC (EC, 2002 – as amended) of 0.75 ng WHO-PCDD/F-TEQ kg, 1.0 ng WHO-PCDD/F-PCB-TEQ/kg and 10 µg/kg, respectively.

[2] (b) (4) as set a maximum limit for cadmium of 2 mg/kg which is consistent with feed materials of mineral origin in the EU (rather than feed supplements) on the basis that the levels achieved analytically can meet the lower value.

All methods of analysis follow internationally recognized standards or are validated in-house. Copies of the methods of analysis for the determination of bulk density, moisture, heavy metals, and dioxins and PCBs are provided in Appendices 04B, C and E.

Table 2.7: Proposed Product Specifications for X-Zelit®		
Parameter	**Specification**	**Method of Analysis**
Composition		
Na_2O	12.7-14.2%	XRD (Internal method)
Al_2O_3	21.0-22.5%	XRD (Internal method)
SiO_2	24.0-25.5%	XRD (Internal method)
Physical Properties		
Color	Gray	Visual inspection (Internal method)
Form	Granular	Visual inspection (Internal method)
Odor	Neutral	Sensory inspection (Internal method)
Moisture (130°C)	Max. 10%	Commission Regulation (EC) No 152/2009
pH (1% solution)	10.0-12.0	Potentiometry (Internal method)
Bulk density	400-700 g/L	DIN ISO 697
Heavy Metals		
Arsenic	Max. 12 mg/kg	EN 13805m:2014; EN ISO 17294m:2016 (ICP-MS)
Cadmium	Max. 2 mg/kg	EN 13805m:2014; EN ISO 17294m:2016 (ICP-MS)
Lead	Max. 15 mg/kg	EN 13805m:2014; EN ISO 17294m:2016 (ICP-MS)
Mercury	Max. 0.2 mg/kg	EN 13805m:2014; EN ISO 17294m:2016 (ICP-MS)
Dioxins and Dioxin-Like PCBs[1]		
Dioxins	Max. 0.75 ng WHO-PCDD/F-TEQ/kg	Commission Regulation (EC) No 152/2009 (GC-MS/MS)
Sum of dioxins and dioxin-like PCB's	Max. 1.0 ng WHO-PCDD/F-PCB-TEQ /kg	Commission Regulation (EC) No 152/2009 (GC-MS/MS)
Non-dioxin-like PCBs (ICES-6)	Max. 10 µg/kg	Commission Regulation (EC) No 152/2009 (GC-MS/MS)

Abbreviations: EN = European Standards; GC = gas chromatography; ICES-6 = sum of PCBs 28, 52, 101, 138, 153 and 180; ICP = inductively coupled plasma; ISO = International Organization for Standardization; MS = mass spectrometry; PCB = polychlorinated biphenyl; PCDD = polychlorinated dibenzo-p-dioxins; PCDF = polychlorinated dibenzofurans; TEQ = toxic equivalency; WHO = World Health Organization; XRD = X-ray diffraction.
[1]Reported as upper-bound concentrations (assumption that all values of the different congeners below the limit of quantification are equal to the limit of quantification).

2.3.5 Analytical Data on X-Zelit®

Analytical data for 3 non-consecutive commercial batches of X-Zelit® confirming compliance with the product specifications are summarized in Table 2.8. The Certificates of Analysis are provided in Appendices 06A to C (CONFIDENTIAL). The results demonstrate that X-Zelit® can be manufactured in compliance with the product specifications and displays acceptable batch to batch variability. Across the 3 batches, the amounts of each of the nominal components varies by less than 1%.

The physical properties of X-Zelit® are comparable across the 3 batches tested with the bulk density reported to be between [redacted] (b) (4) g/L. Likewise, the moisture content is consistently in the region of 9%.

Arsenic, cadmium and mercury were below detection limits in the 3 batches analyzed. The levels of lead identified (0.14 to 0.21 mg/kg) fall well below the maximum limit of 15 mg/kg set by the product specifications. Similarly, the levels of dioxins, sum of dioxins and dioxin-like PCBs, and non-dioxin-like PCBs falls well below the maximum limits laid down in the product specification and by Commission Directive 2002/32/EC (EC, 2002 – as amended).

Table 2.8: Analytical Data on 3 Batches of X-Zelit®

Parameter	Units	Specifications	Analytical Data		
			Batch 332775	Batch 342384	Batch 347568
Date of manufacture		-	[redacted] (b) (4)		
Composition					
Na_2O	%	12.7-14.2			
Al_2O_3	%	21-22.5			
SiO_2	%	24-25.5			
Physical Properties					
Color	-	Gray			
Form	-	Granular			
Odor	-	Neutral			
Moisture (130°C)	%	Max. 10			
pH (1% solution)	-	10.0-12.0			
Bulk density	g/L	400-700			
Heavy Metals					
Arsenic	mg/kg	12			
Cadmium	mg/kg	2			
Mercury	mg/kg	0.2			
Lead	mg/kg	15			
Dioxins and Dioxin-Like PCBs					
Dioxins	ng WHO-PCDD/F-TEQ/kg	0.75			
Sum of dioxins and dioxin-like PCBs	ng WHO-PCDD/F-PCB-TEQ/kg	1			
Non-dioxin-like PCBs	µg/kg	10			

Abbreviations: "<" = below detection limits; ICES-6 = sum of PCBs 28, 52, 101, 138, 153 and 180; PCB = polychlorinated biphenyl; PCDD = polychlorinated dibenzo-p-dioxins; PCDF = polychlorinated dibenzofurans; TEQ = toxic equivalency; WHO = World Health Organization.

2.4 SHELF-LIFE AND STABILITY OF SYNTHETIC SODIUM ALUMINOSILICATE AND X-ZELIT®

Synthetic sodium aluminosilicate is supplied in bulk (*ca*. 22,000 kg) or in big bags (450 kg). As an inorganic (mineral) substance it should not degrade on storage and a shelf-life of 24 months is proposed when stored unopened in the original packaging in the absence of humidity under ambient conditions.

X-Zelit® is supplied in 25 kg bags and also is not expected to degrade on storage on the basis of its composition (synthetic sodium aluminosilicate on a wheat carrier). A shelf-life of 24 months is proposed when stored unopened in the original packaging in the absence of humidity under ambient conditions. The specification for the packaging material is provided in Appendix 07A (CONFIDENTIAL), while a statement on the stability of X-Zelit® prepared by (b) (4)® is provided in Appendix 07B (CONFIDENTIAL).

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5.1 Intended Use, Use Levels and Mode of Administration

Protekta intends to market synthetic sodium aluminosilicate for use in feed as an aid to maintain calcium balance in periparturient (dry) dairy cows. The ingredient will be incorporated into dairy feed as part of a formulation comprising 75 to 80% synthetic sodium aluminosilicate, 17 to 20% wheat (carrier) and 1 to 3% rapeseed oil (anti-dust agent). The formulation will be marketed under the trade name X-Zelit® and is intended for addition to the feed of periparturient dairy cows at levels of 500 g/head/day, delivering up to 400 g synthetic sodium aluminosilicate/cow/day, either as top-dressing or as part of the TMR, for at least 14 days, and no more than 28 days, pre-calving. The duration of feeding reflects the practical time period that dry cows will be separated from the rest of the herd before calving. The use of sodium aluminosilicate in the form of X-Zelit® will be discontinued at calving.

2.5.2 Calcium Homeostasis in Dairy Cattle During the Transition Phase (3 Weeks Before to 3 Weeks After Parturition)

The demand for calcium in pregnant cows increases dramatically during gestation due to fetal development and the production of colostrum which can result in substantial calcium losses even in a single milking (Horst *et al.*, 1997). As an example, a cow producing 10 L of colostrum (2.3 g Ca/kg) will lose around 23 g of calcium in a single milking, representing around nine times the amount of calcium present in the entire plasma pool of the cow (Goff *et al.*, 1991). Calcium lost from the plasma pool must be replaced by increasing intestinal absorption of the mineral, and/or by its re-absorption from the bone. The requirements of periparturient (dry) cows for calcium are significantly lower, estimated to be in the region of 10 to 12 g calcium/day for fetal growth and endogenous fecal losses, and therefore, homeostatic mechanisms for replenishing blood calcium levels are normally not activated (Horst *et al.*, 1997). As a consequence, the increased nutrient needs for synthesis of colostrum combined with reduction in DM intake during late gestation, generally results in cows displaying reduced serum calcium levels to some degree at calving (Martinez *et al.*, 2012; Drackley, 1999; Wilkens *et al.*, 2020).

When cows experience dramatic hypocalcemia after giving birth, the clinical manifestation of this is termed “milk fever” (*paresis puerperalis*) which can have a significant negative impact on the health and welfare of the animal (Horst *et al.*, 1997; Drackley, 1999; DeGaris & Lean, 2008). Normally, blood calcium concentrations in the adult cow are maintained at around 2.1 to 2.5 mmol/L (Goff, 2006); the

levels at which the cow experiences subclinical or clinical hypocalcemia are imprecisely defined, but arbitrary boundaries of 2.0 and 1.4 mmol/L have been proposed (Roche & Berry, 2006). More recently, a blood calcium concentration threshold of <2.15 mmol/L has been defined as the reference point at which subclinical hypocalcemia is observed in cows early postpartum (Neves *et al.*, 2018). For example, Martinez *et al.* (2012) reported an increased risk of metritis in dairy cows in early lactation with a blood calcium level below 2.15 mmol/L. Similarly, Chapinal *et al.* (2012) noted that when cows displayed blood calcium concentrations below 2.1 mmol/L and increased levels of non-esterified fatty acids (>0.5 mEq/L) and β-hydroxybutyrate (>600 µmol/L), a 1.6 to 3.2 kg/day loss in milk production was observed. An increased risk of displaced abomasum in cows with blood calcium levels below 2.1 mmol/L was also described by Rodriguez *et al.* (2017). Thus, the maintenance of normal calcium levels is considered of critical importance to the health and performance of dairy cows and has been the subject of numerous research papers over the past 50 years (e.g., as reviewed by Horst *et al.*, 1997; Thilsing-Hansen *et al.*, 2002a; DeGaris & Lean, 2008; Wilkens *et al.*, 2020).

The parathyroid hormone (PTH) plays a critical role in the regulation of blood calcium homeostasis in mammals including dairy cattle. A decrease in blood calcium concentrations stimulates the secretion of PTH, which has two primary actions: (1) to mobilize skeletal calcium from bone which can then be utilized elsewhere in the body; and (2) to stimulate production of 1,25-dihydroxyvitamin D (calcitriol) production from vitamin D (Barton *et al.*, 1981). Activation of calcitriol increases absorption efficiency of calcium from the digestive tract (mainly in the upper small intestine) and increases calcium reabsorption in the kidney. Calcitriol most importantly stimulates the active transport of dietary calcium across the intestinal epithelium by stimulating the production of calcium binding proteins or transporter proteins (Horst *et al.*, 1997). Thus, stimulation of homeostatic mechanisms in dairy cows during the transition phase is essential to ensure extraction of calcium from the bones early postpartum in order to replace calcium lost to milk production and maintain calcium balance.

2.5.3 Dietary Strategies for Maintaining Calcium Balance

The incorporation of synthetic sodium aluminosilicate in the diet of periparturient cows for at least 14 days, and no more than 28 days, pre-calving is one of a number of dietary strategies for maintaining calcium balance in dry cows (Wilkens *et al.*, 2020). An alternative approach is the feeding of negative dietary cation-anion difference (DCAD) diets, typically 0 > DCAD (mEq/kg DM) > -200 from 3 weeks before calving until calving (Gaynor *et al.*, 1989; Caixeta *et al.*, 2020; EC, 2020). Another method of prevention is to supply rumen protected feed materials rich in phytic acid (>6%) and with a calcium content of <2% to dairy cows for 4 weeks pre-calving in order to achieve a minimum of 28 to 32 g available calcium per cow per day (Reindhardt *et al.*, 1988; EC, 2020). Other strategies employed at the first signs of parturition or a couple of days pre-calving, are to supplement the diet with highly bioavailable levels of calcium (i.e., oral calcium drenches; Goff & Horst, 1993) or waxy-leaf nightshade which releases dihydroxycholcalciferol-glycoside (EC, 2020).

Synthetic sodium aluminosilicate represents an alternative to these existing methods for maintaining calcium balance during the dry period.

2.5.4 *In Vitro* Binding Capacity of Synthetic Sodium Aluminosilicate in Rumen Fluid

Thilsing *et al.* (2006a) conducted an *in vitro* experiment which mimicked the transport of ingested synthetic sodium aluminosilicate in the forestomaches and proximal part of the small intestine to assess the binding capacity of synthetic sodium aluminosilicate to calcium, phosphorus, and magnesium as influenced by changes in pH. Rumen fluid collected from a non-pregnant healthy Jersey cow was adjusted to contain different concentrations of calcium and phosphorus and incubated with and without synthetic sodium aluminosilicate. Calcium carbonate and monosodium phosphate were added to rumen fluid to give 4 different solutions: 1) high calcium and low phosphorus, 2) high calcium and high phosphorus, 3) low calcium and high phosphorus, and 4) low calcium and low phosphorus. Synthetic sodium aluminosilicate was added to the rumen fluids at 0 (control) or 0.06 g per 8 mL of rumen fluid. The amount of synthetic sodium aluminosilicate was chosen to mimic an ongoing *in vivo* experiment by Thilsing *et al.* (2007) and equated to 600 g of synthetic sodium aluminosilicate in approximately 80 kg of digesta (see Section 2.5.5). The experiment was conducted in 3 phases: in phase 1, the rumen fluid solutions were incubated with or without synthetic sodium aluminosilicate at a pH typical of the rumen (approximately between pH 7.8 and 8.0) for 24 hours; in phase 2, hydrochloric acid was added to the rumen fluid, mimicking abomasal conditions (approximately between pH 1.5 and 3.5) for 1 hour; and in phase 3, the pH was increased with sodium bicarbonate to mimic the conditions in the small intestine (approximately between pH 6.8 to 7.2) for an additional 2 hours. Rumen fluid samples were taken at 1 and 24 hours in phase 1, at 5 minutes and 1 hour after the addition of hydrochloric acid in phase 2, and at 5 minutes, 1 hour, and 2 hours after the addition of bicarbonate in phase 3. All samples were centrifuged, and the supernatant analyzed for calcium, magnesium and phosphorus content. Centrifugation before the collection of the supernatant for mineral analysis was performed to separate synthetic sodium aluminosilicate and other particle-bound minerals from non-bound minerals. This separation procedure was chosen because synthetic sodium aluminosilicate precipitates rapidly in aqueous solutions at neutral or alkaline pH. Centrifugation of the sample also removed aluminum phosphate complexes from the supernatant after acid exposure. Therefore, comparing the concentrations of supernatant calcium, magnesium, and phosphorus in synthetic sodium aluminosilicate treated rumen fluid with that of non-treated rumen fluid allowed the amount of mineral bound by synthetic sodium aluminosilicate (or by aluminum if the additive is degraded by ruminal fluid, see Section 6.1.2) at any given stage to be estimated.

There was no significant change in pH during phase 1; however, during phase 2, the addition of acid resulted in a mean pH value of 1.64 in rumen fluid solutions without synthetic sodium aluminosilicate, and a mean pH of 3.47 in samples with synthetic sodium aluminosilicate, the difference being statistically significant ($P<0.0001$). Similarly, the supernatant pH was higher in synthetic sodium aluminosilicate treated samples during phase 3 ($P<0.0001$). The addition of synthetic sodium aluminosilicate to rumen fluid solutions reduced the amount of supernatant calcium and magnesium ($P\leq0.001$) at rumen pH, while phosphorus remained unchanged in phase 1. After adding hydrochloric acid, a large proportion of the sodium aluminosilicate bound calcium and magnesium was released, increasing supernatant calcium ($P\leq0.001$), and magnesium ($P=0.003$) levels. The addition of hydrochloric acid led to a substantial drop in supernatant phosphorus in samples that included sodium aluminosilicate, indicating binding of phosphorus ($P<0.0001$). A low level of supernatant phosphorus

was maintained after carbonate addition. Increasing the pH with carbonate led to a sodium aluminosilicate-induced drop in supernatant calcium and magnesium.

In the authors view, the results of this *in vitro* experiment must be interpreted with caution on the basis that aerobic conditions were employed with no saliva or digesta entering or leaving the system. However, results provide an indication of the binding capacity of synthetic sodium aluminosilicate as influenced by changes in pH. The results of the *in vitro* experiment demonstrated that synthetic sodium aluminosilicate not only bound calcium but also magnesium and phosphorus. The binding of calcium in phases 1 and 3 of the experiment provides evidence that synthetic sodium aluminosilicate is able to decrease the availability of calcium to dairy cows. The small intestine is believed to be the primary site of absorption of calcium, although Schröder *et al.* (1997) showed that some calcium absorption also occurs in the rumen of some ruminants. The removal of calcium from the rumen could therefore be sufficient to cause an overall decrease in the total calcium uptake. The reduction in phosphorus levels in phase 2 (mimicking the stomach) indicate that there may be reduction in inorganic phosphate available from the diet also. Magnesium was reduced with the addition of synthetic sodium aluminosilicate during phase 1 and phase 3. The decrease in magnesium compared with the decrease of calcium was much slower and this was likely due to the cation exchange property of sodium aluminosilicate. The sodium aluminosilicate used in this study has high selectivity for calcium binding, therefore calcium is bound rapidly; when the "free calcium" ions decrease, other competing ions such as magnesium become bound as well.

2.5.5 Studies in Dairy Cattle to Evaluate the Utility of Synthetic Sodium Aluminosilicate

Ten studies were identified in the published literature in which synthetic sodium aluminosilicate was fed to periparturient dairy cows in order to evaluate the utility of the additive to bind dietary calcium and influence plasma calcium concentrations early postpartum (Thilsing-Hansen & Jørgensen, 2001; Thilsing-Hansen *et al.*, 2002b, 2003; Thilsing *et al.*, 2007; Grabherr *et al.*, 2008; Grabherr *et al.,* 2009a; Pallesen *et al.*, 2008; Kerwin *et al.*, 2019; Khachlouf *et al.,* 2019; Crookenden *et al.*, 2020). The studies were performed in a mixture of research and commercial farms in the U.S., Europe and North Africa, and the TMR comprised a range of forages. Together the studies are considered to cover a variety of different conditions which may be considered relevant to U.S. commercial feeding practices. Detailed summaries of each of the studies is provided in Table 2.9. The overall findings of the studies with respect to calcium binding and balance in periparturient dairy cows are summarized and critically evaluated below. Another two study reports were identified in the public domain which are not published in peer reviewed journals (Thilsing-Hansen *et al*., 2006b; Zoltán, 2019). On the basis that the robustness and validity of the trials cannot be verified, these studies are not included in the discussion. However, it is noteworthy that the conclusions of these studies were consistent with those of the published data summarized herein.

In addition to evaluating the ability of synthetic sodium aluminosilicate to influence serum calcium levels in periparturient cows, the studies identified also considered endpoints relevant to safety including the effect on other mineral levels (phosphorus, magnesium and aluminum) and performance [dry matter intake (DMI), incidence of disease and milk production]. The findings of the studies with respect to the safety of synthetic sodium aluminosilicate are evaluated in Section 6.1.3.

2.5.5.1 Synthetic Sodium Aluminosilicate Test Articles

Only studies in dairy cows using synthetic sodium aluminosilicate with the chemical formula $Na_{12}Al_{12}Si_{12}O_{48}.27H_2O$ were included in the utility evaluation. One of the studies was conducted using X-Zelit® as the source of synthetic sodium aluminosilicate (Kerwin *et al.*, 2019). A similar formulated product, marketed as (b) (4) in which synthetic sodium aluminosilicate (*ca*. 80%) is mixed on a carrier (*ca*. 20%; not defined) was used in the study by Crookenden *et al.* (2020). The majority of other studies use products marketed under the trade names (b) (4) (Thilsing-Hansen & Jørgensen, 2001; Thilsing-Hansen *et al.*, 2002b, 2003) and Zeoline[3] (Thilsing *et al.*, 2007; Pallesen *et al.*, 2008; Khachlouf *et al.,* 2019) which appear to comprise only synthetic sodium aluminosilicate without the addition of carriers or other components. The remaining studies by Grabherr *et al.* (2008, 2009a) identify synthetic sodium aluminosilicate to be manufactured by (b) (4) (b) (4)) but do not identify the trade name or specific commercial product. Although the products may vary in crystal structure, compositionally they are all of the chemical formula $Na_{12}Al_{12}Si_{12}O_{48}.27H_2O$. As a result, their ability to bind calcium in the feed was anticipated to be similar under the conditions of intended use and all of these sources of synthetic sodium aluminosilicate were considered relevant to the utility assessment. The source of the synthetic sodium aluminosilicate and level of supplementation in the diet of dairy cattle is clearly identified in each of the study summaries.

It is also noteworthy that other zeolites (e.g., Clinoptilolite) have been investigated for similar purposes in the feed of dairy cattle (e.g., Stojić *et al.*, 2020). Due to their compositional and structural differences to synthetic sodium aluminosilicate, studies on other Zeolites were not considered directly relevant to the utility assessment.

2.5.5.2 Feeding Studies in Periparturient Dairy Cows

As mentioned above, 10 studies were identified in which dairy cows were fed diets supplemented with synthetic sodium aluminosilicate over the transition period and are summarized in Table 2.9. The period of supplementation varied from 14 days (Thilsing-Hansen *et al.*, 2003; Thilsing *et al.*, 2007; Pallesen *et al.,* 2008; Grabherr *et al.*, 2008, 2009a; Crookenden *et al.*, 2020), to 21 days (Kerwin *et al.*, 2019; Thilsing-Hansen *et al.*, 2002b), 28 days (Thilsing-Hansen & Jørgensen, 2001; Thilsing-Hansen *et al.*, 2002b) and 40 days (Khachlouf *et al.*, 2019) before the expected day of calving. Likewise, the level of synthetic sodium aluminosilicate supplementation ranged from 100 to 200 g/head/day (Grabherr *et al.*, 2009a; Khachlouf *et al.*, 2019) to 400 g/head/day (Kerwin *et al.*, 2019; Crookenden *et al.*, 2020), 500 g/head/day (Thilsing-Hansen *et al.*, 2002b and 2003; Pallesen *et al.*, 2008; Grabherr *et al.*, 2008), 700 g/head/day (Thilsing-Hansen *et al.*, 2003) and 1,000 g/head/day (Thilsing-Hansen & Jørgensen, 2001). Under all of the experimental conditions, serum calcium concentrations were significantly higher in dairy cows fed synthetic sodium aluminosilicate-supplemented diets relative to control diets in the early postpartum period (typically day of calving to around 4 or 7 days later). Furthermore, serum calcium concentrations were reported to stay above established hypocalcemia limits (*ca*. 2 mmol/L; see Section 2.5.2) in the synthetic sodium aluminosilicate-supplemented cows. While serum calcium levels in both

[3] (b) (4)) is specifically identified by Pallesen *et al.*, 2008 as synthetic sodium aluminosilicate and is identified as synthetic on the European Zeolites Producers Association (EUZEPA); the other studies which use this test article only refer to the material as sodium aluminosilicate. However, for the purposes of this assessment, they are assumed to be similar synthetic products.

the experimental (synthetic sodium aluminosilicate-supplemented) and control cows generally exhibited a linear decrease before and after calving, the drop in serum concentrations was more pronounced in the control animals. Thus, a number of the authors concluded that plasma calcium levels were stabilized by synthetic sodium aluminosilicate supplementation around calving (e.g., Thilsing-Hansen & Jørgensen, 2001; Thilsing *et al.*, 2007 ; Pallesen *et al.*, 2008; Grabherr *et al.*, 2008; Khachlouf *et al.*, 2019).

The incidence of subclinical hypocalcemia or milk fever in dairy cows postpartum was reported in most of the studies. Fewer cases of subclinical hypocalcemia or milk fever were reported in cows fed diets supplemented with synthetic sodium aluminosilicate in the early postpartum period in a number of the studies (e.g. Thilsing-Hansen & Jørgensen, 2001; Thilsing-Hansen *et al.*, 2003; Pallesen *et al.,* 2008; Kerwin *et al.*, 2019; in other studies too few cases were reported in either the control or experimental cows for any conclusions to be drawn (e.g., Thilsing-Hansen *et al.*, 2002b; Crookenden *et al.*, 2020 – no cows contracted milk fever).

Thilsing-Hansen *et al.* (2003) evaluated the results of 6 separate studies in which dairy cows were fed 500 or 700 g/head/day of synthetic sodium aluminosilicate for 14 or 28 days prepartum. Synthetic sodium aluminosilicate was observed to significantly increase the mean serum calcium concentrations on the day of calving in all experiments, although the effectiveness in reducing hypocalcemia also appeared to be influenced by the additive-calcium ratio in the diet. The studies were conducted at a number of different locations (extensive farming and intensively-driven farms) and the endpoints measured were not consistent between experiments, making direct comparison of the findings difficult. From the results, it appeared that the effect of feeding synthetic sodium aluminosilicate for 28 days is comparable to that of 14 days pre-partum, with no statistically significant adverse effects on the effectiveness of the ingredient, or the health of the cows, observed with an increased period of feeding.

It is recognized that Grabherr *et al.* (2009a) and Khachlouf *et al.* (2019) indicate that levels of synthetic sodium aluminosilicate in the diet in the range of 200 g/head/day may be effective in increasing serum calcium levels around parturition. However, Grabherr *et al.* (2009a) also noted that levels of synthetic sodium aluminosilicate above 200 g/head/day significantly reduced DMI intake relative to the other experimental groups (131 or 215 g synthetic sodium aluminosilicate/head/day) which may have confounded the results of this study. The majority of studies, including the study by Kerwin *et al.* (2019) using Protekta's formulation, X-Zelit®, indicate that synthetic sodium aluminosilicate in the range 400 to 500 g/head/day is effective in influencing serum calcium levels in periparturient cows.

The studies were conducted in a mixture of Holstein, Holstein-Friesian and Jersey cows which were normally multiparous. The exceptions were studies by Grabherr *et al.* (2009a) and Khachlouf *et al.* (2019) in which a mixture of primiparous and multiparous cows were used. Khachlouf *et al.* (2019) did not assess the impact of lactation number on any study variable, but the effect of synthetic sodium aluminosilicate supplementation on the serum mineral (calcium, phosphorus and magnesium) levels in cows appeared in the study by Grabherr *et al.* (2009a) to be significant only in older cows (greater than 2 lactations). The conclusions that can be drawn from one study are limited, but as the authors note, the findings are consistent with other reports of younger cows exhibiting a greater ability to mobilize calcium stores than older ones (Moore *et al.*, 2000; Chan *et al.*, 2006; Martinez *et al.*, 2016; Stojić *et al.*, 2020).

In a few studies, the response of homeostatic mechanisms in periparturient cows on supplementation with synthetic sodium aluminosilicate was also evaluated. Serum 1,25-dihydroxyvitamin D concentrations were observed to increase in the synthetic sodium aluminosilicate-supplemented cows relative to the control cows in the study by Thilsing-Hansen *et al.* (2002). Similarly, Thilsing *et al.* (2007) reported that serum 1,25-dihydroxyvitamin D concentrations in animals fed diets supplemented with synthetic sodium aluminosilicate were well above the levels normally observed in periparturient cows and the levels in periparturient cows fed diets supplemented with both synthetic sodium aluminosilicate and calcium carbonate ($P<0.05$). These findings are consistent with stimulation of renal reabsorption of calcium from the bone and renal vitamin D metabolism leading to production of 1,25-hydroxyvitamin D.

Taken together, the body of available evidence summarized above supports the utility of synthetic sodium aluminosilicate to positively impact serum calcium concentrations in early postpartum cows when fed for at least 14 days, and no more than 28 days, pre-calving. Kerwin *et al.* (2019) in particular demonstrated that serum calcium concentrations were significantly increased in cows fed diets supplemented with X-Zelit® at 500 g/head/day, equating to 400 g synthetic sodium aluminosilicate/head/day for 21 days before the expected calving date relative to control cows as parturition approached and during the early postpartum period.

As mentioned above, the effect of synthetic sodium aluminosilicate on other parameters relevant to safety, such as serum phosphorus and magnesium levels, DMI intake and milk production, were also measured in the studies. These findings are evaluated separately in Section 6.1.3.

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

Reference – Kerwin *et al*., 2019

Objective – Effects of feeding synthetic Zeolite A during the prepartum period on serum mineral concentration, oxidant status, and performance of multiparous Holstein cows

Test Article – SSA (as X-Zelit®)

Animals – Late-gestation multiparous Holstein cows (n=26-29/treatment)

Location – (b) (4)

Relevant Endpoints Measured

Plasma Analysis (weekly until 7 days before expected calving, then daily until 7 DIM, then 3 x /week until 28 DIM): mineral status (Ca, P, Mg), oxidative stress indices (AOP, RONS, OSi)

Milk Production and Composition: milk yield (wk 1-4 and wk 1-9), milk composition wk 1-4 (fat, protein, lactose, fatty acids)

Performance: DMI, rumination, BW, BCS, health, reproductive performance

Dietary Treatments

21 days before expected parturition:

- **CON** – corn silage + wheat straw + canola meal + soybean meal
- **EXP** – corn silage + wheat straw + canola meal + soybean meal + X-Zelit® (500 g/head/day equivalent to 400 g SSA/head/day)

Postpartum:

Corn silage + Alfalfa silage + steam-flaked corn + soybean meal + canola meal. SSA supplementation ceased. Cows were milked 3x/day for 63 days of lactation

Results

Plasma Analysis:

- ↑ plasma Ca in EXP cows vs. CON cows as parturition approached and during early postpartum period ($P<0.05$)
- ↓ plasma P in EXP cows vs. CON cows during prepartum period and 1st 2 days of lactation ($P<0.05$)
- ↓ plasma Mg in EXP cows vs. CON cows only during immediate postpartum period ($P<0.05$)
- NSD in oxidative stress markers between treatment groups (RONS, AOP and OSi)

(b) (4)

(b) (4)

Performance:

- Tendency to ↓ prepartum DMI ($P<0.1$) and ↓ rumination ($P<0.05$) in EXP cows vs. CON cows; but NSD in postpartum DMI, rumination, milk yield, milk component yield and colostrum measurements

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows
General Health: ↓ prevalence of subclinical hypocalcemia (P<0.05) from days -1 to 3 with largest difference occurring within the 1st day postpartum • Tendency to ↑ hazard of pregnancy (P<0.1) by 150 days in milk in EXP cows vs. CON cows • Tendency to ↑ incidence of displaced abomasum in EXP cows vs. CON cows (P=0.06) and ↑ incidence clinical mastitis in CON cows vs. EXP cows (P=0.09) [Low #s of animal means caution should be used when interpreting health/reproductive parameters]
Conclusions – Treatment with SSA during the prepartum period resulted in improved serum Ca concentrations around parturition and similar postpartum performance vs. controls and reduced incidence of hypocalcaemia

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows
Reference – Crookenden *et al.*, 2020
Objective – Feeding synthetic Zeolite to transition dairy cows alters neutrophil gene expression
Test Article – SSA (b) (4)
Animals – Late-gestation multiparous Holstein-Friesian (n=18) and Holstein-Friesian x Jersey (n=2) cows (n=10/treatment)
Location (b) (4)
Relevant Endpoints Measured Plasma Analysis: Mineral status (Ca, P, Mg), energy status, liver function, inflammatory markers Blood Analysis: Neutrophil isolation for respiratory burst assay and gene expression analysis [days -19, -14, -7 (pre-calving), 0, 1, 4, 7 and 28 (post-calving)]
Dietary Treatments Min. 14 days (17±3 days) pre-calving, cows were fed to achieve a BCS of 5.0: • **CON** – grazed fresh pasture + maize silage + MgO (80-100 g/head/day) • **EXP** – grazed fresh pasture + maize silage + MgO (80-100 g/head/day) + Optimate MF+ (500 g/head/day equivalent to SSA at 400 g/head/day) SSA supplementation ceased at calving 4-days post-calving (during colostrum phase): • >35 kg DM (grazed fresh pasture) + $CaCO_3$ (200 g/head/day) Cows were milked 2x/day for 1st 12 weeks of lactation (FCM yield 25.9 ± 0.75 kg/day)
Results Plasma Analysis: • ↓ plasma P in EXP cows vs. CON cows over period of SSA supplementation (P<0.01) • ↓ plasma Mg on day of calving in EXP cows vs. CON cows (P<0.05) • ↑ plasma Ca in EXP cows vs. CON cows during the 1st 4 days of lactation (P<0.05) (b) (4) (b) (4) Blood Analysis: • NSD on neutrophil respiratory burst between treatment groups over the transition period (P>0.10) • Few effects were observed on expression of genes involved in neutrophil function; 2 genes (CXCR4 and S100A8) were down regulated (P≤0.05) and 4 genes tended to be downregulated (P≤0.13) over the transition period in EXP cows vs. CON cows (affected genes included those encoding proteins involved in neutrophil inflammatory response and reliant on Ca availability) General Health: No clinical cases of milk fever were observed in EXP or CON cows
Conclusions – Treatment with SSA increased plasma Ca concentrations at the onset of lactation

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows
Reference – Pallesen *et al.*, 2008
Objective – Effect of pre calving zeolite, magnesium and phosphorus supplementation on periparturient serum mineral concentrations
Test Article – SSA (b) (4)
Animals – Late-gestation multiparous Danish Jersey cows (n=10/treatment)
Location – (b) (4)
Relevant Endpoints Measured Serum analysis (before supplementation, 6-10 days after start of supplementation, 0-2 days postpartum and 13-17 days postpartum): minerals (Ca, Mg, P)
Dietary Treatments TMR = corn silage + concentrate + dried beet pulp + barley straw + urea 2 weeks before expected parturition: • **Group A** – TMR + 200 g/cow/day high Mg & P supplement + 500 g/cow/day SSA • **Group B** – TMR + 45 g/cow/day low Mg & P supplement + 500 g/cow/day SSA • **Group C** – TMR + 200 g/cow/day high Mg & P supplement SSA, Mg & P supplementation ceased at calving
Results Serum Analysis: • Less variable serum Ca around calving in Group B cows vs. Group A cows (P=0.021 between mean parturient levels in these 2 groups; NSD at other sampling points) and no cases of clinical hypocalcaemia or hypomagnesaemia • ↓ serum Ca in Group A cows vs. Group B cows; 1 case of hypocalcaemic (1.4 mmol/L) at calving (which developed milk fever) but no cases of hypomagnesaemia • ↓ serum Ca in Group C cows vs. Group B cows; 12 cases of hypocalcaemia (of which 3 developed milk fever) but no cases of hypomagnesaemia • Serum Ca levels between Group A and C cows differed significantly at all sampling points (P=0.039, P=0.001 and P=0.001, respectively); NSD in serum Mg levels among groups
(b) (4)
Performance Feed refusals were <1% of total amount fed in all groups during period of SSA supplementation (with prolonged eating times) General Health: Milk fever was identified in 1 cow in Group A, no cows in Group B and 3 cows in Group C
Conclusions – Combined P and Mg supplementation in addition to SSA supplementation did not increase serum Mg, but reduced SSA-induced hypophosphatemia as well as the stabilizing effect of SSA on parturient serum Ca

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

Reference – Grabherr *et al*., 2008 – paper written in German

Objective – Influence of Zeolite A supplementation during the dry period of dairy cows on feed intake, on the macro and trace element metabolism around calving and milk yield in the following lactation

Test Article – SSA manufactured by (b) (4)

Animals – Late-gestation multiparous German Holstein cows (n=23/treatment)

Location – (b) (4)

Relevant Endpoints Measured

Blood Analysis (1st day of trial, 7 days before calving, day 0, 1, 2 & 7): minerals (Ca, Mg, P, Fe), FFA, BHB, trace elements (Cu, Zn, Mn)

Milk Production and Composition: yield (daily; FCM), composition (2x/week; fat, protein, lactose, urea)

Dietary Treatments

2-3 weeks before expected calving date:

TMR = SBM + barley + wheat + dried pulp + soybean oil + mineral mix

- **CON** – TMR
- **EXP** – TMR + 90 g SSA/kg DM/day (approx. 558 g SSA/cow/day)

SSA supplementation ceased at calving

Postpartum TMR = maize + grass + concentrate

Results

Serum Analysis:

- ↑ serum Ca in EXP cows vs. CON cows from 7 days before calving (P=0.017); serum Ca in CON cows fell below limit for hypocalcaemia (1.87±0.34 mmol/L vs. 2 mmol/L) at calving, whereas serum Ca was stabilized in EXP cows (2.3±0.15 mmol/L on calving; 2.41±0.18 mmol/L 7 days postpartum)
- ↓ serum Mg on day of calving and 1 day postpartum in EXP cows vs. CON cows (P<0.05)
- ↓ serum P after 1st week of SSA supplementation in EXP cows vs. CON cows (P<0.05)
- ↑ serum FFA 1 week after SSA supplementation and serum beta-HB around calving (likely result of depressed DMI) in EXP cows vs. CON cows
- NSD in serum Cu, Zn, Fe and Mn between EXP and CON cows

(b) (4)

Performance

- ↓ DMI intake (P<0.0001) in EXP cows vs. CON cows (6.2±1.3 kg vs. 12.0 ± 1.4 kg; 48% lower) prepartum

NSD in DMI or milk yield postpartum between EXP and CON cows

Conclusions – The reduced DMI and hypophosphatemia observed following SSA supplementation indicates this supplementation level is not suitable for periparturient cows

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

Reference – Grabherr *et al*., 2009a

Objective – Effect of several doses of Zeolite A on feed intake, energy metabolism and on mineral metabolism in dairy cows around calving

Test Article – SSA

Animals – Late-gestation German Holstein cows (n=20/treatment)

Lactation no.	1	2	3	4	5	6	7	∑1 & 2	∑ > 3
n	29	18	0	22	8	2	1	47	33

Location – (b) (4)

Relevant Endpoints Measured

Serum Analysis (day -28, -14, -7, 0, +1, +2, +7): minerals (Mg, Ca, P, K), crea, NEFA & BHB;
Urine Analysis (day -14, +1, +7): minerals (Mg, Ca, P, K), crea
Performance: BCS, FI, milk yield (105 days), milk composition (fat, protein, lactose)

Dietary Treatments

4 weeks before expected calving:
TMR = maize silage + grass silage + concentrate
2 weeks before expected calving:

- Group I – TMR
- Group II – TMR + 12 g SSA/kg DM/day (131 g SSA/cow/day)
- Group III – TMR + 23 g SSA/kg DM/day (215 g SSA/cow/day)
- Group IV – TMR + 43 g SSA/kg DM/day (310 g SSA/cow/day)

SSA supplementation ceased at calving
Postpartum:
TMR = maize silage + grass silage + concentrate

Results

Serum Analysis:

- NSD in serum Ca among groups on younger cows (1st and 2nd lactation); in all group serum Ca at calving remained above the limit of hypocalcaemia (2 mmol/L)
- In older cows (3rd or more lactation), ↑ serum Ca in Group III and IV cows which was well above hypocalcaemia limit (2.27±0.20 mmol/L and 2.31±0.20 mmol/L, respectively), but ↓ serum Ca in Group I and II cows (1.85±0.26 mmol/L and 1.94±0.21 mmol/L, respectively); compared to the serum Ca 14 days pre-calving the effect in Group I and II cows was significant
- Hypocalcaemia incidences were 6/8 cows in Group I and II, 2/9 cows in Group III and 0/8 cows in Group IV; reduction rate of hypocalcaemia was 71% for Group III vs. 100% for Group IV
- NSD in serum Mg among groups on younger cows (1st and 2nd lactation)
- In older cows (3rd or more lactation), ↓ serum Mg in Group IV cows (0.94±0.08 mmol/L) vs. Group I and II cows 1.30±0.12 mmol/L and 1.21±0.20 mmol/L); serum Mg remained within reference ranges (0.75 to 1.30 mmol/L) in all groups
- ↑ serum P in Group IV cows (0.75±0.22 mmol/L in younger cows; 0.47±0.11 mmol/L in older cows) was below reference range of 1.25 mmol/L at 7 days pre-calving; serum P in Group IV cows was ↓ vs. Group I (1.90±0.23 mmol/L; 1.80±0.33 mmol/L) and II (1.88±0.25 mmol/L; 1.68±0.16 mmol/L); NSD in serum P between Group III cows (1.32±0.42 mmol/L; 1.17±0.30 mmol/L) vs. Group I and II cows
- In older cows, serum P ↓ in Group I and II on day of calving (1.17±0.28 mmol/L and 1.02±0.43 mmol/L, respectively) but remained around limit of reference range (1.25 mmol/L)
- Negative correlation between serum P and SSA supplementation level prepartum (P<0.0001)
- NSD on serum K among groups
- ↑ serum NEFA on day of calving in all groups; apparent but not significant ↑ in Group IV cows serum NEFA at 7 days pre-calving vs. Groups I and II cows

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

- ↑ serum beta-HB in Groups I, III and IV cows after calving which was significant on day 7 postpartum in Groups I and III cows, and also on days 1 and 2 postpartum in Group IV cows; mean serum beta-HB ↑ from 0.57 mmol/L 14 days pre-calving to 1.17 mmol/L 1 day postpartum

(b) (4)

[redacted]

Acid-Base Status and Urinary Excretion of Ca, Mg, P and K

- 7 days post postpartum, NABE correlated negatively with DMI during last 2 weeks ante partum (P=0.0058)
- ↑ urinary Ca excretion in Group IV cows vs. other groups (P<0.05) from 1 day after parturition to day 7 vs. Group I and II; Ca excretion in Groups I to III cows was below physiological ranges (0.2 to 0.9%) at day 1 after parturition
- ↓ urinary Mg excretion in Group IV cows vs. Group I and II cows at 1 day postpartum
- ↓ urinary P excretion in Group IV cows vs. Group I and III cows (and tended to be lower vs. Group II) at 1 day postpartum
- ↓ fractional urinary K excretion in Groups I, II and IV cows at 1 day postpartum, and Group IV at 7 days postpartum, correlating with NABE

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows
<u>Performance</u> • Reduction in DMI intake in Group IV (-4.8 kg) cows vs. Group 1 (-1.4 kg), Group II (-1.1 kg) and Group III (-1.7 kg) cows • ↓ DMI intake (7.3 kg/day) in Group IV cows vs. Groups I, II and III (10.1, 10.9 and 9.5 kg/day; P<0.05) cows; reduction in Group IV cows DMI was from the start of SSA supplementation through entire experimental period • NSD in BCS among groups • NSD in DMI, milk yield and milk composition during lactation among groups (milk yield 2550-3216 kg FCM)
Conclusions – Supplementation of periparturient cows with 23 g SSA/kg DM TMR (215 g/head/day) was adequate to reduce subclinical hypocalcaemia without significant effects on DMI and serum P

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows
Reference – Khachlouf *et al*., 2019
Objective – Effects of dietary Zeolite supplementation on milk yield and composition and blood mineral status in lactating dairy cows
Test Article – SSA (b) (4)
Animals – Holstein primiparous (n=20) and multiparous (n=22) cows (n=21/treatment)
Location – (b) (4))
Relevant Endpoints Measured Plasma Analysis: mineral status (Ca, P, Mg) Milk Production and Composition: milk yield, (days 7, 20, 40 & 60 post-calving), composition (fat, protein, lactose, minerals)
Dietary Treatments 40 days before expected lactation (DMI 11.5 kg/cow/day): • **CON** – TMR • **EXP** – TMR + Zeoline (SSA; 200 g/head/day) [# days of SSA supplementation not clearly specified during prepartum period] SSA supplemented ceased at calving 1st 60 days of lactation (DMI 19.0 kg/cow/day): • TMR TMR consisted of oat hay, oat silage, alfalfa pellets and concentrate; mineral balancer added to TMR to meet Ca, P and Mg requirements
Results Plasma Analysis: • ↑ plasma Ca in EXP cows vs. CON cows pre- and post-calving • Ca levels were stabilized in EXP cows vs. CON cows around calving • Plasma Ca levels remained above hypocalcaemia limit (85.0 mg/L) in both groups • NSD in plasma P between groups and mean value remained above lower limit of reference interval (55-65 mg/L) • NSD in plasma Mg between groups and mean values remained within reference ranges (18-24 mg/L)
(b) (4)

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows
Performance: • ↑ milk production in EXP cows vs. CON cows during 1st 60 days of lactation (+2.18 kg/cow/day; P<0.001) • NSD in milk composition (total solids, fat, protein, lactose and ash) between groups (P>0.05) • NSD in Ca, P or Mg contents of milk between groups (P>0.05)
Conclusions Treatment of periparturient cows with 200 g SSA/head/day was effective at increasing plasma calcium levels before and after calving. Additionally, SSA supplementation was not associated with any adverse effects on milk production or composition.

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows
Reference – Thilsing-Hansen and Jørgensen, 2001
Objective – Prevention of parturient paresis and subclinical hypocalcemia in dairy cows by Zeolite A administration in the dry period
Test Article – SSA (b) (4)
Animals – Late-gestation multiparous Jersey cows (n=8-9/treatment)
Location – Not stated
Relevant Endpoints Measured Serum Analysis (day 0, 1 & 2): Ca
Dietary Treatments TMR = barley & pea wholecrop silage + concentrate + barley + sugar beet pulp + beet molasses + seed grass straw + silaged mash + mineral mix 4 weeks before expected parturition: • **CON** – 10 kg TMR per head per day + seed grass straw *ad libitum* • **EXP** – 10 kg TMR per head per day + seed grass straw *ad libitum* + Dedima 100 (SSA; 1 kg/head/day) 1 week before expected parturition: • **CON** – TMR *ad libitum* + seed grass straw *ad libitum* • **EXP** – TMR *ad libitum* + seed grass straw *ad libitum* + Dedima 100 (SSA; 1 kg/head/day) SSA supplementation ceased at calving Postpartum: • 250 g/head $CaCO_3$ given as a chalk suspension drench on the day 0 (calving), day +1 and day +2 • All animals were fed TMR and seed grass straw *ad libitum.*
Results Serum Analysis: • Serum Ca <2.0 mmol/L in 6/8 CON cows vs. none of the EXP cows
(b) (4)
General health 3/8 CON cows contracted milk fever vs. none of the EXP cows
Conclusions – Treatment with SSA prevented parturient paresis and subclinical hypocalcaemia

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows
Reference – Thilsing-Hansen *et al.*, 2002b
Objective – The effect of Zeolite supplementation in the dry period on periparturient Ca, P and Mg homeostasis
Test Article – SSA (b) (4)
Animals – Late-gestation multiparous Jersey cows (n=15-16/treatment)
Location – (b) (4)
Relevant Endpoints Measured Blood Analysis (1 week before calving, day 0, 1, 2 & 7): plasma minerals (Ca, Mg, P), serum $1,25(OH)_2D$ Urine Analysis (1 week before calving): DPD, creatine Milk Production (weekly): yield, composition (fat, protein) Performance: DMI
Dietary Treatments TMR = grass & clover silage + beet pulp silage + beet molasses + barley straw + grain mixture + concentrate • **CON** – TMR • **EXP** – TMR + Dedima 100 • Dedima 100 incrementally increased from 0 to 700 g SSA/head/day during the third week before expected parturition, then maintained at 700 g SSA/head/day until calving. SSA supplementation ceased at calving Postpartum: • 250 g/head $CaCO_3$ given as a chalk mixed into ration on the day 0 (calving), day +1 and day +2
Results Plasma Biochemical Analysis: • ↑ plasma Ca in EXP cows on day of calving ($P<0.0001$), but no difference 1 week before calving ($P=0.27$) vs. CON cows • ↓ plasma Mg in EXP cows on day of calving ($P=0.0068$), but no difference 1 week before calving ($P=0.83$) vs. CON cows • ↓ plasma P in EXP cows on day of calving ($P=0.015$) and 1 week before calving ($P=0.0004$) vs. CON cows; plasma P of EXP cows increased to within reference interval 1 week after calving • ↑ serum $1,25(OH)_2D$ in EXP cows 1 week before expected date of calving ($P=0.0409$) vs. CON cows (64.7 vs. 43.7 pg/mL, respectively)
(b) (4)

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

(b) (4)

[redacted]

Urinalysis:

- NSD in urinary excretion of deoxypyridinoline between EXP and CON groups (P=0.6123)

Performance

- ↓ DMI in EXP cows during last 2 weeks of pregnancy vs. CON cows (12% and 3% feed refusals, respective)
- NSD in mean accumulated milk yield (P=0.448), milk fat (P=0.089) or milk protein (P=0.483) 105 days after calving, although there was a tendency to ↓ milk fat yield in EXP cows

General Health:

- 2 EXP cows and 1 CON cow received Ca drenches postpartum (at time of drenching all cows were normocalcaemic at 2.57, 2.34 and 2.39 mmol/L)
- No incidences of milk fever

Conclusions – Treatment with SSA increased plasma Ca concentrations at the onset of lactation

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

Reference – Thilsing-Hansen *et al.*, 2003

Objective – The effect of Zeolite A supplementation in the dry period on the blood calcium, magnesium and phosphorus level around calving

Test Article – SSA (b) (4)

Animals – Late-gestation multiparous cows (n=117, treatment groups sizes shown below). Breed not stated.

Location – (b) (4) (6 separate studies)

Relevant Endpoints Measured

Blood Analysis:

Farm	A (intensive)	B (intensive)	C (extensive)
Exp. No.	(b) (4)		
Blood sample days			
Minerals analyzed			

Health: Hypocalcaemia

Dietary Treatments

SSA supplementation of the TMR for 14 to 28 days prepartum:

Farm	A (intensive)	B (intensive)	C (extensive)
Exp. No.	(b) (4)		
No. of cows (EXP + CON)			
g SSA/cow/day			
Days of SSA supplementation			
SSA:calcium ratio[1]			
Diet			
Postpartum supplement			

[1] SSA:calcium ratio = g of zeolite per cow per day/g of dietary calcium per cow per day

SSA supplementation ceased at calving

Results

Plasma Analysis:

- ↑ serum Ca in EXP cows vs. CON cows on days 0, 1 and 2 post-calving (P<0.0001); NSD in serum Ca 1 week post-calving (Exp. 3 & 4) or 3 weeks pre-calving (Exp. 1 & 2) (P>0.1)
- ↑ plasma Mg in CON cows vs. EXP cows (P=0.0006) on day of calving; plasma Mg remains within reference ranges over entire postpartum period; NSD in plasma Mg 3 weeks before (Exp. 1 & 2) or 1 week after calving (Exp. 3 & 4) (P>0.1)
- Plasma P below lower limit of reference interval in EXP and CON cows throughout transition period; ↓ plasma P lower in EXP cows vs. CON cows on day of calving as well as 3 weeks pre-calving (Exp. 1 & 2); NSD in plasma P between EXP and CON cows (Exp. 3 & 4) (P=0.2245) and level effectively normalized

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

(b) (4)

(b) (4)

General Health:

- ↓ incidence of hypocalcaemia (serum Ca<2.0 mmol/L) on day of calving in EXP cows vs. CON cows

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Total
EXP cows	0/9	0/6	1/4	3/14	13/17	2/13	19/63
CON cows	5/8	4/4	3/4	6/11	14/18	7/9	39/54

Conclusions – Treatment with SSA prepartum significantly increased serum Ca and plasma Mg levels (which remained within reference levels) on the day of calving but significantly decreased plasma P before and after calving (normalizing within 1 week); SSA to Ca ratios of 10-20 appear effective at reducing hypocalcaemia. There was apparently no additional effect from feeding SSA for 4 weeks instead of 2 weeks prepartum.

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

Reference – Thilsing *et al.*, 2007

Objective – The effect of dietary calcium and phosphorus supplementation in Zeolite A treated dry cows on periparturient calcium and phosphorus homeostasis

Test Article – SSA (b) (4))

Animals – Late-gestation multiparous Holstein cows (n=4-6/treatment)

Location – (b) (4)

Relevant Endpoints Measured

Blood Analysis (3 per week from 3 weeks before to 3 weeks after calving, plus samples on day 0, 1 & 2):
$1,25(OH)_2D$, minerals (Ca, Mg, P) & PTH;
1 serum sample per cow before SSA supplementation started and 1 sample per cow 4.8 (±0.3SEM) days after SSA supplementation was analyzed for Al
Urine Analysis (3 per week from 3 weeks before to 3 weeks after calving): Ca, DPD & creatine
Milk Production (weekly): yield, composition (fat, protein)

Dietary Treatments

Pre-partum TMR = corn silage + barley + rapeseed cake + palm cake + barley straw + shelled soy meal + NaH_2PO_4 + $CaCO_3$ + MgO + urea

Weeks before the expected date of calving	n	Experimental group	Dietary Ca (g/cow/day)	Dietary P (g/cow/day)	Dietary SSA (g/cow/day)
-4 and -3	5	1	39	36	0
	6	2 (+P)	39	136	0
	4	3 (+Ca)	100	36	0
	5	4 (+Ca, +P)	100	136	0
-2 and -1	5	1	39	36	600
	6	2 (+P)	39	136	600
	4	3 (+Ca)	100	36	600
	5	4 (+Ca, +P)	100	136	600

SSA, Ca and P supplementation ceased at calving.
Postpartum TMR = corn silage + clover-grass silage + barley + rapeseed cake + palm cake + barley straw + shelled soy meal + lactation mineral mix

Results

Plasma Biochemical Analysis

- **Prepartum dietary Ca** did not affect plasma Ca or P of cows in any of experimental subperiods
- **Prepartum dietary P** ↓ plasma Ca of cows before (P=0.012) and during (P<0.0001) SSA supplementation as well as postpartum (days 0-3) (P=0.003), but had no effect on plasma Ca in early lactation (days 4-21) (P=0.713); plasma Ca postpartum (days 0-3) in cows fed diets with and without supplemental P prepartum were 2.25±0.06 and 1.9±0.07 mmol/L, respectively
- **Prepartum dietary P** ↓ plasma P (P=0.015) of cows
- **Prepartum dietary P and Ca** had no effect on plasma Mg; plasma Mg was higher after calving (days 0-21) than before calving (before and during SSA supplementation) but remained above hypomagnesaemia limit (0.8 mmol/L)
- **Prepartum dietary P** ↑ plasma PTH (P<0.0001) during SSA supplementation and the same tendency was observed postpartum (days 0-3)
- **Prepartum dietary Ca** has no effect on plasma PTH during the 1st 3 periods, but ↑ plasma PTH early lactation (days 4-21)

Table 2.9: Summary of Utility Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

Serum $1,25(OH)_2D$ and Al Analysis

- **Prepartum dietary Ca** ↓ serum $1,25(OH)_2D$ (P=0.0038) during SSA supplementation, and mean circulating values in all groups were above those previously reported in aging cows during late pregnancy; no effect was observed on serum $1,25(OH)_2D$ in other periods
- **Prepartum dietary P** did not affect serum $1,25(OH)_2D$ in any of the experimental subperiods
- **Prepartum dietary P and Ca** had no effect on serum Al which was ↑ in all cows during SSA supplementation (P<0.0001); mean serum Al was 85.29±9.06 µg/L during SSA supplementation vs. 13.24 µg/L before SSA supplementation

Urinary Ca/Crea, DPD/Crea and pH Measurements

- **Prepartum dietary P** ↑ urinary Ca/crea ratio (P<0.0001) before as well as during SSA supplementation
- **Prepartum dietary P and Ca** did not affect urinary DPD/crea ratio
- **Prepartum dietary P and Ca** affected urine pH (↓ with dietary P and ↑ with dietary Ca) before but not during SSA supplementation

Performance

- Prepartum dietary P ↑ feed refusals before and during SSA supplementation; ↑ feed refusals during SSA supplementation
- Prepartum dietary P and Ca did not influence mean accumulated milk yield which was comparable to previous lactation

General Health

- 6 cows diagnosed with milk fever; 3/6 cows were hypocalcaemic (plasma Ca <2.0 mmol/L), 5/6 cows displayed plasma P below lower limit of reference interval (<1.81 mmol/L) and no hypomagnesaemia was detected (plasma Mg<0.8 mmol/L); 1/6 cows exhibited high serum PTH and $1,25(OH)_2D$

Prepartum dietary P and Ca did not affect disease incidence

Conclusions – The effect of prepartum SSA supplementation on periparturient Ca homeostasis depends on the level of Ca and P in the dry cow ration; a major part of milk fever prevention on SSA supplementation is likely to arise from a SSA (or Al) induced decrease in dietary P availability; however, serum differences in 1,25(OH)2D during SSA supplementation between cows supplemented and unsupplemented with Ca indicates that part of the SSA induced effect on Ca homeostasis may be the anticipated reduction in Ca availability

Abbreviations: ↑ increased; ↓ decreased; AOP = antioxidant potential; BCS = body condition score; BHB = β-hydroxybutyrate; BW = body weight; CON = control; crea = creatine; DM = dry matter; DMI = dry matter intake; DPD = deoxypyridinoline; EXP = experimental; FCM = fat corrected milk; FFA = free fatty acid; FI = feed intake; HB = hemoglobin; NABE = net acid-base excretion; NEFA = non-esterified fatty acid; NSD = no significant difference; OSi = oxidant status index; PTH = parathyroid hormone; RONS = reactive oxygen and nitrogen species; SBM = soybean meal; SSA = synthetic sodium aluminosilicate; TMR = total mixed ration.

PART 3. §570.235. TARGET ANIMAL AND HUMAN EXPOSURES

3.1 TARGET ANIMAL EXPOSURE

3.1.1 Conditions of Intended Use

As mentioned in Section 2.5.1, synthetic sodium aluminosilicate will be incorporated into dairy feed as part of a formulation on a wheat carrier marketed under the trade name X-Zelit®. Dry cows will be provided with X-Zelit® at levels of 500 g/head/day, delivering up to 400 g synthetic sodium aluminosilicate/cow/day, either as top-dressing or as part of the TMR, for at least 14 days, and no more than 28 days, pre-calving. The duration of feeding reflects the practical time period that dry cows will be separated from the rest of the herd before calving and supplied feed designed to meet their nutritional needs.

3.1.2 Existing Regulatory Status in Feed

3.1.2.1 Existing Regulatory Status of Aluminosilicates as Anti-Caking Agents in Feed in the U.S.

Sodium aluminosilicate and the structurally-related substance sodium calcium aluminosilicate are permitted for use as anti-caking agents in the feed of animals at levels not exceeding 2% in the diet in accordance with 21 CFR §582.2727 and §582.2729 (U.S. FDA, 2020). Examples of aluminosilicates currently marketed for use as anti-caking agents in the feed of livestock including dairy cattle are provided in Appendices 08A to C (CONFIDENTIAL). These examples indicate that there is a history of use of exposure by dairy cattle to sodium and calcium aluminosilicates as anti-caking agents at levels of up to 2% in the diet.

Assuming a dairy cow weighs 650 kg and consumes 25 kg DM in the form of a TMR (Poncheki *et al.*, 2015; EFSA, 2017; University of Minnesota, 2020), the exposure by animals to sodium aluminosilicate from its use as an anti-caking agent at the maximum level of 2% in the diet (TMR as-fed, containing between 45 and 55% DM) will range from 910 to 1,110 g/cow/day. However, it is recognized that during the period of up to 28 days pre-calving, DM intake by dairy cows is reduced to around 12 kg/head/day (University of Minnesota, 2020), and on this basis, a 650 kg cow will consume between 436 and 534 g/head/day of sodium aluminosilicate from its presence as an anti-caking agent in feed at the maximum permitted level of 2% in the diet (TMR as-fed; 45 to 55% DM).

As mentioned above, Protekta wishes to extend the current scope of use of sodium aluminosilicate in feed in the U.S. to include addition to feed as an aid to maintain calcium balance in periparturient cows for at least 14 days, and no more than 28 days, pre-calving. Dairy cows will be provided with up to 500 g X-Zelit®/head/day equivalent to between 375 and 400 g synthetic sodium aluminosilicate/head/day. Thus, the potential exposure of periparturient dairy cows to synthetic sodium aluminosilicate from its presence as an anti-caking agent in the feed is in the same range (436 to 534 g/head/day) as from the proposed use as an aid to maintain calcium balance (375 to 400 g/head/day).

The label of X-Zelit® will include a statement that the product should not be used in conjunction with sodium or hydrated sodium calcium aluminosilicate as an anti-caking agent in feed. Therefore, no additional exposure by dairy cattle to aluminosilicates is anticipated under the intended use of the additive.

3.1.2.2 Existing Regulatory Status of Synthetic Sodium Aluminosilicate for Use in Feed for Periparturient Dairy Cows in the EU and Canada

The formulated feed product, X-Zelit® is currently marketed for use in feed for periparturient cows in the EU by (b)(4) in accordance with Commission Regulation (EU) 2020/354 establishing a list of intended uses of feed intended for particular nutritional purposes (EC, 2020). The regulation includes sodium aluminosilicate as one of a number of dietary strategies to help reduce the risk of milk fever and subclinical hypocalcemia in periparturient dairy cows. Sodium aluminosilicate is permitted for use at levels ranging from 250 to 500 g/day for a maximum duration of 2 weeks from 3 weeks before calving. Full details of the provisions of use of sodium aluminosilicate as laid down in Commission Regulation (EU) 2020/354 are presented in Table 3.1.

Notably, the intended use of synthetic sodium aluminosilicate in the EU differs from that in the U.S. and Canada. In both the U.S. and Canada, synthetic sodium aluminosilicate is intended for use only to maintain normal calcium levels and not to prevent nutritional deficiencies. However, the mode of action and dietary supplementation levels of synthetic sodium aluminosilicate to achieve the desired effects in periparturient cows in the EU, U.S. and Canada are largely the same.

Table 3.1: Summary of the Provisions of Use of Synthetic Sodium Aluminosilicate for Dairy Cows in the EU

Particular nutritional purpose: *Reduction of the risk of milk fever and subclinical hypocalcemia*

Essential Nutritional Characteristic	Labeling Declarations	Recommended Length of Time	Other Provisions
Zeolite (sodium aluminosilicate): 250 to 500 g/day	Sodium aluminosilicate	Dairy cows: From 3 weeks before calving until calving	Indicate in the instructions for proper use: – "The amount of feed shall be restricted to ensure that a daily intakes of 500 g sodium aluminosilicate is not exceeded" – The duration of use shall be restricted to a maximum of 2 weeks – "Stop feeding after calving"

Likewise, X-Zelit® is marketed by Protekta for use in feed for periparturient cows in Canada in accordance with its approval by the CFIA and listing under Entry 8.91 of Schedule IV, Part I of the Feed Regulations (1983) (CFIA, 2020). The entry is as follows:

Synthetic sodium aluminosilicate (or synthetic sodium aluminum silicate or sodium aluminosilicate, synthetic) is a form of sodium aluminosilicate which has been chemically synthesized. It is produced by mixing silicates and aluminates together, which then undergo gelation and crystallization processing. The resulting product is then filtered, washed and spray-dried. It has the chemical formula () and a pore size of 4 Ångström. It consists predominately of aluminum oxide and silicon dioxide and to a less extent sodium oxide. If a facilitating agent or carrier is used, it must be approved for use in livestock feed. It shall be used at the approval rate and the common name or names shall be indicated on the label. This ingredient is approved for use as an aid to maintain calcium balance in periparturient dairy cows. It is to

be fed only to dry dairy cows for a period of up to two weeks pre-calving, in an amount not to exceed 400 grams of synthetic sodium aluminosilicate per head per day.

It shall be labeled with a guarantee for actual g/kg of synthetic sodium aluminosilicate.

It shall also be labeled with the following statements: "This product is only approved for use in diets for dry dairy cows for a period of up to two weeks pre-calving, in an amount not to exceed 400 grams of synthetic sodium aluminosilicate per head per day" and "Caution: Do not use in association with anionic supplements".

Thus, synthetic sodium aluminosilicate in the form of X-Zelit® has an established history of use as an aid to maintain calcium balance in periparturient dairy cattle under equivalent conditions of use to those proposed by Protekta in the U.S.

It is recognized that in both the EU and Canada, synthetic sodium aluminosilicate is permitted for use for 14 days pre-calving. In Protekta and (b) (4) experience in these other markets, in practice X-Zelit® is administered from 14 to 21 days to periparturient cows and discontinued at the point of calving. Based on U.S. commercial feeding practices, where periparturient cows can be separated from the main herd up to 28 days pre-calving, Protekta proposes the use of synthetic sodium aluminosilicate in the form of X-Zelit® for a period of at least 14 days, but no more than 28 days pre-calving, rather than the 14 days specified by the EU and Canada.

Notably, in Canada the use of alternative strategies of dietary intervention to maintain calcium balance in dairy cattle during the transition phase, defined as the period 3 weeks before and after parturition, in parallel is specifically contra-indicated by use of a label statement. These alternative strategies are discussed in Section 2.5.3.

3.2 HUMAN EXPOSURE

3.2.1 Potential Exposure of Dairy Cattle to Synthetic Sodium Aluminosilicate from Other Sources

As previously highlighted, sodium aluminosilicate (synthetic or mined/natural) is currently permitted for use as an anti-caking agent in the feed of animals at levels of up to 2% in the feed. Assuming a dairy cow weighs 650 kg and consumes 25 kg DM in the form of a TMR (Poncheki *et al.*, 2015; EFSA, 2017; University of Minnesota, 2020), the exposure by animals to sodium aluminosilicate from its use as an anti-caking agent at the maximum level of 2% in the diet (TMR as-fed; 45 to 55% DM) will range from 910 to 1,110 g/cow/day. However, it is recognized that during the period 14 to 21 days pre-calving, DM intake by dairy cows is reduced to around 12 kg/head/day (b) (4)), and on this basis, a 650 kg cow will consume between 436 and 534 g/head/day of sodium aluminosilicate from its presence as an anti-caking agent in feed at the maximum permitted level of 2% in the diet (TMR as-fed; 45 to 55% DM).

Protekta wishes to extend the current scope of use of sodium aluminosilicate in feed in the U.S. to include use in the diets of periparturient cows for a period of at least 14 days, and no more than 28 days, pre-calving in order to help maintain calcium balance. Dairy cows will be provided with up to 500 g X-Zelit®/head/day equivalent to between 375 and 400 g synthetic sodium aluminosilicate/head/day. Thus, the potential exposure of periparturient dairy cows to synthetic sodium aluminosilicate from its

presence as an anti-caking agent in the feed is in the same range (436 to 534 g/head/day) as from the proposed use (375 to 400 g/head/day).

The TMR will not be simultaneously supplemented with sodium or hydrated sodium calcium aluminosilicate as an anti-caking agent and X-Zelit® as an aid to maintain calcium balance, and therefore, no additional exposure by dairy cattle to aluminosilicates is anticipated under intended use of the additive.

Thus, the potential for deposition of synthetic sodium aluminosilicate or its components in the milk of dairy cows under the intended use as an aid to maintain calcium balance in periparturient dairy cows will not exceed that from the existing potential use as an anti-caking agent.

PART 4. §570.240. SELF-LIMITING LEVELS OF USE

The use of synthetic sodium aluminosilicate will be self-limiting on the basis that there are detrimental effects on the nutritional status and health of dry cows on increasing the levels of dietary supplementation beyond that required to achieve the desired effect.

PART 5. §570.245. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Not applicable.

PART 6. §570.250. NARRATIVE

6.1. INFORMATION TO ESTABLISH SAFETY FOR THE TARGET ANIMAL

6.1.1 Introduction

Protekta intends to market synthetic sodium aluminosilicate as an aid to maintain calcium balance in periparturient (dry) dairy cows. The ingredient is a hydrated form of sodium aluminosilicate with the chemical formula $Na_{12}Al_{12}Si_{12}O_{48}.27H_2O$ which is produced by reacting aluminum sulfate with sodium silicate. It will be incorporated into dairy feed as part of a formulation comprising 75 to 80% synthetic sodium aluminosilicate, 17 to 20% wheat (carrier) and 1 to 3% rapeseed oil (anti-dust agent). The formulation will be marketed under the trade name X-Zelit® and is intended for use in the feed of periparturient dairy cows at levels of 500 g/head/day, delivering up to 400 g synthetic sodium aluminosilicate/cow/day, either as top-dressing or as part of the TMR for a period of at least 14 days, and no more than 28 days, pre-calving. The use of sodium aluminosilicate in the form of X-Zelit® will be discontinued at calving.

Synthetic sodium aluminosilicate is intended to lower the availability of calcium to dairy cows from the diet during the prepartum phase. Lowering the availability of the mineral in the feed leads to activation of homeostatic mechanisms in the cow pre-calving, which in turn help maintain calcium balance over the final stages of pre-natal growth and colostrum production.

The safety of synthetic sodium aluminosilicate for the intended use in periparturient cows is primarily based on the following: (a) the known metabolic fate in ruminants (see Section 6.1.2); and (b) a number of published studies in which synthetic sodium aluminosilicate was used to supplement the diet of periparturient cows for periods of 14 to 40 days before the expected calving date (see Section 6.1.3). Together these data form the pivotal body of evidence to support the safe use of synthetic sodium aluminosilicate for use in periparturient cows.

It is also recognized that synthetic sodium aluminosilicate has an established history of use as an anti-caking agent in the feed of all animals in the U.S., EU and Canada. Furthermore, synthetic sodium aluminosilicate is currently permitted for use in periparturient cows in the EU and Canada under comparable conditions of use to those presented herein. The existing history of use of synthetic sodium aluminosilicate in feed provides corroborative evidence of safety and is supported by a scientific opinion published by EFSA (see Section 6.1.4).

Additionally, synthetic sodium aluminosilicate has a history of use as a food additive and has been the subject of reviews by authoritative bodies including EFSA and the Joint Expert Food and Agricultural Organization (FAO)/World Health Organization (WHO) Committee on Food Additives (JECFA). As such a limited body of toxicological information is available to support the safety of synthetic sodium aluminosilicate which provides corroborative evidence of the safety for periparturient cows (see Section 6.1.5).

6.1.2 Absorption, Distribution, Metabolism and Excretion (ADME) of Synthetic Sodium Aluminosilicate in Dairy Cattle

Cook *et al.* (1982) studied the hydrolysis and degradation of synthetic sodium aluminosilicate of the chemical formula $Na_{12}Al_{12}Si_{12}O_{48}.27H_2O$ in various aqueous solutions ranging from pH 3 to 9. Synthetic sodium aluminosilicate was found to be partially degraded to release silicic acid, amorphous aluminum silicates and aluminum. Similar behavior is expected in the digestive tract of all animals, including dairy cattle, and is supported by studies in dairy cattle as well as in laboratory animals as summarized below.

6.1.2.1 Study in Dairy Cows to Evaluate Changes in the Rumino-Intestinal Tract

Grabherr *et al.* (2009b) conducted a study in dairy cows to evaluate the effect of synthetic sodium aluminosilicate on rumen fermentation and mineral metabolism. In the study, 8 double fistulated (rumen and proximal duodenum) cows were fed maize silage, grass silage and concentrate supplemented with 0, 10 or 20 g/kg DM synthetic sodium aluminosilicate (manufactured (b) (4) (b) (4) for a period of 3 weeks (days 1 to 21). Daily feed amounts were adjusted to reflect the performance of the cow and varied between 3.9 and 15.5 kg/day. Rumen fluid, duodenum chyme and feces were sampled for analysis in order to evaluate nutrient digestibility. Short chain fatty acid (SCFA) concentrations were analyzed in rumen fluid, and soluble aluminum, calcium, magnesium and phosphorus levels in rumen fluid as well as duodenal ingesta. Blood samples were taken to analyze the concentration of inorganic phosphate and aluminum at day 1 (before supplementation) and day 7 (during 21-day supplementation period).

Synthetic sodium aluminosilicate supplementation of cows was observed to significantly reduce ruminal DM digestibility and fermentation of organic matter ($P<0.05$). The apparent ruminal DM digestibility was 45.4% in cows without synthetic sodium aluminosilicate supplementation, and 41.3 and 39.6% for cows provided with 10 or 20 g sodium aluminosilicate/kg DM, respectively.

Relative molar proportions of acetate were reported to increase in the rumen on synthetic sodium aluminosilicate supplementation (10 or 20 g/kg DM), and propionate and valerate levels to decrease ($P<0.05$). Overall, there was no effect of synthetic sodium aluminosilicate supplementation on total SCFAs levels or ruminal pH.

Cows that received 20 g/kg DM displayed the lowest concentrations of soluble calcium, magnesium, and phosphorus in the rumen fluid, but these concentrations were not significantly different compared to controls. The fecal excretion, as well as the digestibility of total calcium and magnesium, was not affected by the addition of synthetic sodium aluminosilicate to the diet. Phosphorus concentrations in the rumen fluid correlated negatively with synthetic sodium aluminosilicate supplementation ($r2 = 0.75$; $P=0.0003$). Further, the fecal excretion of phosphorus increased significantly in cows with the highest sodium aluminosilicate dosage (equating to 36.9 g phosphorus/day) compared to the control group (equating to 29.9 g phosphorus/day). This in turn caused a significant ($P<0.05$) decrease of serum inorganic phosphate in cows who received the highest dose of sodium aluminosilicate (20 g/kg DM). The serum inorganic phosphate concentration decreased significantly after three days of synthetic sodium aluminosilicate supplementation from 2.05 mmol/L on day 1 (before the beginning of synthetic sodium aluminosilicate supplementation), to 1.51 mmol/L on day 3, and reaching nadir of 1.16 mmol/L by day 6, which was below the lower limit of the reference range (1.55 to 2.29 mmol/L; Fürll & Moritz,

2005). Cows supplemented with 10 g synthetic sodium aluminosilicate/kg DM also exhibited a statistically significant decrease in serum inorganic phosphate from 2.03 down to 1.40 mmol/L on day 5. However, on day 7 the serum inorganic phosphate concentration was 1.64 mmol/L, which was within the reference range (1.55 to 2.29 mmol/L; Fürll & Moritz, 2005).

The concentration of aluminum in rumen fluid was increased significantly in groups that received 10 or 20 g/kg DM of synthetic sodium aluminosilicate ($P<0.05$) relative to the control. The mean ruminal aluminum concentration in the control cows was 6.3 µmol/L, whereas aluminum concentrations reached 14.3 µmol/L in cows receiving 10 g synthetic sodium aluminosilicate/kg DM and 13.83 µmol/L in animals that received 20 g/kg DM. Soluble aluminum was increased in the duodenum in groups receiving 10 or 20 g of synthetic sodium aluminosilicate ($P=0.011$), with a mean flow of 0.3 g/day in the control compared to cows that received 10 g/kg DM having a flow of 7.3 g/day and in animals who received 20 g/kg DM a flow of 11.5 g/day. Serum aluminum concentrations increased in all animals on day 7, with the highest increase observed in animals that received 20 g sodium aluminosilicate/kg DM (129 µg/L), compared to 85 µg/L in controls; however, this increase was not statistically significant. The increase in aluminum in control animals was unexpected and the authors suggested that the chromium oxide marker used in the digestibility study may have partly contributed to the increase in aluminum as it contained 0.09 g aluminum in the 100 g of marker used in the study.

The authors postulated that the reduced DM and organic matter was in part due to the indigestibility of synthetic sodium aluminosilicate. Furthermore, phosphorus is an important factor for ruminal microbial growth, and the reduced availability of this mineral in the rumen of animals supplemented with synthetic sodium aluminosilicate may have depressed microbial activity. Consistent with findings in vivo (see Section 6.1.3), synthetic sodium aluminosilicate is partially broken down through the digestive tract causing release of aluminum. Dietary phosphate can bind with aluminum released from synthetic sodium aluminosilicate to form non-absorbable compounds, most likely aluminum phosphate. The results of this study support the degradation of synthetic sodium aluminosilicate in the rumen or abomasum rather than further along the digestive tract. There was no correlation between phosphorus flow at the duodenum and sodium aluminosilicate supplementation, providing further evidence that binding of phosphorus by aluminum takes place in the rumen. This conflicts with studies by Thilsing *et al.* (2006a; 2007) who in both *in vivo* and *in vitro* work suggest that degradation takes place after synthetic sodium aluminosilicate has been exposed to acidic conditions. The authors did not propose an explanation for the differences observed in these *in vitro* and *in vivo* studies.

6.1.2.2 Study in Periparturient Dairy Cows using Synthetic Sodium Aluminosilicate and Evaluating Aluminum Uptake

Of the studies summarized in Section 2.5.5 in periparturient cows to evaluate the utility of synthetic sodium aluminosilicate, only one included analysis of serum aluminum levels in the animals as briefly outlined below. Further details of the study are also presented in Table 2.9.

In a study conducted by Thilsing *et al.* (2007) and summarized in Table 2.9, twenty-one pregnant non-lactating cows who had completed ≥ 2 lactations were assigned into one of 4 groups according to parity and expected date of calving, to achieve a balance of these characteristics. Four weeks prior to parturition, cows in group 1 remained unsupplemented, cows in group 2 received additional phosphorus (417 g monosodium phosphate, equating to an additional 100 g of phosphorus/head/day), cows in

group 3 received additional calcium (171 g calcium carbonate, equating to an additional 100 g of calcium/head/day), and cows in group 4 received additional calcium and phosphorus. During the last 2 weeks of pregnancy each cow received 600 g of synthetic sodium aluminosilicate per day. Supplementation with synthetic sodium aluminosilicate stopped at calving. Three weekly blood and urine samples were taken from 3 weeks before calving until 3 weeks after calving. Additional blood samples were drawn on the day of calving and 24 and 48 hours after calving. Plasma samples were analyzed for calcium, inorganic phosphate, magnesium and PTH and serum for 1,25-dihydroxyvitamin D. Serum aluminum concentrations also were analyzed from one blood sample taken before synthetic sodium aluminosilicate supplementation started and another on average 4.8 (±0.3) days after supplementation.

The mean serum aluminum concentrations of all synthetic sodium aluminosilicate-supplemented cows were increased significantly during supplementation compared to before supplementation ($P<0.0001$). Serum aluminum concentrations were not significantly affected by prepartum calcium and/or phosphorus level, and the mean concentration of serum aluminum was 85.29 ± 9.06 µg/L during supplementation vs. 13.24 ± 2.17 µg/L before supplementation (corresponding to 0.0032 ± 0.0003 and 0.0005 ± 0.00008 mmol/L, respectively). Comparison measurements after supplementation of synthetic sodium aluminosilicate stopped were not conducted.

Grabherr *et al.* (2009b) postulated in the *in vitro* study summarized above (see Section 6.1.2.1), that phosphorus binds with aluminum released from synthetic sodium aluminosilicate in the rumen of the animal to form non-absorbable compounds which subsequently reduce dietary phosphate availability. Although this may be the case, the presence of aluminum in the serum of the cows during synthetic sodium aluminosilicate supplementation indicates that at least some of the mineral is absorbed from the digestive tract of the animal.

6.1.2.3 Studies in Calves using Synthetic Sodium Aluminosilicate and Evaluating Aluminum Uptake

Although less relevant compared to studies in pregnant cows, Turner *et al.* (2007a; 2007b), evaluated the effects of synthetic sodium aluminosilicate on mineral metabolism and mineral composition in calves. Three day old Holstein bulls (10/group) received in the diet either 0 (control) or 0.05% body weight synthetic sodium aluminosilicate (sodium zeolite A) dissolved in milk-replacer, twice daily, for 60 days. Body weight was recorded weekly to allow for the adjustment of dosage. Blood samples were taken on days 0, 30, and 60, and plasma samples were analyzed for mineral analysis (aluminum, calcium, copper, iron, magnesium, phosphorus, silicon, and zinc). On day 30, urine and feces were collected every 6 hours for 3 days and analyzed for mineral metabolism. Tissue organs (adrenal, aorta, heart, kidney, liver, lung, muscle, pancreas, spleen, tendon and trachea) were harvested on day 60 and analyzed for mineral concentrations.

There were no statistically significant differences between control and synthetic sodium aluminosilicate-treated animals in terms of body weight. At day 30, aluminum concentrations in feces were significantly increased ($P<0.0001$; 34 mg/g in treated calves vs. 2.5 mg/g in control calves). There was no significant difference in urinary concentrations of aluminum, calcium, copper, iron, magnesium, phosphorus, silicon, or zinc compared to controls. Plasma analysis revealed an increase in silicon concentration in synthetic sodium aluminosilicate supplemented calves relative to controls, and phosphorus levels tended to be lower on day 30 but not significantly. No other plasma mineral concentrations were

affected by synthetic sodium aluminosilicate supplementation over the 60 day study period (aluminum, calcium, copper, iron, magnesium and zinc). Calves fed diets containing synthetic sodium aluminosilicate were observed to absorb larger amounts (~15%) of aluminum from the feed than control calves (P=0.001). Although no increase in plasma aluminum concentrations was observed, mineral analysis of tissue organs revealed the aluminum was significantly increased in all tissue organs vs. control (P<0.05). The authors suggested that this was likely a factor of aluminum being rapidly cleared from the blood and deposited into the tissues.

Sodium aluminosilicate supplementation increased cortical bone (P=0.0002) and articular cartilage (P=0.05) aluminum content but did not affect architecture or mechanical properties of bone in calves (Turner *et al.*, 2007a). Phosphorus absorption tended (P=0.09) to be decreased in calves receiving synthetic sodium aluminosilicate and consequently slightly more phosphorus was excreted in the feces of treated calves, in agreement with other studies (Thilsing *et al.*, 2006a; 2007). As noted by the study authors, aluminum may bind to phosphorus reducing its availability from the diet and reflected in the tendency to lower plasma phosphate concentrations on day 30. Aluminum is also reported to compete with iron for absorption but the retention and absorption of iron in the current study appeared to be unaffected by synthetic sodium aluminosilicate supplementation. Silicon was not significantly retained but an increase in plasma concentration was observed on day 30 in animals who received sodium aluminosilicate *vs*. control (P<0.1); however, by day 60 this had declined to concentrations similar to control. Although there were significant differences in silicon intake between the control and sodium aluminosilicate-supplemented calves, there was no significant difference in absorption. The authors postulated that the increase in plasma concentration could be due to the slight decrease in silicon excretions observed in treated calves (total excretion in urine and feces of control animals was 786 vs 722 mg). This elevation in plasma silicon could be deposited and explain why an increase in silicon concentrations was observed in the aorta, spleen, lung, muscle, and kidney in comparison to control (P<0.05).

6.1.2.4 Studies in Cattle using Related Compounds and Evaluating Aluminum Uptake

Notably, studies conducted using sedimentary (natural) sodium aluminosilicates and different aluminum salts in cattle report similar findings to those summarized above. In a study using clinoptilolite (which differs in structure and cation exchange capacity to synthetic sodium aluminosilicate), Karatzia *et al.* (2010) added 0 or 0.2 kg/day of clinoptilolite to the rations of non-pregnant dairy cows for 12 weeks and did not measure any significant differences in serum or ruminal concentrations of aluminum when compared to controls. There were no corresponding detectable changes in serum or ruminal phosphorus concentration. In a study conducted by Valdivia *et al.* (1978) beef steers received up to 1,200 mg/kg of aluminum chloride in the diet for 84 days, the authors reported a non-significant increase in aluminum in liver, kidney, muscle, and brain, with no differences in iron, manganese, phosphorus, calcium and magnesium concentrations in tissues in relation to increasing levels of aluminum in the ration. Contrary to the studies in periparturient cows using synthetic sodium aluminosilicate, there were no significant changes in plasma concentrations of calcium, phosphorus, or magnesium.

6.1.2.5 ADME of Synthetic Sodium Aluminosilicate in Laboratory Animals

The metabolic fate of synthetic sodium aluminosilicate has been evaluated in both rat and dog studies. Although the studies took place in single stomached species similarities in behavior are expected after acid digestion in either ruminant or monogastric species and therefore, the data were considered relevant to the safety assessment of synthetic sodium aluminosilicate for periparturient dairy cows. As mentioned above, synthetic sodium aluminosilicate is expected to be partially degraded in the digestive tract with the release of silicic acid, amorphous aluminum silicates and aluminum (Cook *et al.*, 1982) in all animals. In all of the studies outlined below, a single oral dose of synthetic sodium aluminosilicate resulted in absorption of a small but quantifiable level of silicon but negligible amounts of aluminum.

Gloxhuber *et al.* (1983) performed a preliminary absorption study in a group of 5 male Wistar rats that received 1,000 mg synthetic sodium aluminosilicate/kg body weight via oral administration (presumed to be gavage). Urine and feces were monitored for 24 hours post-dosing. The lungs, liver, spleen, heart, blood, kidney, small intestine, stomach, and large intestine were analyzed for silicon and aluminum content, the results are as summarized in Table 6.1. Less than 1% of the administered dose of silicon was retained by the kidneys or eliminated in the urine, with 82.3% recovered in the feces (Gloxhuber *et al.*, 1983). The recovery of aluminum in the experiment was poor as only 42.5% of the total dose of aluminum was recovered. Of the recoverable amount of aluminum, 34.2% was collected in the feces followed by 5.3% in the small intestine and 3% in the stomach. The lack of recovery of the remaining ~57% was addressed by the authors as being due to poor absorption of aluminum from synthetic sodium aluminosilicate. No further details were available in the publication regarding the aluminum recovery.

Table 6.1: Recovery of Administered Dose of Synthetic Sodium Aluminosilicate

Organ	Aluminum (%)	Silicon Dioxide (SiO_2) (%)
Lungs	-	0.02
Heart	-	0.05
Spleen	0.006	-
Stomach	3	9.89
Small Intestine	5.3	5.25
Large Intestine	-	1.33
Liver	-	0.04
Kidney	0.0018	0.22
Blood	-	0.02 (in 10 mL)
Urine	0.031	0.65
Feces	34.2	82.3
Total recovery	42.5	99.8

The rate and amounts of urinary excretion of silicon and aluminum following oral administration of food grade magnesium trisilicate, sodium silicate, or synthetic sodium aluminosilicate was determined in groups of adult male Sprague-Dawley rats (4/group) by Benke & Osborne (1979). Rats were administered doses of 0 (control), 40, 200, or 1,000 mg/kg body weight via a single oral administration. Urine was collected over 24-hour periods up to 96 hours after dosing and was analyzed for silicon and aluminum. Urinary silicon excretion increased rapidly after dosing and peak excretion rates occurred within 24 hours in all test groups. Synthetic sodium aluminosilicate exhibited the most rapid urinary

excretion rate (half-life of 6 to 8 hours), followed by magnesium trisilicate (half-life of 16 to 20 hours), sodium silicate (half-life of 24 hours), and sodium aluminosilicate (half-life of 38 hours). The half-lives were independent of dose within 40 to 1,000 mg/kg body weight. Benke & Osborne (1979) concluded that differences in half-life were due to the production of absorbable silicon in the digestive tract, consistent with synthetic sodium aluminosilicate being broken down by acid hydrolysis. The urine of rats dosed with synthetic sodium aluminosilicate did not display any detectable increase in aluminum. The detection limit of the analytical method would have permitted the detection of 0.01 to 0.2% of the dose and was considered valid for the study by the investigators.

Yokoi & Enomoto (1979) studied the excretion of silicic acid in rats orally treated with different preparations of sodium aluminum silicate gels[4] with known distributions of molecular forms of silicic acid. Groups of rats (4/group) were administered silicate preparations suspended in 1 mL of 0.5% carboxymethyl cellulose solution and given at doses of 20, 50, 100, 250, or 500 mg of SiO_2/ kg body weight/day by means of a stomach tube. Urine was collected for a 24-hour period before and after silicate administration. The volume and pH of urine were measured, and silicic acid content was determined colorimetrically as a measure of absorption. The authors concluded from the results that in the digestive tract, the various silicic acids formed upon acid hydrolysis are absorbed through the lipoid membrane pore route. This mechanism was noted by the authors to be common in the permeating of hydrophilic compounds although molecular weight will also influence absorption. A possible mechanism was proposed involving formation of renal and urinary calculi, where silicic acids are absorbed from the GI tract, largely by physical or diffusion processes. The silicic acids then concentrate in the urine to exceed the saturated concentration and undergo polymerization. The polymer formed is converted into insoluble precipitates via colloidal silicic acids which can lead to the formation of stones when in the presence of promoters such as urinary proteins.

Cefali *et al.* (1996) investigated the pharmacokinetics and bioavailability of silicon and aluminum from synthetic sodium aluminosilicate administered as either a capsule, an oral suspension or an oral solution, relative to an intravenous (*i.v.*) bolus infusion administered over a 1- to 1.5-minute period. Twelve (12) female beagle dogs, 6 to 8 months of age, weighing between 7.3 and 8.7 kg were used in the study. The dogs were individually housed in an environmentally controlled room (72 ± 1.4°F, humidity 42 ± 7.9%) with a 12-hour light/dark cycle. Feed was available for 2 to 3 hours per day and was available approximately 4 hours after dosing of synthetic sodium aluminosilicate. A 7-day control phase was utilized before dosing started. Each animal was dosed with either, an oral capsule (30 mg/kg body weight), oral solution (30 mg/kg body weight), oral suspension (30 mg/kg body weight), or an *i.v.* solution (20 mg/kg body weight) in a randomized five-way crossover design. The oral solution and suspension were administered by gavage, followed by an oral gavage of deionized water to ensure that all the test article was delivered into the stomach. The *i.v.* dose was administered into a vein in the leg. Blood was obtained at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 36 hours after dosing.

Plasma samples, drawn between 0 and 36 hours, were analyzed for silicon and aluminum concentrations by graphite furnace atomic absorption. Silicon concentrations in the plasma were observed to increase substantially after 4 hours during the control period, which corresponded with the start of feeding and an apparent uptake of the mineral from the diet. Values for silicon were corrected against the values

[4] Produced synthetically but exact chemical formula not reported.

collected during the control period to accurately establish the amount of silicon absorbed from synthetic sodium aluminosilicate. The same procedures had been intended for aluminum; however, uncorrected data was used due to the low aluminum values collected during treatment and the irregular pattern collected in the control period. The data for the dose corrected silicon absorption are described in Table 6.2.

Table 6.2: Silicon Pharmacokinetic and Bioavailability Estimates for Synthetic Sodium Aluminosilicate from Oral Capsules, Oral Solution and Oral Suspension Using Baseline Corrected Data

Endpoint	Oral Capsule	Oral Solution	Oral Suspension	Control
Dose (mg/kg)	4.62	4.198	3.96	0
AUC∞ (mg/hour/L)	81.18 ± 3.9 **	11.0 ± 3.31 **	8.21 ± 3.30 **	8.89 ± 1.64
C_{max} (mg/L)	1.28 ± 1.13**	2.87 ± 0.37 **	1.44 ± 0.99 **	0.44 ± 0.12
T_{max} (hours)	3.67 ± 4.00 **	0.83 ± 0.25 **	5.88 ± 7.44	11.3 ± 7.97
% absorption (relative to *i.v.*)	2.33	3.44	2.73	N/A

Abbreviations: AUC∞ = Area under the curve infinity; C_{max} = maximum concentration; T_{max} = time of maximum concentration.
Significance is denoted by **(P<0.001).

The mean elimination half-life and clearance of silicon from the dose were 17.5 hours and 0.221 ± 0.0192 mL/min/kg. The dose-corrected percentages for silicon absorption for the oral capsule, oral solution, and oral suspension relative to the *i.v.* dose were 2.33, 3.44, and 2.73%, respectively. Both the C_{max} and T_{max} data from dogs receiving suspension and capsules were highly variable making it difficult to draw definitive conclusions from the data. Five dogs receiving capsules exhibited C_{max} plasma levels which were nearly an order of magnitude lower than the values obtained for the other seven dogs. Two of these five had a T_{max} of 12 hours, in contrast to T_{max} values ranging from 1 to 4 hours for the other 10 dogs. Similarly, four of the 12 dogs receiving suspension manifested low C_{max} values. All four demonstrating low C_{max} values, had T_{max} values greater than or equal to 12 hours. All dogs receiving the oral solution had similar C_{max} values ranging from 2.33 to 3.66 mg/L, and all had T_{max} values ranging from 0.5 to 1.0 hour. The data supports that the absorption of silicon following ingestion of synthetic sodium aluminosilicate was low, with all oral forms resulting in less than 3.5% absorption. The absorption from the oral solution was substantially faster than the suspension and capsule forms.

While the plasma aluminum concentrations after oral doses were not substantially different compared to control concentrations, plasma concentrations of aluminum after the *i.v.* dose were high. The mean elimination half-life and clearance of aluminum from the *i.v.* dose was 91.2 hours and 0.0497 ± 0.0082 ml/min/kg body weight. Due to the high plasma aluminum concentrations following the *i.v.* synthetic sodium aluminosilicate dose, and the apparent long half-life of aluminum, a distinct sequence effect was observed which affected the oral doses of synthetic sodium aluminosilicate and made comparison difficult. The group mean AUC values for the non-confounded oral dosages are compared with group control means and the results summarized in Table 6.3.

Table 6.3: Aluminum Bioavailability Estimates for Synthetic Sodium Aluminosilicate from Oral Capsules, Oral Solution and Oral Suspension Using Baseline Corrected Data				
Endpoint	**Oral Capsule**	**Oral Solution**	**Oral Suspension**	***i.v.***
AUC (µg per hour per L)	222	312*	262	648969**
% absorption (relative to *i.v.*)	0.023	0.032	0.028	

Abbreviations: AUC = Area under the curve; *i.v.* = intravenous.
Significance is denoted by * (P<0.05) and **(P<0.001).

The absolute extent of aluminum absorption from synthetic sodium aluminosilicate observed after doses of the oral capsule, oral solution and oral suspension were 0.023, 0.032 and 0.028% of the *i.v.* dose, respectively. The plasma aluminum AUC values from the oral capsule (*P*=0.6328) and suspension (*P*=0.1331) were not statistically different from those during the control period. However, the aluminum AUC of the oral solution was statistically greater than the AUC of the corresponding control period (*P*=0.0154). When considering both sets of data from silicon and aluminum a single-dose oral administration of synthetic sodium aluminosilicate results in low but measurable absorption of silicon with negligible absorption of aluminum.

In a follow up study, the oral bioavailability of silicon and aluminum in synthetic sodium aluminosilicate was studied in dogs by Cefali *et al.* (1995). Groups of female Beagle dogs (12/group) were administered either a single 30 mg/kg body weight dose of synthetic sodium aluminosilicate, 16 mg/kg body weight sodium aluminosilicate, or 20 mg/kg body weight magnesium trisilicate via capsule. Aluminum hydroxide was administered by gavage at 675 mg/animal (5 mL total), the gavage was rinsed with deionized water to ensure all the test article was received. Blood was sampled via the jugular vein at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 hours after dosing. The plasma samples were assayed for silicon and aluminum.

The mean bioavailability parameters for silicon are summarized in Table 6.4. The plasma silicon concentrations from the aluminum hydroxide treatment group served as the control (i.e., background dietary absorption). The absorption of silicon was found to be elevated (*P*<0.5) relative to baseline with no significant differences in C_{max} and T_{max} among the compounds or sampling times, respectively. The findings of the study suggest that a small but measureable amount of silicon was absorbed from sodium aluminosilicate, synthetic sodium aluminosilicate or aluminum hydroxide.

Table 6.4: Silicon Bioavailability Estimates for Synthetic Sodium Aluminosilicate, Sodium Aluminosilicate, Magnesium Trisilicate and Aluminum Hydroxide				
Endpoint	**Synthetic Sodium Aluminosilicate**	**Sodium Aluminosilicate**	**Magnesium Trisilicate**	**Aluminum Hydroxide**
Silicon Dose (mg/kg bw)	4.66	4.75	4.63	0.0
AUC∞ (mg.hour/L)	9.5 ± 4.5*	7.7 ± 1.6	8.8 ± 3.0	6.1 ± 1.9
C_{max} (mg/L)	1.07 ± 1.06	0.67 ± 0.27	0.75 ± 0.31	0.44 ± 0.17
T_{max} (hours)	7.9 ± 6.4	5.8 ± 4.6	6.9 ± 6.3	8.5 ± 3.4

Abbreviations: AUC∞ = Area under the curve infinity; C_{max} = maximum concentration; T_{max} = time of maximum concentration.
Significance is denoted by *(P<0.05).

The mean bioavailability parameters of aluminum are described in Table 6.5. The plasma aluminum concentrations during magnesium trisilicate treatment served as the control treatment (i.e., background

dietary absorption). The findings of the study suggest that absorption of aluminum from sodium aluminosilicate, synthetic sodium aluminosilicate or aluminum hydroxide was not greater than that absorbed from dietary sources. There was no statistically significant difference in the C_{max} and T_{max} values among compounds.

Table 6.5: Aluminum Bioavailability Estimates for Synthetic Sodium Aluminosilicate, Sodium Aluminosilicate, Magnesium Trisilicate and Aluminum Hydroxide

Endpoint	Synthetic sodium aluminosilicate	Sodium Aluminosilicate	Magnesium Trisilicate	Aluminum Hydroxide
Aluminum Dose (mg/kg)	3.36	0.9	0	28
AUC (mg.hour/L)	342 ± 111	338 ± 167	315 ± 69	355 ± 150
C_{max} (mg/L)	29 ± 9	27 ± 14	24 ± 5	29 ± 11
T_{max} (hours)	3.5 ± 4.1	4.2 ± 4.3	5.7 ± 7.3	5.0 ± 4.7

Abbreviations: AUC = Area under the curve; C_{max} = maximum concentration; T_{max}= time of maximum concentration.

Overall, the authors of the studies in rats and dogs above concluded that a single dose oral administration of synthetic sodium aluminosilicate was associated with a small but appreciable absorption of silicon but little absorption of aluminum.

6.1.2.6 Summary of ADME of Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows

Synthetic sodium aluminosilicate is expected to be largely excreted intact in the feces of the periparturient cows under the conditions of intended use of 400 g/head/day for at least 14 days, and no more than 28 days, pre-calving. A small portion of synthetic sodium aluminosilicate is expected to partially degrade in the digestive tract of dairy cattle with the release of silicic acid, amorphous aluminum silicates and aluminum (Cook *et al.*, 1982). The available studies in dairy cattle indicate that following partial hydrolysis in the digestive tract under the low pH conditions of the abomasum (Thilsing *et al.*, 2006a; 2007) or within the rumen (Grabherr *et al.*, 2009a), aluminum is released and at least some is absorbed resulting in a small increase in the serum concentration of the mineral (Thilsing *et al.*, 2007 and Grabherr *et al.*, 2009a). None of the studies evaluated the uptake of silicon by periparturient cows but availability of dietary phosphorus was reported to be impacted by synthetic sodium aluminosilicate supplementation, likely the result of the formation of non-absorbable compounds on combination with aluminum.

After oral ingestion, synthetic sodium aluminosilicate was mainly excreted in the feces in rats and dogs. A small fraction of the additive appeared to be hydrolyzed in the digestive tract of the animals and silicon (presumably as silicic acid) was absorbed. The absorbed silicon was ultimately excreted via the urine within 24 hours of administration. By comparison, negligible absorption of aluminum was observed. The metabolic fate is not expected to differ significantly between species and these results can be extrapolated to dairy cattle.

6.1.3 Studies in Dairy Cattle to Evaluate the Safety of Sodium Aluminosilicate

As mentioned in Section 2.5.5, 10 studies were identified in the published literature in which synthetic sodium aluminosilicate was fed to periparturient dairy cows in order to evaluate the utility of the additive to bind dietary calcium and influence plasma calcium concentrations early postpartum (Thilsing-Hansen & Jørgensen, 2001; Thilsing-Hansen *et al.*, 2002b, 2003; Thilsing *et al.*, 2007; Grabherr *et al.*,

2008; Grabherr *et al.,* 2009a; Pallesen *et al.*, 2008; Kerwin *et al.*, 2019; Khachlouf *et al.,* 2019; Crookenden *et al.*, 2020). In addition to evaluating the ability of synthetic sodium aluminosilicate to influence serum calcium levels in periparturient cows, the studies identified also considered endpoints relevant to safety including the effect on other mineral levels (phosphorus, magnesium and aluminum) and performance (DMI, incidence of disease and milk production). The findings of the studies with respect to the safety of synthetic sodium aluminosilicate are evaluated below, organized by endpoint (mineral levels, feed intake, milk production and incidence of disease). To aid the evaluation, a summary of the pertinent findings of each study with respect to safety is provided in Table 6.6.

Table 6.6: Summary of Safety-Related Endpoints using Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows				
Reference	**SSA Use Level**	**Duration SSA Pre-Calving**	**Specific Design Considerations**	**Key Safety-Related Findings**
Kerwin *et al.*, 2019	400 g/head/day	21 days	X-Zelit®	↓ plasma P prepartum and 2 days post-calving ↓ plasma Mg immediately postpartum NSD in oxidative stress markers (blood) Tendency to ↓ DMI prepartum NSD milk yield, milk component, colostrum measurements
Crookenden *et al.*, 2020	400 g/head/day	14 days	MgO pre-calving	↓ plasma P prepartum ↓ plasma Mg on day 0 NSD in neutrophil respiratory burst assay
Pallesen *et al.*, 2008	500 g/head/day	14 days	Mg and P supplements	NSD in plasma Mg NSD in DMI No incidences of hypomagnesaemia
Grabherr *et al.*, 2008	Approx. 558 g/head/day	14-21 days		↓ serum P prepartum (after 1 week of SSA supplementation) ↓ serum Mg on day 0 and 1 postpartum NSD in serum Cu, Zn, Fe and Mn ↓ DMI prepartum NSD in DMI or milk yield postpartum
Grabherr *et al.*, 2009a	131-310 g/head/day	14 days	Primiparous and multiparous cows	↓ plasma P in older cows but remained around lower limit of reference range ↓ plasma Mg in older cows but remained within reference range ↓ urinary P in cows fed 213 or 310 g SSA/head/day ↓ urinary Mg in cows fed 213 or 310 g SSA/head/day ↓ fractional urinary K ↓ DMI in cows fed 310 g SSA/head/day NSD in BCS
Khachlouf *et al.*, 2019	200 g/head/day	40 days		NSD in plasma P and remained within reference values NSD in plasma Mg and remained within reference values ↑ milk production but NSD in Ca, P or Mg milk contents
Thilsing-Hansen & Jørgensen, 2001	1 kg/head/day	28 days		No adverse events reported
Thilsing *et al.*, 2002b	700 g/head/day	21 days		↓ plasma P prepartum; within reference values within 1 week postpartum ↓ plasma Mg day 0 ↓ DMI 2 weeks prepartum

Table 6.6: Summary of Safety-Related Endpoints using Studies on Synthetic Sodium Aluminosilicate in Periparturient Dairy Cows				
Reference	**SSA Use Level**	**Duration SSA Pre-Calving**	**Specific Design Considerations**	**Key Safety-Related Findings**
				NSD in milk yield, milk fat or milk protein
Thilsing-Hansen *et al.*, 2003	500-1 kg/head/day	14-28 days	Varying SSA to Ca ratio Mineral mix prepartum	↓ plasma P prepartum (3 weeks) and day 0 ↓ plasma Mg on day 0 (remained within reference limits) and returned to normal within 1 week
Thilsing *et al.*, 2007	600 g/head/day	14 days	MgO, and NaH_2PO_4, and/or $CaCO_3$ pre-calving	Dietary Ca: no effect on plasma Ca or P Dietary P: ↓ plasma Ca and P, no effect Mg Dietary Ca and P: no effect plasma Mg or Al ↑ serum Al ↓ DMI prepartum Dietary Ca and/or P: NSD in milk yield P levels below reference limits No hypomagnesaemia detected Dietary Ca and/or Mg: NSD in disease incidence

Abbreviations: ↑ increased; ↓ decreased; BCS = body condition score; DMI = dry matter intake; NSD = no significant difference; SSA = synthetic sodium aluminosilicate.

6.1.3.1 Effect of Synthetic Sodium Aluminosilicate Supplementation on Phosphorus Levels

In all but one of the studies in periparturient dairy cows, plasma phosphorus concentrations were observed to decrease during the period of synthetic sodium aluminosilicate supplementation relative to control cows irrespective of supplementation levels and durations (Thilsing-Hansen & Jørgensen, 2001; Thilsing-Hansen *et al.*, 2002b, 2003; Thilsing *et al.*, 2007; Grabherr *et al.*, 2008; Grabherr *et al.,* 2009a; Pallesen *et al.*, 2008; Kerwin *et al.*, 2019; Crookenden *et al.*, 2020). The plasma phosphorus levels were generally observed to fall below the lower limit of the reference range for dairy cows of 1.3 to 2.6 mmol/L (Goff, 2006) around parturition but to return to normal levels within 1 to 2 weeks post-calving. The exception was the study Khachlouf *et al.* (2009) in which 200 g synthetic sodium aluminosilicate/day was administered to periparturient cows for 40 days with only a numerical drop in plasma phosphorus levels between experimental and control animals. Plasma phosphorus levels in both the experimental and control animals in this study remained within reference ranges throughout the pre- and postpartum periods. Hypophosphatemia commonly observed in parturient cows and the overall trend to decreased plasma phosphorus levels which normalized within weeks of calving was reported in experimental and control cows in all of the studies.

As mentioned previously, a number of the study authors associate the lower plasma prepartum and early postpartum phosphorus concentrations in plasma observed on synthetic sodium aluminosilicate with reduced dietary phosphate availability. Partial degradation of synthetic sodium aluminosilicate is thought to release aluminum which forms insoluble aluminum phosphate complexes in the intestinal lumen (e.g., Thilsing-Hansen *et al.*, 2002b). Grabherr *et al.* (2008; 2009b) suggested that the transient hypophosphatemia was solely an unwanted side effect of synthetic sodium aluminosilicate supplementation. However, Thilsing and *et al.* (2007) postulated that because of the complex interaction between calcium and phosphorus homeostasis in ruminants, at least part of the effect on calcium status around calving may be attributed to the sodium aluminosilicate-induced hypophosphatemia, and that some degree of hypophosphatemia was probably necessary to activate calcium homeostatic mechanisms in cows. Elevated plasma phosphorus concentrations greater than 2.0 mmol/L (Goff, 2006) can inhibit the conversion of 1,25-dihydroxyvitamin D from 25-hydroxyvitamin D. Without this conversion, calcium absorption activity is not increased in the small intestine, regardless of PTH synthesis activation (Goff, 2006). The study by Pallesen *et al.* (2008) further supports the hypothesis that hypophosphatemia is necessary to initiate calcium homeostasis; combined phosphorus and magnesium supplementation reduced the hypophosphatemia induced by 500 g synthetic sodium aluminosilicate/head/day but also reduced the stabilizing effect of synthetic sodium aluminosilicate on the parturient blood calcium level. As such, it is reasonable to conclude that the additional phosphorus diminished the effect of synthetic sodium aluminosilicate in blood calcium levels at calving.

Chronic hypophosphatemia can lead to depressed feed intake and lactation performance (NRC, 2001). A reduction in DMI by periparturient cows during the period of synthetic sodium aluminosilicate supplementation was recorded in some (Thilsing *et al.*, 2002b; Thilsing *et al.*, 2007; Grabherr *et al.*, 2009a) but not all of the studies (Grabherr *et al.*, 2008; Pallesen *et al.*, 2008). However, milk performance and composition was not negatively impacted in any of the studies and no other adverse effects on the health of the animals was noted (Thilsing *et al.*, 2002b; Kerwin *et al.*, 2019; Khachlouf *et al.*, 2019). Moreover, DMI appears to return to normal levels in cows postpartum (Grabherr *et al.*, 2008).

Overall, supplementation of the diet with synthetic sodium aluminosilicate at 400 g/head/day in the form of X-Zelit® for at least 14 days, and no more than 28 days, pre-calving is expected to induce hypophosphatemia for a transient period pre- and post-calving. However, levels are expected to return to within normal levels within a short period (7 to 14 days) postpartum and not to pose a safety concern to dairy cattle.

6.1.3.2 Effect of Synthetic Sodium Aluminosilicate Supplementation on Magnesium Levels

In a number of the studies in periparturient dairy cows, plasma magnesium concentrations were observed to decrease around parturition during the period of synthetic sodium aluminosilicate supplementation relative to control cows (Thilsing-Hansen *et al.*, 2002b, 2003; Grabherr *et al.*, 2008, 2009a; Kerwin *et al.*, 2019; Crookenden *et al.*, 2020). However, in three other studies, there was no significant effect of synthetic sodium aluminosilicate supplementation on plasma magnesium levels (Thilsing *et al.*, 2007; Pallesen *et al.*, 2008; Khachlouf *et al.*, 2019). In some instances, plasma magnesium continued to remain within reference values of 0.75 to 1.0 mmol/L (Goff, 2006) even when depressed in experimental cows compared to control animals (Thilsing-Hansen *et al.*, 2003; Grabherr *et al.*, 2009a). The exact mechanisms for the observed decrease in plasma magnesium concentrations in some studies has not been conclusively elucidated, but it has been suggested that synthetic sodium aluminosilicate could directly bind the magnesium causing a decrease in availability to the cow (Thilsing *et al.*, 2006a; 2007). In the study by Pallesen *et al.* (2008), dietary supplementation prepartum with magnesium appeared not to be necessary, with incidences of hypomagnesaemia observed in animals fed magnesium supplemented diets and not in those fed the unsupplemented rations. According to Thilsing-Hansen *et al.* (2002b, 2003) and Pallesen *et al.* (2008), plasma magnesium levels are less well-controlled than calcium in periparturient cows and are principally a balance between ruminal and intestinal absorption, and renal excretion. Consequently, plasma magnesium levels can be controlled in a well-managed herd through provision of normal diets meeting NRC recommendations (NRC, 2001) during the period of synthetic sodium aluminosilicate supplementation prepartum.

Together, the data indicate that while minor changes in plasma magnesium concentration may occur following supplementation of the diet of periparturient cows with 400 g synthetic sodium aluminosilicate/head/day for at least 14 days, and no more than 28 days, pre-calving, the effect is only transient around parturition and is not associated with any adverse effects on health.

6.1.3.3 Effect of Synthetic Sodium Aluminosilicate Supplementation on Aluminum Levels

As described in Section 6.1.2, available studies in dairy cattle indicate that following partial hydrolysis in the digestive tract of periparturient cows (Thilsing *et al.*, 2006a; 2007; Grabherr *et al.*, 2009a), aluminum is released. Aluminum is known to play a role in the utilization of minerals in animals including ruminants, both by complexation to form non-absorbable compounds and through effects of absorbed aluminum on mineral metabolism (Allen, 1984). The levels of aluminum detected in the plasma of periparturient cows fed 600 g of synthetic sodium aluminosilicate for 14 days pre-calving were significant compared to controls (85.29 ± µg/L vs. 13.24 ± 2.17 µg/L, corresponding to 0.0032 ± 0.0003 and 0.0005 ± 0.00008 mmol/L) but were not at concentrations which resulted in any adverse effects on DMI, general health or milk production by the animal.

In a study by Cefali *et al.* (1996) in dogs, aluminum from synthetic sodium aluminosilicate was not absorbed to any substantial degree after administration of a single dose and was efficiently cleared from the body via the kidneys. No significant species differences are anticipated with regards to aluminum metabolism and the mineral should be efficiently excreted in the urine of periparturient dairy cows following administration of synthetic sodium aluminosilicate at levels of 400 g/head/day for at least 14 days, and no more than 28 days, pre-calving without any significant accumulation in tissues (Allen, 1984; Ganrot, 1986; Krewski, 2007). Thus, the slight transient increase in plasma aluminum concentrations under the intended conditions of use of synthetic sodium aluminosilicate are not expected to pose a safety concern to the target animal.

6.1.3.4 Effect of Synthetic Sodium Aluminosilicate Supplementation on Other Mineral Levels

As mentioned in Section 6.1.2, synthetic sodium aluminosilicate remains largely intact in the digestive tract of animals following ingestion, but a small portion appears to be partially degraded with the release of silicic acid, amorphous aluminum silicates and aluminum (Cook *et al.*, 1982). Although synthetic sodium aluminosilicate should preferentially bind calcium it may interact with other dietary trace elements. Moreover, aluminum released from synthetic sodium aluminosilicate is known to form non-absorbable complexes with various bivalent cations such as copper(II), zinc(II), iron and manganese (II) affecting their availability to animals from the diet (Allen, 1984). The potential effect of dietary supplementation of periparturient cows with approximately 558 g/head/day of synthetic sodium aluminosilicate for 14 to 21 days pre-calving on serum copper, zinc, iron and manganese concentrations was evaluated by Grabherr *et al.* (2008) at day of calving, as well as days 1, 2 and 7 post-calving. There was no effect of synthetic sodium aluminosilicate supplementation on serum levels of these trace elements.

Thus, it may be concluded that under the intended conditions of use of synthetic sodium aluminosilicate by Protekta in the diet of periparturient cows at similar levels of 400 g/head/day under equivalent conditions of use (at least 14 days, and no more than 28 days, pre-calving), there will be no impact on trace elements in the diet.

6.1.3.5 Effect of Synthetic Sodium Aluminosilicate Supplementation on Voluntary Feed Intake

DMI was evaluated in the majority of studies conducted in periparturient cows using synthetic sodium aluminosilicate. As mentioned above, a reduction in DMI by periparturient cows during the period of synthetic sodium aluminosilicate supplementation was recorded in some (Thilsing *et al.*, 2002b; Thilsing *et al.*, 2007; Grabherr *et al.*, 2009a) but not all of the studies (Grabherr *et al.*, 2008; Pallesen *et al.*, 2008). Thilsing *et al.* (2007) note that, although poor palatability of synthetic sodium aluminosilicate may contribute to the reduced voluntary feed intake by the animals, the depletion in available phosphate or other dietary minerals from interactions with the additive or aluminum released by the additive, may play a role. The composition of the TMR may also influence DMI intake and potentially any effect will not be consistent between herds. Reductions in DMI appear to be transient for the period of synthetic sodium aluminosilicate supplementation and were not associated with any adverse effects on body condition score or general health (Grabherr *et al.*, 2008; Kerwin *et al.*, 2019).

Overall, any impact of synthetic sodium aluminosilicate on DMI under the conditions of intended use by Protekta at levels of 400 g/head/day for at least 14 days, and no more than 28 days, pre-calving is not expected to pose a safety concern to the target animal.

6.1.3.6 Effect of Synthetic Sodium Aluminosilicate Supplementation on Milk Yield

A number of the studies conducted in periparturient cows using synthetic sodium aluminosilicate evaluated parameters related to milk production, such as milk yield (or fat-corrected milk yield), milk composition and colostrum content (Thilsing *et al.*, 2002; Grabherr *et al.*, 2008; 2009b; Kerwin *et al.*, 2019; Khachlouf *et al.*, 2019). There was no impact of synthetic sodium aluminosilicate supplementation on the yield or composition of milk when measured up to 105 days post-calving. Khachlouf *et al.* (2019) also reported that synthetic sodium aluminosilicate supplementation of the diet at 200 g/head/day for 40 days has no effect on calcium, magnesium or phosphorus content of milk.

Thus, synthetic sodium aluminosilicate supplementation of periparturient cows for at least 14 days, and no more than 28 days, pre-calving at 400 g/head/day in the form of X-Zelit® by Protekta is not expected to adversely affect lactation.

6.1.3.7 Summary of Studies in Periparturient Cows

Taken together, the data above indicate that although synthetic sodium aluminosilicate administration to periparturient cows for at least 14 days, and no more than 28 days, pre-calving at a level of 400 g/head/day may have an impact on plasma phosphorus and magnesium levels, and may reduce DMI, these effects are transient and do not result in any detrimental effects on general health, performance of milk production by the animals. Plasma mineral levels and DMI return to with normal ranges within a short period postpartum with no long-term effects on the health of the animal.

6.1.4 Information to Support the Existing Use of Synthetic Sodium Aluminosilicate in Animal Food

6.1.4.1 History of Use of Synthetic Sodium Aluminosilicate in Animal Food

As mentioned in Section 3.1.2, sodium aluminosilicate and the structurally-related substance sodium calcium aluminosilicate are permitted for use as anti-caking agents in the feed of animals at levels not exceeding 2% in the diet in accordance with 21 CFR §582.2727 and §582.2729 (U.S. FDA, 2020). The existing history of use of these substances as an anti-caking agent in feed provide supporting evidence for the safety of synthetic sodium aluminosilicate in periparturient cows for at least 14 days, and no more than 28 days, pre-calving at a level of 400 g/head/day (as X-Zelit®).

Assuming a dairy cow weighs 650 kg and consumes 25 kg DM in the form of a TMR (Poncheki *et al.*, 2015; EFSA, 2017; University of Minnesota, 2020), the exposure by animals to sodium aluminosilicate from its use as an anti-caking agent at the maximum level of 2% in the diet (TMR as-fed; 45 to 55% DM) will range from 910 to 1,110 g/cow/day. However, it is recognized that during the period 14 to 21 days pre-calving, DM intake by dairy cows is reduced to around 12 kg/head/day (University of Minnesota, 2020), and on this basis, a 650 kg cow will consume between 436 and 534 g/head/day of sodium aluminosilicate from its presence as an anti-caking agent in feed at the maximum permitted level of 2% in the diet (TMR as-fed; 45 to 55% DM).

As mentioned above, Protekta wishes to extend the current scope of use of sodium aluminosilicate in feed in the U.S. to include use in the diets of periparturient cows for a period of at least 14 days, and no more than 28 days, pre-calving in order to help maintain calcium balance. Dairy cows will be provided with up to 500 g X-Zelit®/head/day equivalent to between 375 and 400 g synthetic sodium aluminosilicate/head/day. Thus, the potential exposure of periparturient dairy cows to synthetic sodium aluminosilicate from its presence as an anti-caking agent in the feed is in the same range (436 to 534 g/head/day) as from the proposed use (375 to 400 g/head/day). Synthetic sodium aluminosilicate will not be used in parallel as an anti-caking agent and as an aid to maintain calcium balance, and therefore, overall exposure by dairy cows will not increase under the conditions of intended use by Protekta.

6.1.4.2 Previous Scientific Evaluations of Synthetic Sodium Aluminosilicate for Use in Feed

As described in Section 3.1.2, sodium aluminosilicate (synthetic or mined/natural form) has a long and established history of use as an anti-caking agent in the U.S., Canada and EU. Furthermore, the formulated feed product, X-Zelit® is currently marketed for use as an aid to maintain calcium balance in periparturient cows in the EU by ViloFoss® in accordance with Commission Regulation (EU) 2020/354 establishing a list of intended uses of feed intended for particular nutritional purposes (EC, 2020). Likewise, X-Zelit® is marketed by Protekta for use as an aid to maintain calcium balance in periparturient cows in Canada in accordance with its approval by the CFIA and listing under Entry 8.91 of Schedule IV, Part I of the Feed Regulations (1983) (CFIA, 2020).

The authorization of synthetic sodium aluminosilicate for use as an aid to maintain calcium balance in the EU under equivalent conditions of use to those proposed herein by Protekta is supported by two scientific opinions by EFSA (2004, 2007). In the first opinion (EFSA, 2004), the Panel concluded:

"*Synthetic sodium aluminosilicate has the potential to reduce the risk of milk fever in dairy cows, but optimal dosage and duration of treatment are not well established. The FEEDAP Panel is aware of the short term intended use of synthetic sodium aluminosilicate but needs to know the potential longer term (3-month consequences) in the view of animal health and welfare.*

The risk for the health of the dairy cow cannot be fully assessed because there is insufficient data on magnesium supply and bioavailability of trace elements in treated cows.

No data are given on the potential influence of synthetic sodium aluminosilicate on composition and quality of milk, including the possibility that aluminium content in milk may be increased by release from synthetic sodium aluminosilicate at pH <4.0."

These data gaps were addressed, and a second opinion subsequently issued by EFSA (2007) in which the Panel concluded:

"*In the former opinion on synthetic sodium aluminosilicate, the FEEDAP Panel stated that this product has the potential to reduce the risk of milk fever. Recent data confirm this conclusion, particularly in older cows with three or more calvings. Synthetic sodium aluminosilicate gradually prevents the decrease in serum calcium occurring after calving.*

Although only 500 g/day of synthetic sodium aluminosilicate was shown to significantly reduce the milk fever incidence, a dose range of 250 to 500 g/day of synthetic sodium aluminosilicate leads to a dramatic depression of feed intake. Even the effective dose reduces feed intake and induced hypophosphatemia; however, these effects are considered transient. Synthetic sodium aluminosilicate may reduce serum magnesium but this is without physiological significance. Serum levels of copper and zinc as well as milk yield and composition are not affected by sodium aluminosilicate treatment.

The FEEDAP Panel concludes that (i) the observed side effects after a two-week treatment with synthetic sodium aluminosilicate do not have long lasting consequences on the health of cows; that (ii) aluminum from synthetic sodium aluminosilicate does not lead to any safety concern for the dairy cow provided the appropriate use level and duration are followed, and that (iii) synthetic sodium aluminosilicate treatment of the dry cow does not result in an adverse effect in calves.

Milk aluminum concentration was not affected by the use of synthetic sodium aluminosilicate but serum aluminum significantly increased. The Panel concluded that, considering the range of aluminum found in commercial milk samples, the treatment of dry cows with synthetic sodium aluminosilicate will not measurably increase consumer exposure to aluminum."

The same body of published evidence used by EFSA to support its conclusion on the safety and utility of synthetic sodium aluminosilicate for use in periparturient cows have been summarized in this GRAS dossier.

6.1.5 Toxicological Information on Synthetic Sodium Aluminosilicate

6.1.5.1 History of Use of Synthetic Sodium Aluminosilicate in Human Food

Sodium aluminosilicate (synthetic or mined/natural form) has a long and established history of use as an anti-caking agent in human food in the U.S., EU, and Canada. Details of the regulatory status of the ingredient in these different jurisdictions is provided in Table 6.7.

Table 6.7: Regulatory Status of Sodium Aluminosilicate (Synthetic or Mined) for Use in Human Food in the U.S., EU and Canada		
Jurisdiction	**Regulatory Status**	**Reference**
U.S.	GRAS for use as an anti-caking agent in food at levels not exceeding 2% in accordance with GMP	21 CFR §182.2727 (U.S. FDA, 2020)
EU	Permitted for salt intended for the surface treatment of ripened cheese at a carry-over maximum of 20 mg/kg (Annex II; direct food additives). Permitted for use in nutrients (except those for infant or young children foods) at a level not to exceed 15,000 mg/kg in fat-soluble preparations (Annex III; additives in food additives)	Commission Regulation (EC) No 1333/2008 (EC, 2008 – Annexes II and III)
Canada	Permitted for use as an anti-caking agent in salt at a level of 1.0% (or 2.0% in fine grain salt); icing sugar singly or in combination with calcium phosphate tribasic, calcium silicate, magnesium carbonate, magnesium silicate, magnesium stearate and silicon dioxide at a total level of 1.5%; in dried egg white, dried whole egg, dried whole egg mix, dried yolk and dried yolk mix at a level of 2.0%; in garlic or onion salt at a level of 2.0%; and in unstandardized dry mixes in accordance with GMP.	List of Permitted Anti-caking Agents as part of the Marketing Authorization for Food Additives that may be Used as Anticaking Agents (Health Canada, 2020)

Abbreviations: GMP = good manufacturing practice.

6.1.5.2 Previous Scientific Evaluations of Synthetic Sodium Aluminosilicate for Use in Human Food

The safety of the series of amorphous hydrated sodium aluminum silicates referred to as synthetic sodium aluminosilicate and comprising various proportions of Na_2O, Al_2O_3 and SiO_2 have been evaluated by a number of scientific bodies to support the existing use as additives in food. A summary of these evaluations is provided in Table 6.8. These evaluations primarily evaluated the safety of the structurally-related group of additives on the basis of the potential exposure by humans to the aluminum content, taking into account established safe limits for humans for this element (e.g., EFSA, 2008, 2011). Notably in the most recent evaluation of synthetic sodium aluminosilicate for use as a food additive in the EU, EFSA concluded that the current body of limited toxicological data were insufficient to establish safety at the estimated levels of intake.

Table 6.8: Scientific Evaluations of Sodium Aluminosilicate by Scientific Bodies	
Reference	**Key Conclusions**
EFSA, 2020	In the re-evaluation of sodium aluminosilicate and potassium aluminosilicate for use as food additives in the EU, the EFSA Panel were unable to conclude on safety on the basis of insufficient characterization and only limited toxicological information available; based on an exposure estimate, the Panel concluded that the TWI for aluminum may be exceeded based on current industry practices.
SCF, 1991	In the evaluation of sodium aluminosilicate and potassium aluminosilicate for use as food additives in the EU, the Panel concluded that use was acceptable and established a PTWI of 7 mg Al/kg bw from all proposed intakes.
EFSA, 2008	In a follow-up evaluation to the SCF (1991) opinion, the EFSA Panel established a TWI of 1 mg Al/kg bw.
EFSA, 2011	In a statement on the evaluation of a new study related to the bioavailability of Al in food, EFSA considered that the previous TWI (EFSA, 2008) should be maintained.
JECFA, 2011	The Committee evaluated Al-containing food additives including sodium aluminosilicate and established at PTWI of 2 mg Al/kg bw for Al from all Al compounds in foods, including food additives.

Abbreviations: PTWI = provisional tolerable weekly intake; TWI = tolerable weekly intake.

Toxicological information is not considered critical to the safety evaluation of synthetic sodium aluminosilicate for use by Protekta in the form of X-Zelit® as a supplement for periparturient cows at 400 g/head/day for at least 14 days, and no more than 28 days, pre-calving. For completeness however, the available published and unpublished data evaluated previously by EFSA and JECFA, is summarized below to provide corroborative evidence that synthetic sodium aluminosilicate should not pose a concern to the health of dairy cattle under the conditions of intended use.

The primary body of toxicity information available on synthetic sodium aluminosilicate was conducted by Gloxhuber *et al.* (1983). Several studies were performed including metabolism (see Section 6.1.2), sub-chronic, as well as a combined chronic and carcinogenicity study from sodium aluminum silicate in rats and mice. Several other available studies conducted in rats or mice were unpublished summaries and not always conducted according to current OECD testing guidelines as many were conducted pre-1981; these are described in detail for consideration of validity.

6.1.5.3 Repeated Dose Toxicity Studies

Short Term Studies

In 2 unpublished studies cited in HERA (2004), groups of Fischer-344 rats (5/group, sex not reported) and BSC3F1 mice (5/group, sex not reported) were administered sodium aluminum silicate at 0 (control), 0.625. 1.25, 2.5, 5, or 10% (w/w) via the diet for 14 days (unpublished study conducted by Henkel, R 0100197 – 1979 and R 0100196 – 1979, cited in HERA, 2004). There were no compound-related effects on body weights or food consumption in any of the animals and necropsy findings showed no signs of toxicity. No further study details were available.

Subchronic Studies

In a 90-day repeat dose study conducted by Gloxhuber *et al.* (1983), groups of SPF maintained Wistar rats (20/sex/group) were administered synthetic sodium aluminosilicate (zeolite A; $Na_{12}Al_{12}Si_{12}O_{48}.27H_2O$) via the diet at concentrations of 0 (control), 1,000, 5,000 or 10,000 mg/kg diet. A

satellite group was used to provide initial biochemical measurements. At the end of the 90-day feeding period, urine samples from all surviving test and control animals were evaluated for volume, pH, protein, glucose, urobilinogen, ketones and specific gravity. Blood samples were taken for hemoglobin determinations, red cell and total white cell counts, determination of blood sugar, serum alkaline phosphatase, serum glutamic pyruvic transaminase, serum glutamic-oxalacetic transaminase, urea and total serum proteins. Organ weights (brain, heart, kidney, liver, gonads, adrenal glands, thyroid, pituitary and thymus) were recorded and underwent histological examination.

At 10,000 mg/kg diet the function and histopathology of kidneys and bladder were found to be altered when compared to control. Alterations included diminished urine secretion, hematuria, and ketone bodies in the urine. In 12 of 20 male animals, urinary calculi of different size and composition, mainly composed of silicon, were observed. Histological examination showed a hyperplastic reaction of the transitional bladder epithelium in rats that displayed calculi. In male animals without calculi, in the females of the 10,000 mg/kg group and in all other groups, no hyperplasia of the bladder epithelium was observed. The elemental analysis of blood, liver and kidneys is described in Table 6.9 for rats dosed with 10,000 mg/kg zeolite A. At 10,000 mg/kg diet, silicon concentrations of kidneys were higher than in controls (No *P* value given). No significant differences were found compared to control regarding the concentrations of iron in blood, copper and cobalt in the liver, or in zinc, aluminum and copper in the kidneys. The No-Observed-Adverse-Effect-Level (NOAEL) of synthetic sodium aluminosilicate was determined to be 5,000 mg/kg diet or approximately 250 mg/kg body weight/day.

Table 6.9: Elemental Analysis of Blood, Liver and Kidneys of Rats Fed 10,000 mg/kg of Synthetic Sodium Aluminosilicate for 90 Days

Organ	Element	Sex	Control Group	Zeolite A
Blood	Iron	M	461.0 ± 57.0	478.0 ± 20.0
		F	477.0 ± 49.0	486.0 ± 63.0
Liver	Cobalt	M	0.190 ± 0.04	0.226 ± 0.007
		F	0.202 ± 0.07	0.154 ± 0.02
	Copper	M	23.6 ± 3.3	26.9 ± 1.8
		F	28.9 ± 4.4	26.3 ± 4.4
Kidney	Zinc	M	103.0 ± 6.43	101.0 ± 3.47
		F	105.0 ± 5.74	107.0 ± 3.09
	Aluminum	M	12.1 ± 4.5	13.0 ±7.6
		F	21.7 ± 40.2	17.2 ± 17.2
	Silicon	M	528.0 ± 99.0	1688.0 ± 1021.0
		F	552.0 ±139.0	843.0 ± 535.0

Initial 90-day Study Extended to 163 days

In a repeat dose study groups of COX-SD rats (20/sex/group), 0% (control), 0.5%, 1.0% or 2.0% (w/w) of sodium aluminum silicate (form not specified) was administered via the diet, initially for 90 days then extended to 163 days (Henkel unpublished data, Henkel, TBD EX 0143-1975. Cited in: HERA 2004). Interim terminations were made at 28 and 91 days. The 28-day sacrifice did not reveal any indication of toxicity. At 91 days three animals had displayed signs of urinary bladder complications. One died on day 84 and another on day 85 both displayed evidence of bladder toxicity. The sacrifice on day 91 had bladder stones. The study was extended to 163 days and bladder stones were observed in one animal at

0.5% and one animal at 1.0% with more being reported at 2.0% (actual number not provided). A NOAEL cannot be determined from this study.

Urinary toxicity was further explored in a follow up study (Henkel unpublished data. Henkel, TBD EX 0127-1976 Cited in: HERA, 2004). In a repeat dose study, groups of COX-SD rats (40/sex/group), 0 (control), 0.125, or 2.0% (w/w) (approximately 69 and 110 mg/kg body weight/day, respectively) of sodium aluminum silicate (zeolite A) was administered via the diet for 160 or 200 days. Urinalysis did not find significant differences between control or treated groups. In the treated groups a white crystalline material was present in the urine. In rats treated with 2% sodium aluminosilicate (zeolite A), an increase in the incidence of bladder and kidney stones relative to control was observed. No other signs of urinary toxicity or kidney function impairment were noted. Pathological examination found histologic changes of the kidneys and urinary bladders in the 2% dose group; no differences were observed between other groups. In the kidneys, microscopic alterations were typified by an increase in the severity of interstitial nephritis, regenerative epithelium and pelvic epithelial hyperplasia. A non-staining crystalline material was frequently present in the kidneys of the 2% dose group. In the urinary bladder, in the 2% dose group, an increase in the incidence and severity of transitional epithelial hyperplasia was observed. Microscopic sodium aluminum silicate related alterations were not observed in animals of the lower dose groups. A NOAEL of 0.125% (approximately 69 mg/kg body weight/day) was determined from this study. In a 24-week oral toxicity study Long-Evans rats (10/sex/group) were fed via the diet either 0 (control), 0.125, 0.5 or 2.0% of sodium aluminum silicate (zeolite A-type) (Unpublished data Henkel, Henkel, TBD EX 0129-1976, TBD EX 0137-1976. Cited in: HERA, 2004). Evaluation of mortality, physical appearance, feed efficiency, body weights, organ weights and organ/body weight ratios did not reveal evidence of toxicity relative to control at any dose. In both sexes of 0.5 and 2.0% dose groups, pathology revealed compound related microscopic alterations in the kidneys. No compound related microscopic changes of the kidneys, relative to control, were observed in the 0.125% diet. The NOAEL in this study can therefore considered to be 0.125% in the diet (approximately 69 mg/kg body weight/day).

6.1.5.4 Chronic and Carcinogenicity Toxicity Study

Gloxhuber *et al.* (1983) conducted a 2-year (104 week) joint chronic and carcinogenicity study in rats. Groups of Wistar rats (50/sex/group) were administered via the diet 0 (control), 10, 100 or 1,000 mg/kg diet of synthetic sodium aluminosilicate (zeolite A; $Na_{12}Al_{12}Si_{12}O_{48}.27H_2O$). These dietary concentrations were equivalent to 0, 0.62, 6.1, and 58.5 mg/kg body weight/day in males and 0.65, 6.53, and 62.2 mg/kg body weight/day in females. A satellite group (15/sex/group) was used to provide biological samples for initial and interim investigations. Feed and water intake, mortality, morbidity, and body weights were recorded. After 6, 26, 78 and 104 weeks, clinical chemistry analysis was performed. Urinary volume, pH, protein, urobilinogen, ketones, blood and aluminum and silicon in the spun deposit were determined. Iron, cobalt and copper were measured in the liver and zinc, aluminum and silicon were measured in the kidney. The authors only provide a small extract on the clinical data recorded, the main findings were in the highest treatment dose of 1,000 mg/kg and the data is described. The hematology and blood biochemistry are detailed in Table 6.10. The glutathione activity in female rats was lowered (P<0.05) and serum calcium (P<0.05) was higher in treated animals, however these observations were not manifested clinically and can be considered minor. The number of leucocytes was significantly decreased in the treatment group of male rats. The authors concluded that the

observation was not treatment-related because other parameters, such as bone marrow cell counts and differential blood cell count, showed no significant differences to support that the change in leucocytes was connected to synthetic sodium aluminosilicate exposure.

Urinary excretion of aluminum and silicon was slightly increased in treated animals versus controls but was not considered statistically significant. No other urinary measurements were found to significantly differ between control and treated animals.

Table 6.10: Analysis of Blood, Liver and Kidneys of Rats Fed 1,000 mg/kg of Synthetic Sodium Aluminosilicate for 104 Weeks

Hematology	**Males**		**Females**	
Parameter	Control	Zeolite A	Control	Zeolite A
Hemoglobin (g/100 ml)	14.8 ± 1.4	15.0 ± 1.2	14.4 ±0.6	14.3 ± 0.4
Hematocrit (%)	49.8 ± 2.0	50.4 ± 2.9	47.4 ± 2.2	49.7 ± 3.8
Erythrocytes ($x10^6/mm^3$)	6.4 ± 0.9	6.1 ± 0.8	6.3 ± 0.5	6.0 ± 1.0
Leucocytes ($x10^3/mm^3$)	5.1 ± 1.9	2.3 ±1.1*	8.3 ± 2.7	6.8 ± 1.2
Thrombocytes ($x10^3/mm^3$)	335.0 ± 140.0	332.0 ± 94.0	364.0 ± 188.0	457.0 ± 205.0
Blood Biochemistry				
Iron (µg/100 ml blood)	154.1 ± 26.3	157.7 ± 39.3	232.8 ± 28.3	233.5 ± 47.7
Glutamic pyruvic transaminase (U/100 ml Serum)	60.1 ± 22.0	72.0 ± 28.6	73.1 ± 28.4	40.7 ± 7.99**
Glutamic oxalacetic Tranaminase (U/100 ml Serum)	85.1 ± 20.7	126.1 ± 49.7	129 ±44.2	85.2 ± 34.6*
Alkaline phosphatase (U/100 ml Serum)	129.3 ± 19.2	145.7 ± 39.3	73.9 ± 11.8	70.4 ± 24.8
Glucose (mg/100 ml blood)	148.8 ± 16.2	148.0 ± 46.8	127.8 ± 23.1	127.6 ± 24.9
Total Protein (g/100 ml serum)	7.0 ± 0.2	7.2 ± 0.4	6.7 ± 0.3	7.0 ± 0.5
Urea (mg/100 ml serum)	44.6 ± 3.3	47.7 ± 5.4	48.8 ± 9.0	60.7 ± 12.8
Sodium (mg/100 ml Serum)	339.0 ± 3.2	337.2 ± 3	349.8 ± 3.7	351.2 ± 10.1
Calcium (mg/100 ml serum)	9.4 ± 1.2	10.8 ± 0.4 **	10.3 ± 0.8	9.6 ± 0.4 *
Potassium (mg/100 ml serum)	19.1 ± 2.7	18.8 ± 2.0	19.2 ± 1.6	18.0 ± 2.7

Significance determined by students T-test: *P<0.05; **P<0.01.

Similar to the 90-day study by Gloxhuber *et al.* (1983), silicon concentrations were found to have increased in the kidneys of test animals (Table 6.11), no significant differences were found regarding the concentrations of copper or cobalt in the liver, and zinc or aluminum in the kidneys. The organ weights of males displayed no significant differences when compared to control values. In the females, the relative weights of the adrenal glands of the 10 mg/kg group as well as of the thymus of the 100 and 1,000 mg/kg groups differed significantly (P<0.05) from those of the controls. Histopathology revealed no significant treatment-related effects in any of the organs examined. No neoplastic changes were observed. No significant incidence of a specific tumor type or of spontaneous mortality was evident in any group. Comparison of the separate sums of the tumorous changes observed in the 1,000 mg/kg and control groups by the statistical method of Kastenbaum & Bowman (1970) showed no significant difference between the groups in the frequency of tumors. From this conclusion a NOAEL of 60 mg/kg body weight/ day in the rat can be allocated for synthetic sodium aluminosilicate.

Table 6.11: Elemental Analysis of Blood, Liver and Kidneys of Rats Fed 1,000 mg/kg of Synthetic Sodium Aluminosilicate for 104 Weeks				
Organ	**Element**	**Sex**	**Control Group**	**Zeolite A**
Liver	Copper	M	12.9 ± 3.8	15.4 ± 5.3
		F	16.2 ± 4.1	19.0 ± 11.1
	Cobalt	M	0.21 ± 0.06	0.18 ± 0.07
		F	0.20 ± 0.03	0.16 ± 0.04
Kidney	Zinc	M	90.0 ± 3.0	92.8 ± 8.8
		F	99.1 ± 3.4	105.0 ± 6.9
	Aluminum	M	19.6 ± 21.0	28.1 ± 23.6
		F	11.1 ± 5.7	8.4 ± 2.7
	Silicon	M	586.0 ± 470.0	841.0 ± 1004.0
		F	552.0 ±139.0	308.0 ± 411.0

6.1.5.5 Genotoxicity

In vitro Studies

The mutagenic potential of sodium aluminosilicate (form not specified) was investigated by Prival *et al.* (1991) using a standard bacterial reverse mutation assay using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA 1538 and *Escherichia coli* WP2 (uvrA) both in the absence and presence of 10% Aroclor 1254-induced rat liver S9. Sodium aluminosilicate was suspended in 0.067 M potassium or sodium phosphate buffer (pH 7.0) and was tested using concentrations of 0 (control), 0.033, 0.10, 0.33, 1.0, 3.3, or 10 mg per plate. All platings were performed in duplicate and all tests were repeated and run concurrently with positive controls with direct acting mutagens as well as mutagens requiring S9 activation. The following positive control compounds were used in the absence of S9: 2-nitrofluorene (5.0 or 10 µg per plate) for strains TA98 and TA1538; sodium azide (0.50 or 1.0 µg) for TA100 and TA1535; 9-aminoacridine (50 or 100 µg) for TA1537; and AF-2 (furylfuramide; 0.1 µg) or N-methyl-N'-nitro-N-nitrosoguanidine (10 µg) for *E. coli* strain WP2. Anthramine was the positive control compound requiring S9 metabolic activation used for all bacterial strains. Anthramine was used at 1.0 or 2.5 µg per plate for strains TA98, TA100 and TA1538, at 2.5 µg per plate for strains TA1535 and TA1537, and at 10 µg per plate for *E. coli* strain WP2. Sodium aluminosilicate was noted as being inorganic by the investigators and was likely tested as suspension and was found not to have mutagenic potential.

In vivo Studies

The cytogenic effect of sodium aluminosilicate (form not specified) *in vivo* was evaluated. Groups of male Albino rats (15/group) were administered 0 (control), 4.25, 42.5 or 425 mg/kg body weight orally by intubation as a single dose or a repeated dose study (conducted over 5 days). A parallel study was performed where male albino rats (15/group) were administered 5,000 mg/kg body weight orally by intubation as a single dose or as a repeated dose over 5 consecutive days (Unpublished data, Litton Bionetics INC., 1974. Cited: Hera, 2004). Observations were made at 6, 24 and 48 hours after dosing. Triethylene melamine was used a positive control and saline as a negative control. Metaphase chromosome spreads were prepared from the bone marrow and scored for chromosomal aberrations. No significant differences in the variety or number of chromosome aberrations were observed between

animals treated with sodium aluminum silicate and control. The expected positive response was elicited in bone marrow from animals treated with triethylene melamine.

To evaluate chromosomal aberrations of germ cells in the dominant lethal assay, male Albino rats (15/group) were administered 0 (control), 4.25, 42.5 or 425 mg/kg body weight orally by intubation as a single dose or a repeated dose study (conducted over 5 days). A parallel study was performed where male albino rats (15/group) were administered 5,000 mg/kg body weight orally by intubation as a single dose or as a repeated dose over 5 consecutive days (Unpublished data, Litton Bionetics INC., 1974. Cited: Hera, 2004). Triethylene melamine was used a positive control and saline as a negative control. Following treatment, the males were mated with two females per week for 8 weeks. Pregnant females were sacrificed at 14 days and the uterus examined for early deaths, late fetal deaths and the total numbers of implantation. From mating weeks 4 and 5 a non-dose dependent decrease in average corpora lutea and preimplantation losses were observed in animals administered sodium aluminosilicate when compared to negative controls but not when compared to historical controls. In the study where rats received 4.25, 42.5 or 425 mg/kg body weight of sodium aluminosilicate a significant not dose dependent decrease in average corpora lutea and preimplantation losses were observed in experimental groups from mating week 4 and 5 compared to negative controls, but not when compared to historical controls. Average resorptions showed significant but not dose dependent increases in the experimental group from mating week 3 in all dose groups when compared to the negative control (zero value), but not when compared to historical controls. In the study where rats received 5,000 mg/kg body weight no significant differences between dosed animals and controls were observed.

To evaluate chromosomal aberrations of germ cells in the dominant lethal assay, in a sub-acute study male Albino rats (15/group) were administered 0 (control), 4.25, 42.5 or 425 mg/kg body weight orally by intubation as a single dose or a repeated dose study (conducted over 5 days). A parallel study was performed where male albino rats (15/group) were administered 5,000 mg/kg body weight orally by intubation as a single doe or as a repeated dose over 5 consecutive days (Unpublished data, Litton Bionetics INC., 1974. Cited: Hera, 2004). Triethylene melamine was used a positive control and saline as a negative control. Following treatment, the males were mated with two females per week for 7 weeks. Significant dose related increases at 42.5 and 425 mg/kg body weight in average implantations and corpora lutea were observed in the experimental group from mating week 4 in comparison to the negative control. However, both the negative and experimental responses were significantly different when compared to historical controls. Significant dose-related increases in average resorptions were observed at 42.5 and 425 mg/kg body weight dose groups from mating week 6 when compared to the negative controls. However, no differences were observed when these groups were compared with the historical control. Positive controls elicited the expected response in preimplantation loss and embryo resorption from the first 5 mating weeks. The authors of the study concluded that sodium aluminosilicate does not induce dominant lethal mutations as measured in this study. The conclusion was made as no dose response or time trend pattern were revealed in the assay.

6.1.5.6 Carcinogenicity

As previously described in the chronic oral chronic toxicity study by Gloxhuber *et al.* (1983) in which groups of Wistar rats (50/sex/group) were administered via the diet 0 (control), 10, 100 and 1,000 mg/kg of sodium aluminosilicate (approximately equivalent to 0.6, 6.0 or 60 mg/kg body weight/day) for

104 weeks. No significant treatment-related effects relative to control on the types or incidences of any neoplastic changes was observed in this study.

6.1.5.7 Developmental Toxicity and Teratogenicity

Reproductive toxicity and teratogenicity were assessed by Nolen & Dierckman (1983) using groups of pregnant Sprague-Dawley rats (20/group) that were administered 0 (control) 74 or 1,600 mg/kg body weight synthetic sodium aluminosilicate (zeolite A; $Na_{12}Al_{12}Si_{12}O_{48}.27H_2O$) on days 6-15 and groups of pregnant New Zealand rabbits (20/group) were administered 0 (control) 74, 345 or 1,600 mg/kg body weight synthetic sodium aluminosilicate (zeolite A) by gavage on gestation days 6-18. Vehicle controls were included but no details were provided. Synthetic sodium aluminosilicate produced no adverse effects on the dam, embryo, or fetus in either the rats or rabbits at any dose. The NOAEL was reported as 1,600 mg/kg body weight (the highest dose tested) for both maternal toxicity and teratogenicity.

In another study groups of pregnant Wistar rats were administered with 0 (control), 16, 74, 345 or 1,600 mg/kg body weight of sodium aluminosilicate on gestation days 6-15 via gavage (unpublished data, FDRL, 1973. Cited in: HERA 2004). The dams were sacrificed on gestation day 20. The administration of the test compound had no clear effect on implantation or on maternal or fetal survival. The number of abnormalities recorded in the test group in either soft or skeletal tissues did not differ from the number observed in the control group. Sodium aluminosilicate was not teratogenic in rats at the dose levels tested. The NOAEL was 1,600 mg/kg body weight for maternal toxicity and for teratogenicity.

Effects in mice were examined in the following study where groups of pregnant CD-1 mice were administered with 0 (control), 16, 74, 345 or 1,600 mg/kg body weight of sodium aluminosilicate on gestation days 6-15 via gavage (unpublished data, FDRL, 1973. Cited in: HERA, 2004). The dams were sacrificed on gestation day 17. The administration of the test compound had no clear effect on implantation or on maternal or fetal survival. The number of abnormalities recorded in the test group in either soft or skeletal tissues did not differ from the number observed in the control group. Sodium aluminosilicate was not teratogenic in rats at the dose levels tested. The NOAEL was 1,600 mg/kg body weight for maternal toxicity and for teratogenicity.

In another study in rabbits, pregnant Dutch rabbits were treated daily with sodium aluminosilicate (unpublished data FDRL, 1973. Cited in HERA, 2004) with 0, 16, 74, 345 or 1,600 mg/kg body weight on gestation days 6-18 per gavage. The dams were sacrificed on gestation day 29. The administration of the test compound had no clearly discernible effect on implantation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues in the test groups did not differ from the number occurring spontaneously in the control group. These data show that the test compound was not teratogenic in rabbits at the dose levels tested. The NOAEL determined for synthetic sodium aluminosilicate was 1,600 mg/kg body weight for maternal toxicity and for teratogenicity.

6.1.5.8 Summary of the Toxicological Information on Synthetic Sodium Aluminosilicate

Sodium aluminosilicate (primarily synthetic sodium aluminosilicate) was not associated with any adverse systemic effects after oral administration. The only adverse effect reported was the formation of calculi in the bladder, which were consistently reported in several studies. In a urinary toxicity study these findings were explored, and it was reported that a white crystalline substance was apparent in the urine and as deposits in the kidney. Calculi formation could be due to absorption of silicon after oral ingestion

of sodium aluminosilicate. The NOAEL from this study was reported by the authors to be 69 mg/kg body weight/day.

Protekta intends to supplement the diet of periparturient cows with X-Zelit® at a level of 500 g/head/day, equating to 400 g/head/day of synthetic sodium aluminosilicate. Assuming a dairy cow weighs around 650 kg, the anticipated exposure by the animal will be in the region of 0.615 g/kg body weight/day which exceeds the NOAEL of 69 mg/kg body weight/day derived from the urinary toxicity study. However, synthetic sodium aluminosilicate will only be administered to animals for a period of 14 to 28 days and this short-term administration is not anticipated to be associated with any accumulation of deposits in the kidney or other long-term adverse effects.

The results *in vitro* mutagenicity assays indicate that sodium aluminosilicate is without mutagenic potential, either in the presence or absence of metabolic activation. The *in vitro* assays were only partially performed according to current OECD guidelines but are considered to be valid. The results of the *in vivo* test systems corroborated the results from the in vitro assays. Sodium aluminosilicate was tested in a cytogenetic assay in rats, a dominant lethal assay in rats, and a host mediated assay in mice. Doses ranged from 4.25 to 5,000 mg/kg body weight and an acute and subacute dosing regime was employed. No genotoxic effects were reported in these studies.

The potential teratogenicity of sodium aluminosilicate was investigated in Sprague-Dawley rats, CD-1 mice, and New Zealand white rabbits. These studies were not performed to current OECD guidelines, or GLP. However, the data from these studies indicated that sodium aluminosilicate was not associated with any toxicological effects on the mother, embryonic or fetal development. The findings provide corroborative evidence that the feeding of synthetic sodium aluminosilicate to periparturient cows for a 14 to 28 day period pre-calving should not have any adverse effect on the offspring.

6.2 HUMAN FOOD SAFETY EVALUATION

6.2.1 Potential Exposure of Dairy Cattle to Synthetic Sodium Aluminosilicate from Other Sources

As previously highlighted, sodium aluminosilicate (synthetic or mined/natural) is currently permitted for use as an anti-caking agent in the feed of animals at levels of up to 2% in the feed. Assuming a dairy cow weighs 650 kg and consumes 25 kg DM in the form of a TMR (Poncheki *et al.*, 2015; EFSA, 2017; University of Minnesota, 2020), the exposure by animals to sodium aluminosilicate from its use as an anti-caking agent at the maximum level of 2% in the diet (TMR as-fed; 45 to 55% DM) will range from 910 to 1,110 g/cow/day. However, it is recognized that during the period 14 to 21 days pre-calving, DM intake by dairy cows is reduced to around 12 kg/head/day (University of Minnesota, 2020), and on this basis, a 650 kg cow will consume between 436 and 534 g/head/day of sodium aluminosilicate from its presence as an anti-caking agent in feed at the maximum permitted level of 2% in the diet (TMR as-fed; 45 to 55% DM).

Protekta wishes to extend the current scope of use of sodium aluminosilicate in feed in the U.S. to include use in the diets of periparturient cows for a period of at least 14 days, and no more than 28 days, pre-calving in order to help maintain calcium balance. Dairy cows will be provided with up to 500 g X-Zelit®/head/day equivalent to between 375 and 400 g synthetic sodium aluminosilicate/head/day. Thus, the potential exposure of periparturient dairy cows to synthetic sodium aluminosilicate from its

presence as an anti-caking agent in the feed is in the same range (436 to 534 g/head/day) as from the proposed use (375 to 400 g/head/day).

The TMR will not be simultaneously supplemented with sodium or hydrated sodium calcium aluminosilicate as an anti-caking agent and X-Zelit® as an aid to maintain calcium balance, and therefore, no additional exposure by dairy cattle to aluminosilicates is anticipated under intended use of the additive.

Thus, the potential for deposition of synthetic sodium aluminosilicate or its components in the milk of dairy cows under the intended use as an aid to maintain calcium balance in periparturient dairy cows will not exceed that from the existing potential use as an anti-caking agent. However, recognizing that synthetic sodium aluminosilicate may not be widely used in dairy cattle feed, the safety for humans is considered further in the below sections.

6.2.2 Effect of Synthetic Sodium Aluminosilicate Supplementation on the Quality of Milk

As mentioned in Section 6.1.3.6, a number of the studies conducted in periparturient cows using synthetic sodium aluminosilicate evaluated parameters related to milk production, such as milk yield (or fat-corrected milk yield), milk composition and colostrum content (Thilsing *et al.*, 2002; Grabherr *et al.*, 2008; 2009b; Kerwin *et al.*, 2019; Khachlouf *et al.*, 2019). There was no impact of synthetic sodium aluminosilicate supplementation on the yield or composition of milk when measured up to 105 days post-calving. Khachlouf *et al.* (2019) also reported that synthetic sodium aluminosilicate supplementation of the diet at 200 g/head/day for 40 days has no effect on calcium, magnesium or phosphorus content of milk.

Thus, synthetic sodium aluminosilicate supplementation of periparturient cows for at least 14 days, and no more than 28 days, pre-calving at 400 g/head/day in the form of X-Zelit® by Protekta is not expected to adversely affect the quality of milk intended for human consumption.

6.2.3 Evaluation of the Effect of Synthetic Sodium Aluminosilicate on the Aluminum Content of Milk

As mentioned in Section 6.1.2, synthetic sodium aluminosilicate remains largely intact in the digestive tract of animals following ingestion, but a small portion appears to be partially degraded with the release of silicic acid, amorphous aluminum silicates and aluminum (Cook *et al.*, 1982; Grabherr *et al.*, 2009b).

Of the studies summarized in Section 2.5.5 in periparturient cows to evaluate the utility of synthetic sodium aluminosilicate, only one included analysis of serum aluminum levels in the animals (Thilsing *et al.*, 2007). The mean serum aluminum concentrations of all synthetic sodium aluminosilicate-supplemented cows were increased significantly during supplementation compared to before supplementation ($P<0.0001$). Serum aluminum concentrations were not significantly affected by prepartum calcium and/or phosphorus level, and the mean concentration of serum aluminum was 85.29 ± 9.06 µg/L during supplementation vs. 13.24 ± 2.17 µg/L before supplementation (corresponding to 0.0032 ± 0.0003 and 0.0005 ± 0.00008 mmol/L, respectively). Comparison measurements after supplementation of synthetic sodium aluminosilicate stopped were not conducted. However, the metabolism of aluminum is well-documented in the published literature, and it is anticipated that the mineral will be efficiently cleared from the body via the kidneys following supplementation with

synthetic sodium aluminosilicate (Allen, 1984; Ganrot, 1986; Cefali *et al.*, 1996; Krewski, 2007). Considering that supplementation of the diet of dairy cattle with synthetic sodium aluminosilicate ceases on the day of calving, no accumulation of aluminum in the milk of the lactating cows will occur from this proposed use as an aid to maintain calcium balance.

Unpublished data on the levels of aluminum deposited in the milk of dairy cows supplemented with synthetic sodium aluminosilicate pre-calving were reported in the most recent opinion by EFSA (2008). Dairy cows (10 cows/group) were each fed diets supplemented with 0, 250 or 500 g synthetic sodium aluminosilicate/head/day for a period pre-calving (not defined). There were no observed significant differences in milk aluminum levels in samples measured between 1 and 2 weeks after parturition. These data were not considered fully robust by EFSA but together with the known metabolic fate of synthetic sodium aluminosilicate in the dairy cattle, the Panel concluded there was no anticipated risk to humans under the conditions of intended use as a supplement for periparturient dairy cows at 500 g/head/day for 14 days pre-calving. These data provide corroborative evidence that the intended use of synthetic sodium aluminosilicate by Protekta will not pose a human food safety concern.

6.2.4 Exposure by Humans to Aluminum from the Food Supply

Humans are exposed to aluminum from a number of sources in the food supply, including drinking water, plant-based foods and food additives, as well as from the use of aluminum salts in anti-acids and some analgesics. Furthermore, the safety of aluminum from food has been evaluated by EFSA and a tolerable weekly intake (TWI) of 1 mg/kg body weight/day was established (EFSA, 2008 and 2011). For a 70 kg individual, the TWI is equivalent to 70 mg aluminum/person/day. Thus, background exposure by humans to aluminum from the normal diet and the use of over-the-counter pharmaceuticals is expected to be significantly greater than any residual levels that as a worst-case scenario, may be present in the milk from dairy cattle in the early postpartum period.

6.2.5 Overall Conclusions on Human Food Safety

Taken together, supplementation of the diet of dairy cattle by Protekta with synthetic sodium aluminosilicate at 400 g/head/day as X-Zelit® for a period of at least 14 days, and no more than 28 days, pre-calving is not expected to impact the quality of milk, production of milk, or composition of milk. In particular, any aluminum absorbed by periparturient dairy cows from synthetic sodium aluminosilicate supplementation is expected to be efficiently excreted in the urine and not to lead to any deposition or accumulation in milk.

6.3 SUMMARY AND BASIS FOR GRAS CONCLUSION

Protekta, Inc. (hereafter referred to as “Protekta”) intends to market synthetic sodium aluminosilicate for use as an ingredient in feed for periparturient (dry) dairy cows. The ingredient will be incorporated into dairy cattle feed as part of a formulation comprising 75 to 80% sodium aluminosilicate, 17 to 20% wheat (carrier) and 1 to 3% rapeseed oil (anti-dust agent), and marketed under the trade name X-Zelit®.

Synthetic sodium aluminosilicate is chemically synthesized by mixing silicates and aluminates which then undergo gelation and crystallization. The resulting product is filtered, washed and spray-dried to yield a white powder which has the chemical formula $Na_{12}Al_{12}Si_{12}O_{48}\cdot 27H_2O$ and is known as Zeolite A.

Synthetic sodium aluminosilicate is combined with wheat and rapeseed oil in order to produce the market formulation, X-Zelit® for use as a supplement in the feed of periparturient cows. All raw materials are considered safe and suitable for the intended use. The production processes to synthetic sodium aluminosilicate and X-Zelit® are conducted in accordance with cGMP and HACCP plans that are in place.

Appropriate feed grade specifications have been established for synthetic sodium aluminosilicate and X-Zelit® which include levels of the nominal components of sodium oxide (Na_2O), alumina (Al_2O_3) and silicon dioxide (SiO_2) to reflect the stoichiometry of the form of Zeolite (known as Zeolite A). Criteria are also defined to control the levels of moisture, heavy metal contaminants, and dioxins and PCBs.

The results of analysis of 3 commercial batches of synthetic sodium aluminosilicate and X-Zelit®, respectively confirm compliance with the product specification and acceptable batch to batch variation.

A shelf-life of 24 months is proposed for synthetic sodium aluminosilicate and X-Zelit® when stored in the original packaging in the absence of humidity under ambient conditions. As an inorganic (mineral) substance, no degradation is anticipated and stability studies were not considered necessary.

X-Zelit® is intended for use in the feed of periparturient dairy cows at levels of 500 g/head/day, delivering up to 400 g synthetic sodium aluminosilicate/cow/day, either as top-dressing or as part of the total mixed ration (TMR) for a period of at least 14 days, and no more than 28 days, pre-calving. Although Protekta normally recommends supplementing the TMR with synthetic sodium aluminosilicate for 14 days pre-calving, recognizing that in the U.S., dry cows are separated from the main herd for up to 28 days pre-calving, in practice exposure by the cows is expected to vary from at least 14 days, to up to 28 days.

Pursuant to Title 21 of the CFR Part 582.2727, sodium aluminosilicate is GRAS for use as an anti-caking agent in animal feed at levels not exceeding 2% in the diet (U.S. FDA 2020). Protekta wishes to extend the current scope of use of sodium aluminosilicate in feed in the U.S. to include the use in the diets of periparturient cows for a period of at least 14 days, and no more than 28 days, pre-calving as an aid to maintain calcium balance. Assuming a dairy cow weighs 650 kg and consumes 25 kg DM in the form of a TMR (Poncheki *et al.*, 2015; EFSA, 2017; University of Minnesota, 2020), the exposure by animals to sodium aluminosilicate from its use as an anti-caking agent at the maximum level of 2% in the diet (TMR as-fed; 45 to 55% DM) will range from 910 to 1,110 g/cow/day. These levels of potential intake of sodium aluminosilicate by dairy cows from its presence in the TMR as an anti-caking agent are approximately 2-fold higher than from the intended use as an aid to maintain calcium balance in periparturient cows for a period of 14 to 21 days pre-calving. However, it is recognized that during the period 14 to 21 days pre-calving, DM intake by dairy cows is reduced to around 12 kg/head/day (University of Minnesota, 2020), and on this basis, a 650 kg cow will consume between 436 and 534 g/head/day of sodium aluminosilicate from its presence as an anti-caking agent in feed at the maximum permitted level of 2% in the diet (TMR as-fed; 45 to 55% DM). These potential levels of exposure of periparturient dairy cows to sodium aluminosilicate from its presence as an anti-caking agent in the feed are in the same range as from the intended use as an aid to maintain calcium balance of 400 g/cow/day. The directions of use on the label of X-Zelit® will include a statement that the formulation should not be used in conjunction with sodium or hydrated sodium calcium aluminosilicate as an anti-caking agent.

Therefore, no additional exposure by dairy cattle to aluminosilicates beyond the current regulated maximum of 2% in the TMR is anticipated under the proposed extension of use of the substance.

The ability of synthetic sodium aluminosilicate to selectively bind calcium was demonstrated in an *in vitro* experiment using rumen fluid (Thilsing *et al*., 2006a). The results of the experiment indicated that synthetic sodium aluminosilicate not only bound to calcium but also to magnesium and phosphorus.

The utility of synthetic sodium aluminosilicate to bind calcium in the diet of periparturient cows has been demonstrated in a number of feeding studies in the target animal (Thilsing-Hansen & Jørgensen, 2001; Thilsing-Hansen *et al.*, 2002b, 2003; Thilsing *et al.*, 2007; Grabherr *et al.*, 2008; Grabherr *et al.* 2009a; Pallesen *et al.*, 2008; Kerwin *et al.*, 2019; Khachlouf *et al.,* 2019; Crookenden *et al.*, 2020). The studies were conducted on test articles considered equivalent to synthetic sodium aluminosilicate in the form of X-Zelit®, and under a range of conditions considered applicable to commercial farming conditions and covering the scope of intended use (i.e., for at least 14 days, and no more than 28 days, pre-calving at a level of 400 g/head/day).

Taken together, the results of the feeding studies support the utility of synthetic sodium aluminosilicate to help maintain calcium levels in dry cows when fed for a period of at least 14 days, and no more than 28 days, pre-calving. Kerwin *et al.* (2019) in particular demonstrated that serum calcium concentrations were significantly increased in cows fed diets supplemented with X-Zelit® at 500 g/head/day, equating to 400 g synthetic sodium aluminosilicate/head/day for 21 days before the expected calving date relative to control cows as parturition approached and during the early postpartum period.

The safety of synthetic sodium aluminosilicate for the intended use in periparturient cows is primarily based on the following: (a) the known metabolic fate in ruminants; and (b) a number of published studies in which synthetic sodium aluminosilicate was used to supplement the diet of periparturient cows for periods of 14 to 40 days before the expected calving date. Together these data form the pivotal body of evidence to support the safe use of synthetic sodium aluminosilicate for use in feed for periparturient cows.

Synthetic sodium aluminosilicate is expected to be largely excreted intact in the feces of the periparturient cows under the conditions of intended use of 400 g/head/day. A small portion of synthetic sodium aluminosilicate is expected to partially degrade in the digestive tract of dairy cattle with the release of silicic acid, amorphous aluminum silicates and aluminum (Cook *et al.*, 1982). The available studies in dairy cattle indicate that following partial hydrolysis in the digestive tract under the low pH conditions of the abomasum (Thilsing *et al.*, 2006a; 2007) or within the rumen (Grabherr *et al.*, 2009a), aluminum is released and at least some is absorbed resulting in a small increase in the serum concentration of the mineral (Thilsing *et al*., 2007 and Grabherr *et al*., 2009a). None of the studies evaluated the uptake of silicon by periparturient cows but availability of dietary phosphorus was reported to be impacted by synthetic sodium aluminosilicate supplementation, likely the result of the formation of non-absorbable compounds on combination with aluminum.

After oral ingestion, synthetic sodium aluminosilicate was mainly excreted in the feces in rats and dogs. A small fraction of the additive appeared to be hydrolyzed in the digestive tract of the animals and silicon (presumably as silicic acid) was absorbed, and ultimately excreted via the urine within 24 hours of administration. By comparison, negligible absorption of aluminum was observed. The metabolic fate is

not expected to differ significantly between species and these results can be extrapolated to dairy cattle.

The feeding studies identified in the published literature in which the diets of periparturient cows were fed diets supplemented with synthetic sodium aluminosilicate in order to help maintain calcium balance, also included parameters related to safety such as the effect on other mineral levels (phosphorus, magnesium and aluminum) and performance (DMI, incidence of disease and milk production). Taken together, the data indicate that although synthetic sodium aluminosilicate fed to dry cows for at least 14 days, and no more than 28 days, pre-calving at a level of 400 g/head/day may have an impact on plasma phosphorus and magnesium levels, and may reduce DMI, these effects are transient and do not result in any detrimental effects on general health, performance of milk production by the animals (Thilsing-Hansen *et al*., 2003). Plasma mineral levels and DMI return to with normal ranges within a short period postpartum with no long-term effects on the health of the animal.

It is also recognized that synthetic sodium aluminosilicate has an established history of use as an anti-caking agent in the feed of all animals in the U.S., EU and Canada. Furthermore, synthetic sodium aluminosilicate is currently permitted for use in feed for periparturient cows in the EU and Canada under comparable conditions of use to those presented herein. The existing history of use of synthetic sodium aluminosilicate in feed provides corroborative evidence of safety and is supported by a scientific opinion published by EFSA.

Additionally, synthetic sodium aluminosilicate has a history of use as a food additive and has been the subject of reviews by authoritative bodies including EFSA, JECFA and the U.S. FDA. As such a limited body of toxicological information is available to support the safety of synthetic sodium aluminosilicate which provides corroborative evidence of the safety for periparturient cows.

The primary toxicological studies were undertaken by Gloxhuber *et al*. (1983) using synthetic sodium aluminosilicate. Gloxhuber *et al*. (1983) performed several toxicology studies including metabolism, sub-chronic, as well as a combined chronic and carcinogenicity study from sodium aluminum silicate in rats and mice. In unpublished studies synthetic sodium aluminosilicate or an unspecified form of sodium aluminum silicate, were administered to rats in 14 day repeat dose and 90 day sub-chronic experiments. These studies investigated the potential for renal toxicity observed in the sub chronic experiment.

Sodium aluminosilicate (primarily synthetic sodium aluminosilicate) was not associated with any adverse systemic effects after oral administration. The only adverse effect reported was the formation of calculi in the bladder, which were consistently reported in several studies. In a urinary toxicity study these findings were explored, and it was reported that a white crystalline substance was apparent in the urine and as deposits in the kidney. Calculi formation could be due to absorption of silicon after oral ingestion of sodium aluminosilicate. The NOAEL from this study was reported by the authors to be 69 mg/kg body weight/day. Protekta intends to supplement the diet of periparturient cows 400 g/head/day of synthetic sodium aluminosilicate, equivalent to around 620 mg/kg body weight/day which exceeds the NOAEL of 69 mg/kg body weight/day derived from the urinary toxicity study. However, synthetic sodium aluminosilicate for use as an aid to maintain calcium balance is fed at comparable levels to those permitted as an anti-caking agent in 21 CFR §582.2727 and only for a short-period of 14 to 28 days

during the dry period. This short-term feeding duration during the dry period is not anticipated to be associated with any accumulation of deposits in the kidney or other long-term adverse effects.

Sodium aluminosilicate was not associated with any toxicological effects on the mother, embryonic or fetal development in teratogenicity studies. The findings provide corroborative evidence that the feeding of synthetic sodium aluminosilicate to dry cows should not have any adverse effect on the offspring.

Feeding studies in periparturient cows fed synthetic sodium aluminosilicate under conditions comparable with the intended use by Protekta indicate that there should be no effect on the composition of milk produced from lactating cows. It is recognized that small amounts of aluminum will be absorbed from synthetic sodium aluminosilicate by periparturient cows for the 14 to 28 days of administration pre-calving. However, considering that supplementation of the diet of dairy cattle with synthetic sodium aluminosilicate ceases on the day of calving, no accumulation of aluminum in the milk of the lactating cows will occur. Aluminum is expected to be efficiently metabolized by periparturient cows and to be excreted efficiently via the kidneys within a few days of supplementation ceasing.

The data and information summarized in this dossier demonstrate that Protekta's synthetic sodium aluminosilicate is produced in accordance with cGMP and meeting appropriate feed-grade specifications, is GRAS based on scientific procedures, under the conditions of intended use in the feed of periparturient cattle at levels of 400 g/head/day for a period of at least 14 days, and no more than 28 days, pre-calving.

PART 7. §570.255. LIST OF SUPPORTING DATA AND INFORMATION

7.1 LIST OF APPENDICES

ALL APPENDICES EXCEPT APPENDICES 04B, 04C, 04E, 07B, 08A, 08B and 08C ARE CONFIDENTIAL

Appendix 01 Manufacturing Information - Confidential
Appendix 02A Specifications Silicon Dioxide - Confidential
Appendix 02B Specifications Aluminum Oxide - Confidential
Appendix 02C Specifications Sodium Hydroxide - Confidential
Appendix 02D Specifications Wheat - Confidential
Appendix 02E Specifications Rapeseed Oil – Confidential
Appendix 03A GMP+ Certificate (Synthetic Sodium Aluminosilicate) - Confidential
Appendix 03B HACCP Plan (Synthetic Sodium Aluminosilicate) - Confidential
Appendix 03C GMP+ Certificate (X-Zelit®) - Confidential
Appendix 03D GMP+ Certificate (X-Zelit®) - Confidential
Appendix 04A Stoichiometry of Synthetic Sodium Aluminosilicate - Confidential
Appendix 04B Method of Analysis ISO 17294 (heavy metals)
Appendix 04C Method of Analysis (dioxins, PCBs & moisture)
Appendix 04D Stoichiometry of formulation - Confidential
Appendix 04E Method of Analysis ISO 697 (bulk density)
Appendix 05A Certificate of Analysis (Composition) 3 batches - Confidential
Appendix 05B Certificate of Analysis (Contaminants) Batch 17100001 - Confidential
Appendix 05C Certificate of Analysis (Contaminants) Batch 17229001 - Confidential
Appendix 05D Certificate of Analysis (Cr, Al) Batch 17100001 - Confidential
Appendix 06A Certificate of Analysis (X-Zelit) Batch 332775 - Confidential
Appendix 06B Certificate of Analysis (X-Zelit) Batch 342384 - Confidential
Appendix 06C Certificate of Analysis (X-Zelit) Batch 347568 - Confidential
Appendix 07A Packaging Material - Confidential
Appendix 07B Shelf Life Statement
Appendix 08A Market Trends Anti-Caking Agents
Appendix 08B (b) (4) PDS
Appendix 08C (b) (4) PDS

7.2 LIST OF ABBREVIATIONS

AAFCO	Association of American Feed Control Officials
ADME	Absorption, Distribution, Metabolism and Excretion
AOP	Antioxidant Potential
AUC	Area Under the Curve
AUC∞	Area Under the Curve Infinity
BCS	Body Condition Score
BHB	β-Hydroxybutyrate
BW	Body Weight
CAS	Chemistry Abstracts Service
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
cGMP	Current Good Manufacturing Practices
C_{max}	Maximum Concentration
CON	Control
Crea	Creatine
DCAD	Dietary Calcium-Anion Difference
DM	Dry Matter
DMI	Dry Matter Intake
DPD	Deoxypyridinoline
EC	European Commission
EFSA	European Food Safety Authority
EN	European Standards
EU	European Union
EXP	Experimental
FAO	Food and Agricultural Organization
FCM	Fat Corrected Milk
FDA	Food and Drug Administration
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FFA	Free Fatty Acid
FI	Feed Intake
FSMA	Food Safety Modernization Act
FSVP	Foreign Supplier Verification Program
GC	Gas Chromatography
GI	Gastrointestinal
GRAS	Generally Recognized as Safe
HACCP	Hazards and Critical Control Points
HB	Hemoglobin
ICES	Sum of PCBs 28, 52, 101, 138, 153 and 180
ICP	Inductively Coupled Plasma
INS	International Numbering System
ISO	International Organization for Standardization
i.v	Intravenous
JECFA	Joint Expert FAO/WHO Committee on Food Additives
MS	Mass Spectrometry
NABE	Net Acid-Base Excretion

NEFA	Non-Esterified Fatty Acid
NOAEL	No Observed Adverse Effect Level
NRC	National Research Council
NSD	No Significant Difference
OSi	Oxidant Status index
OP	Official Publication
PCB	Polychlorinated Biphenyl
PCDD	Polychlorinated Dibenzo-p-Dioxins
PCDF	Polychlorinated Dibenzofurans
PTH	Parathyroid Hormone
PTWI	Provisional Tolerable Weekly Intake
RONS	Reactive Oxygen and Nitrogen Species
SBM	Soybean Meal
SCFA	Short Chain Fatty Acid
SEM	Standard Error of the Mean
SSA	Synthetic Sodium Aluminosilicate
TEQ	Toxic Equivalency
T_{max}	Time of Maximum Concentration
TMR	Total Mixed Ration
TWI	Tolerable Weekly Intake
U.S.	United States
WHO	World Health Organization
XRD	X-Ray Diffraction

Note: Every abbreviation in the text is worded completely the first time and the abbreviation given in (). From then onwards, only the abbreviation is given in the text.

NOMENCLATURE

Synthetic sodium aluminosilicate refers to a range of amorphous and crystalline hydrated sodium aluminosilicates with varying proportions of sodium oxide (Na_2O), aluminum oxide (Al_2O) and silicon oxide (SiO_2) manufactured by reacting aluminum sulfate and sodium silicate followed by precipitation. The subject of the GRAS determination is synthetic sodium aluminosilicate with the chemical formula $Na_{12}Al_{12}Si_{12}O_{48}\cdot 27H_2O$ and which is known as Zeolite A.

7.3 REFERENCES

AAFCO, 2021. Association of American Feed Control Officials (AAFCO) Official Publication (OP). *Chapter Six – Feed Terms and Ingredient Definitions*.

Definition	Ingredient Name
22.3	Grain products
33.2	Vegetable fat, or oils

Allen, V.G., 1984. Influence of dietary aluminum on nutrient utilization in ruminants. *Journal of Animal Science*, *59*(3), pp.836-844.

Barton, B.A., Horst, R.L., Jorgensen, N.A. and DeLuca, H.F., 1981. Concentration of calcium, phosphorus, and 1, 25-dihydroxyvitamin D in plasma of dairy cows during the lactation cycle. *Journal of Dairy Science*, *64*(5), pp.850-852.

Benke, G.M. and Osborn, T.W., 1979. Urinary silicon excretion by rats following oral administration of silicon compounds. *Food and Cosmetics Toxicology*, *17*(2), pp.123-127.

Caixeta, L.S., Weber, W.J., Johnson, D.M., Faser, J., Visser, B.M. and Crooker, B.A., 2020. Effects of anionic supplement source in prepartum negative dietary cation-anion difference diets on serum calcium, feed intake, and lactational performance of multiparous dairy cows. *Journal of Dairy Science*, *103*(5), pp.4302-4314.

Cefali, E.A., Nolan, J.C., McConnell, W.R. and Walters, D.L., 1995. Pharmacokinetic study of zeolite A, sodium aluminosilicate, magnesium silicate, and aluminum hydroxide in dogs. *Pharmaceutical Research*, *12*(2), pp.270-274.

Cefali, E.A., Nolan, J.C., McConnell, W.R. and Walters, D.L., 1996. Bioavailability of silicon and aluminum from Zeolite A in dogs. *International Journal of Pharmaceutics*, *127*(2), pp.147-154.

CFIA. 2020. Administrative Schedules IV and V of the Feed Regulations (1983) in Canada [updated version obtained from the CFIA in June, 2020]. Enforcing regulation: https://laws-lois.justice.gc.ca/eng/regulations/sor-83-593/page-1.html

Schedule	Part	Entry	Single ingredient feed
IV	II	8.91	Synthetic sodium aluminosilicate

Chan, P.S., West, J.W. and Bernard, J.K., 2006. Effect of prepartum dietary calcium on intake and serum and urinary mineral concentrations of cows. *Journal of Dairy Science*, *89*(2), pp.704-713.

Chapinal, N., Carson, M.E., LeBlanc, S.J., Leslie, K.E., Godden, S., Capel, M., Santos, J.E.P., Overton, M.W. and Duffield, T.F., 2012. The association of serum metabolites in the transition period with milk production and early-lactation reproductive performance. *Journal of Dairy Science*, *95*(3), pp.1301-1309.

Cook, T.E., Cilley, W.A., Savitsky, A.C. and Wiers, B.H., 1982. Zeolite A hydrolysis and degradation. *Environmental Science & Technology*, *16*(6), pp.344-350.

Crookenden, M.A., Phyn, C.V.C., Turner, S.A., Loor, J.J., Smith, A.I., Lopreiato, V., Burke, C.R., Heiser, A. and Roche, J.R., 2020. Feeding synthetic zeolite to transition dairy cows alters neutrophil gene expression. *Journal of Dairy Science*, *103*(1), pp.723-736.

DeGaris, P.J. and Lean, I.J., 2008. Milk fever in dairy cows: A review of pathophysiology and control principles. *The Veterinary Journal*, *176*(1), pp.58-69.

Drackley, J.K., 1999. Biology of dairy cows during the transition period: The final frontier?. *Journal of Dairy Science*, *82*(11), pp.2259-2273.

EC, 2002. European Commission (EC). Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. *Official Journal of the European Union,*

L 140, p.10. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02002L0032-20191128

EC, 2008. European Commission (EC). Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. *Official Journal of the European Union,* L 354, p.16. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02008R1333-20201028&qid=1606730916725

EC, 2020. European Commission (EC). Commission Regulation (EU) 2020/354 of 4 March 2020 establishing a list of intended uses of feed intended for particular nutritional purposes and repealing Directive 2008/38/EC. *Official Journal of European Union,* L 67, pp.1-27. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32020R0354&qid=1604050610458

EFSA, 2004. European Food Safety Authority (EFSA). Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the request from the Commission on the use of synthetic sodium aluminium silicate (zeolite) for the reduction of risk of milk fever in dairy cows. *EFSA Journal*, *1*(11), p.160. Available at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2004.160

EFSA, 2007. European Food Safety Authority (EFSA). Opinion of the Scientific Panel on additives and products or substances used in animal feed [FEEDAP] on the safety of Zeolite (sodium aluminosilicate, synthetic) for the reduction of risk of milk fever in dairy cows. *EFSA Journal*, *5*(8), p.523. Available at: https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2007.523

EFSA, 2008. Scientific opinion of the European Food Safety Authority (EFSA) Panel on Food Additives, Flavorings, Processing Aids and Food Contact Materials (AFC). Safety of aluminum from dietary intake. *The EFSA Journal, 754*, pp.1-34. Available at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2008.754

EFSA, 2011. European Food Safety Authority. Statement on the evaluation of a new study related to the bioavailability of aluminum in food. Available at: https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2011.2157

EFSA, 2017. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M.D.L., Bories, G., Chesson, A., Cocconcelli, P.S., Flachowsky, G. and Gropp, J., 2017. Guidance on the assessment of the safety of feed additives for the target species. *EFSA Journal*, *15*(10), p.e05021. Available at: https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2017.5021

EFSA, 2020. European Food Safety Authority (EFSA) Panel on Food Additives and Flavorings (FAF). Re-evaluation of sodium aluminum silicate (E 554) and potassium aluminum silicate (E 555) as food additives. *EFSA Journal*, *18*(6), p.e06152. Available at: https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2020.6152

FDRL, 1973. Food and Drug Research Laboratories, Inc. (FDRL). Teratologic Evaluation of FDA 71-45 (Sodium Silicoaluminate). Prep. for: FDA, U. S. Food and Drug Administration; NTIS, National Technical Information Service, U.S. Department of Commerce, USA, PB 223 810. Cited in Hera, 2004.

Fürll, M. and Moritz, A., 2005. Spezielle Untersuchungen beim Wiederkäuer. *Klinische Labordiagnostik in der Tiermedizin*, *6*, pp.444-474. Cited in Grabherr *et al*., 2009a.

Ganrot, P.O., 1986. Metabolism and possible health effects of aluminum. *Environmental Health Perspectives*, *65*, pp.363-441.

Gaynor, P.J., Mueller, F.J., Miller, J.K., Ramsey, N., Goff, J.P. and Horst, R.L., 1989. Parturient hypocalcemia in Jersey cows fed alfalfa haylage-based diets with different cation to anion ratios. *Journal of Dairy Science*, *72*(10), pp.2525-2531.

Gloxhuber, C., Potokar, M., Pittermann, W., Wallat, S., Bartnik, F., Reuter, H. and Braig, S., 1983. Zeolithe A—A phosphate substitute for detergents: Toxicological investigation. *Food and Chemical Toxicology*, *21*(2), pp.209-220.

Goff, J.P., Reinhardt, T.A. and Horst, R.L., 1991. Enzymes and factors and action in normal controlling vitamin D metabolism and milk fever cows. *Journal of Dairy Science*, *74*(12), pp.4022-4032.

Goff, J.A. and Horst, R.L., 1993. Oral administration of calcium salts for treatment of hypocalcemia in cattle. *Journal of Dairy Science*, *76*(1), pp.101-108.

Goff, J.P., 2006. Macromineral physiology and application to the feeding of the dairy cow for prevention of milk fever and other periparturient mineral disorders. *Animal Feed Science and Technology*, *126*(3-4), pp.237-257.

Grabherr, H., Spolders, M., Flachowsky, G. and Fürll, M., 2008. Einfluss von Zeolith A auf die Futteraufnahme von trockenstehenden Milchkühen, auf den Mengen-und Spurenelementstoffwechsel im peripartalen Zeitraum sowie auf die Milchleistung in der folgenden Laktation. *Berl Munch Tierarztl Wochenschr*, pp.41-52. [Abstract only; DE]

Grabherr, H., Spolders, M., Fürll, M. and Flachowsky, G., 2009a. Effect of several doses of zeolite A on feed intake, energy metabolism and on mineral metabolism in dairy cows around calving. *Journal of Animal Physiology and Animal Nutrition*, *93*(2), pp.221-236.

Grabherr, H., Spolders, M., Lebzien, P., Hüther, L., Flachowsky, G., Fürll, M. and Grün, M., 2009b. Effect of zeolite A on rumen fermentation and phosphorus metabolism in dairy cows. *Archives of Animal Nutrition*, *63*(4), pp.321-336. Lawlor, A.J. and Tipping, E., 2003. Metals in bulk deposition and surface waters at two upland locations in northern England. *Environmental Pollution*, *121*(2), pp.153-167.

Health Canada, 2020. List of Permitted Anti-caking Agents (List of Permitted Food Additives). *Government of Canada*. Available at: https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/1-anticaking-agents.html

Henkel KGaA, unpublished data, Archive-No. R 0100197 (External Report, Tracor-Jitco Inc., 28.06.1979). Cited in Hera, 2004.

Henkel KGaA, unpublished data, Archive-No. R 0100196 (External Report, Tracor-Jitco Inc., 28.06.1979). Cited in Hera, 2004.

Henkel KGaA, unpublished data, Archive-No. TBD EX 0127 (External Report, Procter & Gamble, 06/1976). Cited in Hera, 2004.

Henkel KGaA, unpublished data, Archive-No. TBD EX 0129 (External Report, Procter & Gamble, 05/1976). Cited in Hera, 2004.

Henkel KGaA, unpublished data, Archive-No. TBD EX 0137 (External Pathology Report (13 weeks), Procter & Gamble, 03/1976). Cited in Hera, 2004.

Henkel KGaA, unpublished data, Archive-No. TBD EX 0143 (External Report, Procter & Gamble, 09/1975). Cited in Hera, 2004.

HERA, 2004. Human & Environmental Risk Assessment (HERA). Risk assessment on Sodium aluminium silicate. *Human & Environmental Risk Assessment on Ingredients of European Household Cleaning Products,* 3, pp.1-53. Cited in Hera, 2004.

Horst, R.L., Goff, J.P., Reinhardt, T.A. and Buxton, D.R., 1997. Strategies for preventing milk fever in dairy cattle. *Journal of Dairy Science*, *80*(7), pp.1269-1280.

JECFA, 2011. Joint, F.A.O. and WHO Expert Committee on Food Additives. *Safety evaluation of certain food additives and contaminants*. World Health Organization.

Karatzia, M.A., Pourliotis, K., Katsoulos, P.D. and Karatzias, H., 2010. Effects of in-feed inclusion of clinoptilolite on blood serum concentrations of aluminium and inorganic phosphorus and on ruminal pH and volatile fatty acid concentrations in dairy cows. *Biological Trace Element Research*, *142*(2), pp.159-166.

Kastenbaum, M.A. and Bowman, K.O., 1970. Tables for determining the statistical significance of mutation frequencies. *Mutation Research*, *9*, pp.527-49. [Abstract only]

Kerwin, A.L., Ryan, C.M., Leno, B.M., Jakobsen, M., Theilgaard, P., Barbano, D.M. and Overton, T.R., 2019. Effects of feeding synthetic zeolite A during the prepartum period on serum mineral concentration, oxidant status, and performance of multiparous Holstein cows. *Journal of Dairy Science*, *102*(6), pp.5191-5207.

Khachlouf , K., Hamed, H., Gdoura, R. and Gargouri, A., 2019. Effects of dietary Zeolite supplementation on milk yield and composition and blood minerals status in lactating dairy cows. *Journal of Applied Animal Research*, *47*(1), pp.54-62.

Krewski, D., Yokel, R.A., Nieboer, E., Borchelt, D., Cohen, J., Harry, J., Kacew, S., Lindsay, J., Mahfouz, A.M. and Rondeau, V., 2007. Human health risk assessment for aluminium, aluminium oxide, and aluminium hydroxide. *Journal of Toxicology and Environmental Health, Part B*, *10*(S1), pp.1-269.

Litton Bionetics, 1974. Mutagenic evaluation of compound FDA 71-45, synthetic silica sodium silicoaluminate. Prep. for: FDA, U. S. Food and Drug Administration; NTIS, National Technical Information Service, U. S. Department of Commerce, Springfield, VA, USA, PB 245 468. Cited in Hera 2004.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvão, and J. E. P. Santos., 2012. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in

dairy cows at low or high risk of developing uterine disease. *Journal of Dairy Science, 95*(12), pp.7158-7172.

Martinez, N., Sinedino, L.D.P., Bisinotto, R.S., Daetz, R., Risco, C.A., Galvão, K.N., Thatcher, W.W. and Santos, J.E.P., 2016. Effects of oral calcium supplementation on productive and reproductive performance in Holstein cows. *Journal of Dairy Science*, *99*(10), pp.8417-8430.

Moore, S.J., VandeHaar, M.J., Sharma, B.K., Pilbeam, T.E., Beede, D.K., Bucholtz, H.F., Liesman, J.S., Horst, R.L. and Goff, J.P., 2000. Effects of altering dietary cation-anion difference on calcium and energy metabolism in peripartum cows. *Journal of Dairy Science*, *83*(9), pp.2095-2104.

Neves, R.C., Leno, B.M., Bach, K.D. and McArt, J.A.A., 2018. Epidemiology of subclinical hypocalcemia in early-lactation Holstein dairy cows: The temporal associations of plasma calcium concentration in the first 4 days in milk with disease and milk production. *Journal of Dairy Science*, *101*(10), pp.9321-9331.

Nolen, G.A. and Dierckman, T.A., 1983. Test for aluminosilicate teratogenicity in rats. *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association*, *21*(5), p.697.

NRC, 2001. National Research Council (NRC). Nutrient Requirements of Dairy Cattle, 7th Revised Edition. The National Academies Press, Washington D.C. Available at: https://doi.org/10.17226/9825

NRC, 2005. National Research Council (NRC). Mineral Tolerances of Animals, 2nd Revised Edition. Chapter 2 – Maximum Tolerable Levels. Committee on Minerals and Toxic Substances in Diet and Water for Animals, Board on Agriculture and Natural Resources, Division on Earth and Life Sciences, National Research Council of the National Academies, Division on Earth and Life Sciences, National Research Council of the National Academies (NRC). The National Academies Press, Washington D.C. Available at: www.nap.edu

Pallesen, A., Pallesen, F., Jørgensen, R.J. and Thilsing, T., 2008. Effect of pre-calving zeolite, magnesium and phosphorus supplementation on periparturient serum mineral concentrations. *The Veterinary Journal*, *175*(2), pp.234-239.

Poncheki, J.K., Canha, M.L.S., Viechnieski, S.L. and Almeida, R.D., 2015. Analysis of daily body weight of dairy cows in early lactation and associations withproductive and reproductive performance. *Revista Brasileira de Zootecnia*, *44*(5), pp.187-192.

Prival, M.J., Simmon, V.F. and Mortelmans, K.E., 1991. Bacterial mutagenicity testing of 49 food ingredients gives very few positive results. *Mutation Research/Genetic Toxicology*, *260*(4), pp.321-329.

Reinhardt, T.A., Horst, R.L. and Goff, J.P., 1988. Calcium, phosphorus, and magnesium homeostasis in ruminants. *Veterinary Clinics of North America: Food Animal Practice*, *4*(2), pp.331-350.

Roche, J.R. and Berry, D.P., 2006. Periparturient climatic, animal, and management factors influencing the incidence of milk fever in grazing systems. *Journal of Dairy Science*, *89*(7), pp.2775-2783.

Rodríguez, E.M., Arís, A. and Bach, A., 2017. Associations between subclinical hypocalcemia and postparturient diseases in dairy cows. *Journal of Dairy Science*, *100*(9), pp.7427-7434.

SCF, 1991. Report of the Scientific Committee for Food (twenty-fifth series). First series of food additives of various technological functions. Commission of the European Communities. Directorate General Internal Market and Industrial Affairs. Report EUR 13416. Luxembourg. Available online: http://ec.europa.eu/food/fs/sc/scf/reports/scf_reports_25.pdf

Schröder, B., Rittmann, I., Pfeffer, E. and Breves, G., 1997. In vitro studies on calcium absorption from the gastrointestinal tract in small ruminants. *Journal of Comparative Physiology B*, *167*(1), pp.43-51.

Stojić, M., Ilić, V., Kovačić, M., Gvozdić, D., Stajković, S., Vejnović, B., Savić, O. and Fratrić, N., 2020. Effects of oral supplementation with organically modified clinoptilolite during prepartum period on colostrum quality in primiparous dairy cows. *Journal of Dairy Research*, pp.1-7.

Thilsing-Hansen, T. and Jørgensen, R.J., 2001. Hot topic: prevention of parturient paresis and subclinical hypocalcemia in dairy cows by zeolite A administration in the dry period. *Journal of Dairy Science*, *84*(3), pp.691-693.

Thilsing-Hansen, T., Jørgensen, R.J. and Østergaard, S., 2002a. Milk fever control principles: a review. *Acta Veterinaria Scandinavica*, *43*(1), pp.1-19.

Thilsing-Hansen, T., Jørgensen, R.J., Enemark, J.M.D. and Larsen, T., 2002b. The effect of zeolite A supplementation in the dry period on periparturient calcium, phosphorus, and magnesium homeostasis. *Journal of Dairy Science*, *85*(7), pp.1855-1862.

Thilsing-Hansen, T., Jørgensen, R.J., Enemark, J.M., Zelvyte, R. and Sederevicius, A., 2003. The effect of zeolite A supplementation in the dry period on blood mineral status around calving. *Acta Veterinaria Scandinavica. Supplementum*, *97*, p.87.

Thilsing, T., Jørgensen, R.J. and Poulsen, H.D., 2006a. In vitro binding capacity of zeolite A to calcium, phosphorus and magnesium in rumen fluid as influenced by changes in pH. *Journal of Veterinary Medicine Series A*, *53*(2), pp.57-64.

Thilsing, T., Jørgensen, R.J., Eiland, F. and Hansen, T., 2006b. Testing of a zeolite product for the reduction of the risk of milk fever in 22 private herds. Unpublished.

Thilsing, T., Larsen, T., Jørgensen, R.J. and Houe, H., 2007. The effect of dietary calcium and phosphorus supplementation in zeolite A treated dry cows on periparturient calcium and phosphorus homeostasis. *Journal of Veterinary Medicine Series A*, *54*(2), pp.82-91.

Turner, K.K., Nielsen, B.D., O'Connor-Robison, C.I., Rosenstein, D.S., Marks, B.P., Nielsen, F.H. and Orth, M.W., 2007a. Sodium zeolite a supplementation and its impact on the skeleton of dairy calves. *Biological Trace Element Research*, *121*(2), pp.149-159.

Turner, K.K., Nielsen, B.D., O'Connor-Robison, C.I., Nielsen, F.H. and Orth, M.W., 2007b. Tissue response to a supplement high in aluminum and silicon. *Biological Trace Element Research*, *121*(2), pp.134-148.

University of Minnesota, 2020. Feeding Total Mixed Rations. *University of Minnesota Extension.* Available at: https://extension.umn.edu/dairy-milking-cows/feeding-total-mixed-rations#mixer-scales-calibration-7316110

U.S. FDA, 2020. *United States Food and Drug Administration (U.S. FDA) Title 21 of the Code of Federal Regulations.* Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm

21 CFR Section	**Name**
73.1010	Alumina (dried aluminum hydroxide)
182.2727	Sodium aluminosilicate.
573.940	Silicon dioxide
582.1125	Aluminum sulfate
582.1127	Aluminum ammonium sulfate
582.1129	Aluminum potassium sulfate
582.1131	Aluminum sodium sulfate
582.1763	Sodium hydroxide
582.1781	Sodium aluminum phosphate
582.2122	Aluminum calcium silicate
582.2727	Sodium aluminosilicate
582.2729	Hydrated sodium calcium aluminosilicate.

Valdivia, R., Ammerman, C.B., Wilcox, C.J. and Henry, P.R., 1978. Effect of dietary aluminum on animal performance and tissue mineral levels in growing steers. *Journal of Animal Science*, *47*(6), pp.1351-1356.

Wilkens, M.R., Nelson, C.D., Hernandez, L.L. and McArt, J.A., 2020. Symposium review: Transition cow calcium homeostasis—Health effects of hypocalcemia and strategies for prevention. *Journal of Dairy Science*, *103*(3), pp.2909-2927.

Yokoi, H. and Enomoto, S., 1979. Effect of degree of polymerization of silicic acid on the gastrointestinal absorption of silicate in rats. *Chemical and Pharmaceutical Bulletin*, *27*(8), pp.1733-1739.

Zoltan, S., 2019. X-Zelit feeding experiment. Unpublished.

Study to Investigate the Potential for Deposition of Aluminum in the Milk of Dairy Cows Fed X-Zelit® in the Diet for 14-Days Pre-Calving

Background

X-Zelit® is currently marketed commercially in Canada for use as a calcium binder in dry dairy cows under the conditions of use specified in Schedule IV Part I of the Feed Regulations:

Synthetic sodium aluminosilicate (or synthetic sodium aluminum silicate or sodium aluminosilicate, synthetic) is a form of sodium aluminosilicate which has been chemically synthesized. It is produced by mixing silicates and aluminates together, which then undergo gelation and crystallization processing. The resulting product is then filtered, washed and spray-dried. It has the chemical formula () and a pore size of 4 Ångström. It consists predominately of aluminum oxide and silicon dioxide and to a less extent sodium oxide. If a facilitating agent or carrier is used, it must be approved for use in livestock feed. It shall be used at the approval rate and the common name or names shall be indicated on the label. This ingredient is approved for use as an aid to maintain calcium balance in periparturient dairy cows. It is to be fed only to dry dairy cows for a period of up to two weeks pre-calving, in an amount not to exceed 400 grams of synthetic sodium aluminosilicate per head per day.
It shall be labeled with a guarantee for actual g/kg of synthetic sodium aluminosilicate.
It shall also be labeled with the following statements: "This product is only approved for use in diets for dry dairy cows for a period of up to two weeks pre-calving, in an amount not to exceed 400 grams of synthetic sodium aluminosilicate per head per day" and "Caution: Do not use in association with anionic supplements".

In order to corroborate the conclusion that aluminum from X-Zelit® will not deposit in the milk of cows and pose a human health concern, a short study was conducted at a commercial farm in Guelph, Ontario that routinely uses the additive under commercial conditions in accordance with Schedule IV, Part 1 above.

Study Design

Twenty-one Holstein periparturient dairy cows used in the study are part of a (b) (4) near (b) (4) which routinely uses X-Zelit®. The dairy cows were housed under identical conditions and provided with the same total mixed ration (TMR). Ten of the dry dairy cows were provided with X-Zelit® at levels of 500 g/head/day, delivering up to 400 g synthetic sodium aluminosilicate/cow/day, as top-dressing for 14-days pre-calving (treatment group). The use of synthetic sodium aluminosilicate was discontinued at calving. The diet of the remaining 11 dry dairy cows was unsupplemented for the 14-day period pre-calving (control group).

At Day 7 post-calving, the samples of milk were collected from all of the cows on the study and sent for aluminum analysis at the (b) (4)

Milk samples collected: 7 April 2022

Results

The Certificate of Analysis for the milk samples is provided in Appendix S-1. The results of aluminum levels in milk of dairy cows on Day 7 after calving are presented in Table S-1. For the 10 dairy cows fed X-Zelit® for approximately 14-day pre-calving, the levels of aluminum in milk varied from 27 to 57 µg/kg (mean value of 39 ± 10 µg/L). By comparison, for the 11 dairy cows not fed X-Zelit® pre-calving, but otherwise fed identical total mixed ration (TMR) and housed under the same conditions, the aluminum levels in milk varied from 30 to 160 µg/kg (mean of 62 ± 40 µg/L).

Table S-1: Aluminum Content of Milk from Dairy Cows on Day 7 After Calving

Sample No.	Specimen (Cow) ID	X-Zelit® Supplementation (Pre-Calving)	Milk Al Content (µg/kg)
8	929	Yes	27
9	2237	Yes	30
4	1939	Yes	31
10	1826	Yes	33
5	2229	Yes	36
2	2054	Yes	38
3	1989	Yes	40
7	1905	Yes	48
6	2071	Yes	51
1	1984	Yes	57
14	593	No	30
12	585	No	32
21	2112	No	35
19	411	No	38
13	589	No	39
17	420	No	49
11	480	No	59
16	422	No	62
20	1680	No	66
15	584	No	110
18	61	No	160

Discussion

In 2007, EFSA evaluated the findings of an unpublished study in which periparturient cows were either unsupplemented or supplemented with 500 g/head/day of synthetic sodium aluminosilicate. Under the experimental conditions of the study, there were no significant differences in aluminum levels in milk between the control or treated animals one or two weeks after parturition. The standard deviation in all

groups was high with the treated group displaying mean aluminum levels in milk of 4.4 µg/L with individual values ranging from 0 to 30 µg/L.

Aluminum levels in milk will vary widely depending on the amount present in the feedstuff (e.g., soil levels for grazing animals) and other environmental factors. For example, aluminum levels in commercial milk samples have been reported to vary from 5 to 50 µg/L by Arruda *et al*. (1994), up to 70 µg/L by Lorenzo *et al*. (1999) and 141 ± 157 µg/L by González-Montaňa *et al*. (2019).

Only low levels of aluminum will be absorbed from synthetic sodium aluminosilicate (<1% of the administered amount. On absorption, these low levels are expected to be rapidly excreted via the kidneys, although large dietary aluminum levels can deposition in the bone (JECFA, 2013). Deposition of aluminum in the milk of dairy cows occurs naturally due the presence of aluminum in soil and the environment generally. The levels can vary widely as reported above, and the presence of X-Zelit® in the diet of dry dairy cows for 14-days pre-calving is not expected to result in any measurable increase in aluminum levels in milk. Any consumption of meat from dairy cows will be significantly longer after X-Zelit® administration and thus, no potential safety concerns from aluminum deposition are expected.

Conclusion

Overall, it may be concluded that under representative commercial conditions, inclusion of X-Zelit® in the diet at 500 g/head/day of periparturient dairy cows for 14-days pre-calving was not associated with any increase in aluminum levels in the milk at Day 7 post-calving. Thus, sodium aluminosilicate when fed to dry dairy cows under the conditions of intended use at 400 g/head/day (GRAS substance), equivalent to 500 g/head/day (market formulation) will not increase the levels of aluminum in milk.

The findings of this study corroborate information in the public domain which describes the metabolic fate of aluminum and low likelihood of deposition in edible tissues under the conditions of intended use of X-Zelit®.

References

Arruda, M.A., Quintela, M.J., Gallego, M. and Valcárcel, M., 1994. Direct analysis of milk for aluminium using electrothermal atomic absorption spectrometry. *Analyst*, *119*(8), pp.1695-1699.

EFSA, 2007. European Food Safety Authority (EFSA). Opinion of the Scientific Panel on additives and products or substances used in animal feed [FEEDAP] on the safety of Zeolite (sodium aluminosilicate, synthetic) for the reduction of risk of milk fever in dairy cows. *EFSA Journal*, *5*(8), p.523. Available at: https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2007.523

Fernandez-Lorenzo, J.R., Cocho, J.A., Rey-Goldar, M.L., Couce, M. and Fraga, J.M., 1999. Aluminum contents of human milk, cow's milk, and infant formulas. *Journal of Pediatric Gastroenterology and Nutrition*, *28*(3), pp.270-275.

González-Montaña, J.R., Senís, E., Alonso, A.J., Alonso, M.E., Alonso, M.P. and Domínguez, J.C., 2019. Some toxic metals (al, as, mo, hg) from cow's milk raised in a possibly contaminated area by different sources. *Environmental Science and Pollution Research*, *26*(28), pp.28909-28918.

JECFA, 2011. Joint FAO/WHO Expert Committee on Food Additives. *Safety evaluation of certain food additives and contaminants*. World Health Organization.

(b) (4)

FINAL Report
Case# **G22-028759**
Reported: 2022-Apr-26

Submitted By:
Client ID: **1790209**
PROTEKTA
457 CAMPBELL ST.
BOX 190
LUCKNOW, ON N0G 2H0

Owner:
(b) (4)

Animal ID: **Multiple**
Species/Breed: Bovine, Holstein
Commodity: Cattle, dairy

Phone: 519 357-8454

Specimen Taken Date: 2022-Apr-04
Specimen Sent Date: 2022-Apr-07
Specimen Received Date: 2022-Apr-07
Project #: AHL4

Specimen(s) received: 27 milk samples.

HISTORY
"No history provided"

Other **Method ID:Toxi-064**
Date Authorized: 2022-Apr-26 16:45

Sample ID	Client Sample ID	Specimen type	Sampling date / time	Test	Result	Units	Note
0001	1984	Milk	22-Apr-04	Other	(b) (4)	ug/g	
0002	2054	Milk	22-Apr-04	Other		ug/g	
0003	1989	Milk	22-Apr-04	Other		ug/g	
0004	1939	Milk	22-Apr-04	Other		ug/g	
0005	2229	Milk	22-Apr-04	Other		ug/g	
0006	2071	Milk	22-Apr-04	Other		ug/g	
0007	1905	Milk	22-Apr-04	Other		ug/g	
0008	929	Milk	22-Apr-04	Other		ug/g	
0009	2237	Milk	22-Apr-04	Other		ug/g	
0010	1826	Milk	22-Apr-04	Other		ug/g	
0011	480	Milk	22-Apr-04	Other		ug/g	

(b) (4)

Other **Method ID:Toxi-064**

Date Authorized: 2022-Apr-26 16:45

					(b) (4)	
0012	585	Milk	22-Apr-04	Other		ug/g
0013	589	Milk	22-Apr-04	Other		ug/g
0014	593	Milk	22-Apr-04	Other		ug/g
0015	584	Milk	22-Apr-04	Other		ug/g
0016	422	Milk	22-Apr-04	Other		ug/g
0017	420	Milk	22-Apr-04	Other		ug/g
0018	061	Milk	22-Apr-04	Other		ug/g
0019	411	Milk	22-Apr-04	Other		ug/g
0020	1680	Milk	22-Apr-04	Other		ug/g
0021	1680	Milk	22-Apr-04	Other		ug/g
0022	1680	Milk	22-Apr-04	Other		ug/g
0023	1680	Milk	22-Apr-04	Other		ug/g
0024	2112	Milk	22-Apr-04	Other		ug/g
0025	2112	Milk	22-Apr-04	Other		ug/g
0026	2112	Milk	22-Apr-04	Other		ug/g
0027	2112	Milk	22-Apr-04	Other		ug/g

Comments:

Results in sample as received for Aluminum (ug/g, ppm).

Sample Ids 22-028759-0015, -0018, -0025 and -0027 are reported as an average of replicate analysis.

(b) (4)

Communication History

Report date	Contact	Reported tests
2022-Apr-07 15:33	morten@protekta.com	

Confidential Manufacturing Information: Synthetic Sodium Aluminosilicate

2.2 METHOD OF MANUFACTURE

2.2.1 Raw Materials and Processing Aids for the Manufacture of Synthetic Sodium Aluminosilicate

The raw materials and processing aids used in the production of synthetic sodium aluminosilicate are listed in Table 2.2. All of the raw materials and processing aids are considered safe and suitable for the manufacture of a feed ingredient. Silicon dioxide and sodium hydroxide are recognized technical additives for direct addition to feed, and although there is no listing in the CFR or Association of American Feed Control Officials (AAFCO) Official Publication (OP) for alumina, other aluminum salts such as aluminum sulfate and aluminum calcium silicate are permitted for use. Specifications for the raw materials are provided in Appendices 02A to 02C (CONFIDENTIAL).

Table 2.2: Raw Materials used in the Manufacture of Synthetic Sodium Aluminosilicate

Raw Material	Function	Regulatory Status
Silicon dioxide (silica sand)	Source of silicon dioxide	Permitted for use as a food additive in specific feed components as (a) an anti-caking agent and/or grinding aid; use levels in the feed components range from 0.8% in piperazine, piperazine salts to 3% in vitamins; (b) a carrier at levels of 50% in flavors and 95% in selenomethionine hydroxy analogue (21 CFR §573.940; U.S. FDA, 2020) [Appendix 02A]
Sodium hydroxide	Source of sodium	GRAS for use as a general purpose food additive in accordance with good manufacturing and feeding practice (21 CFR §582.1763; U.S. FDA, 2020) [Appendix 02B]
Aluminum oxide (alumina)	Source of aluminum oxide	Aluminum sulfate, aluminum ammonium sulfate, aluminum potassium sulfate, aluminum sodium sulfate, sodium aluminum phosphate and aluminum calcium silicate are GRAS for use as general purpose food additives in accordance with good manufacturing and feeding practice (21 CFR §582.1125, §582.1127, §582.1129, §582.1131, §582.1781, §582.2122; U.S. FDA, 2020) [Appendix 02C] Note: alumina (aluminum hydroxide) is permitted for use as a color additive for coloring drugs in amounts consistent with good manufacturing practice to color drugs generally (21 CFR§73.1010; U.S. FDA, 2020)
Demineralized water	Processing aid	Not applicable

Abbreviations: CFR = Code of Federal Regulations;

2.2.2 Raw Materials and Processing Aids for Formulation of X-Zelit®

As mentioned in the introduction, synthetic sodium aluminosilicate is marketed for the intended use for periparturient dairy cattle as a formulation under the trade name X-Zelit®. (b) (4) The formulation components are listed in Table 2.3. All components are considered feed-grade,

and safe and suitable for the intended use. Specifications for the formulation components are provided in Appendices 02D and 02E (CONFIDENTIAL).

Table 2.3: Raw Materials used in the Formulation of X-Zelit®

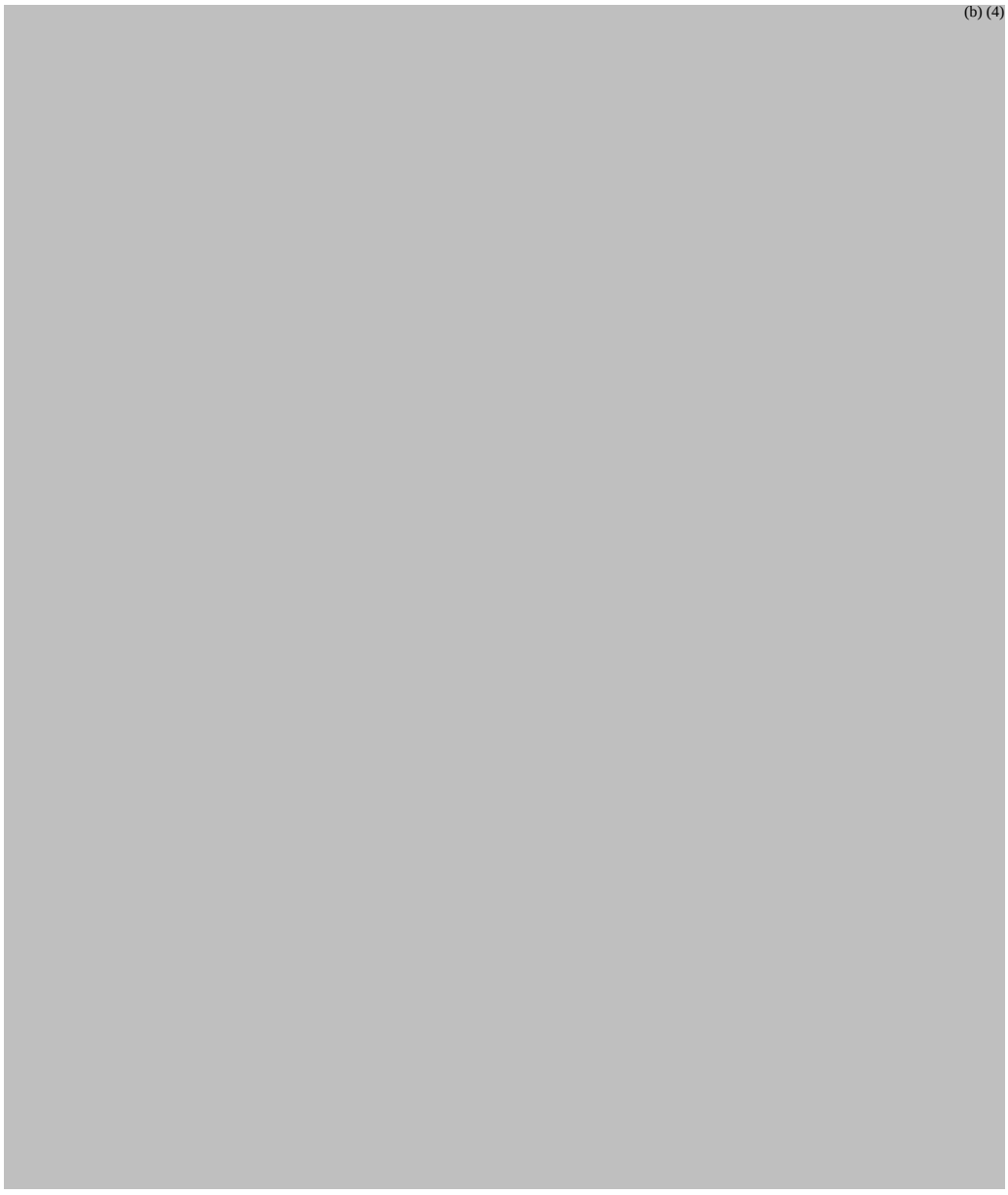
Raw Material	Function	Regulatory Status
Sodium aluminosilicate	Aid to maintain calcium balance	GRAS for use as an anti-caking agent in feed at levels not to exceed 2% in the diet (21 CFR §582.2727; U.S. FDA, 2020)
Wheat (*Triticum aestivum* L., *Triticum durum* Desf. and other wheat cultivars)	Carrier	No official definition but considered a common food and listed within the collective ingredient definition for Grain Products in the AAFCO OP (Collective Term 22.3; AAFCO, 2021) [Appendix 02D]
Rapeseed (*Brassica napus*) oil	Anti-dust agent	Vegetable fat listed in the AAFCO OP (Ingredient definition 33.2; AAFCO, 2021) [Appendix 02E]
Potable water	Processing aid	Not applicable

Abbreviations: AAFCO = Association of American Feed Control Officials; CFR = Code of Federal Regulations; OP = Official Publication.

2.2.3 Manufacturing Process to Synthetic Sodium Aluminosilicate

An overview of the manufacturing process is provided in Figure 2.1. Each stage of the process is detailed in turn below.

Figure 2.1: Flow-Chart of the Manufacture of Synthetic Sodium Aluminosilicate



(b) (4)

2.2.4 Synthetic Sodium Aluminosilicate Production Controls

Synthetic sodium aluminosilicate is manufactured in accordance with cGMP and a Hazard Analysis Critical Control Point (HACCP) system is in place. The site is GMP+ certified and a copy of the certification is provided in Appendix 03A. A copy of the HACCP plan is provided in Appendix 03B. The manufacturer will comply with the requirements for importing feed into the U.S. as laid down by the Food Safety Modernization Act (FSMA) including the foreign supplier verification program (FSVP) and Bioterrorism Act (2002).

2.2.5 Formulation of the Feed Product (X-Zelit®)

An overview of the manufacturing process is provided in Figure 2.2. Each stage of the process is detailed in turn below.

Figure 2.2: Flow-Chart of the Manufacture of X-Zelit®

Mixing

2.2.6 Production Controls

X-Zelit® is manufactured in accordance with cGMP and a HACCP plan is in place. The production facilities are GMP+ Certified and copies of the Certification are provided in Appendix 03C and D.

SILICA SAND SPECIFICATIONS

Technical datasheet silica sand 2a special

The silica sand – 2a special – is, after mining, washed, sieved and classified. This quality is moist available.

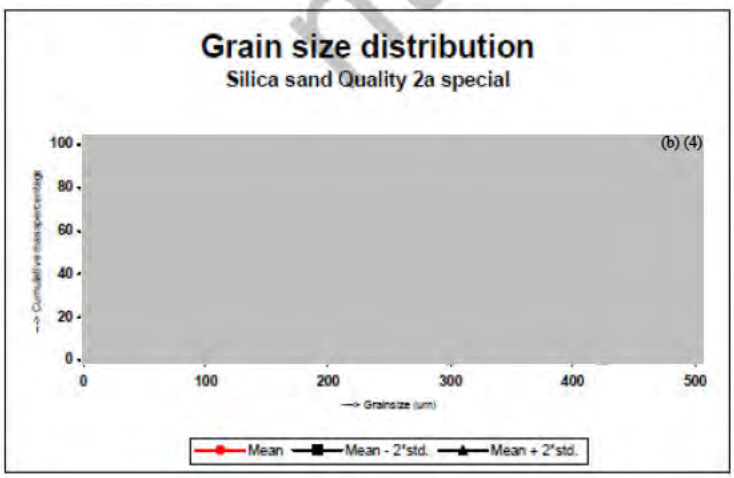
(b) (4)

Chemical analysis (based on dry material)

SiO_2	(b) (4)
Al_2O_3	
Fe_2O_3	
TiO_2	

Granulometric data

fraction	cumulative mass perc.
(µm)	(b) (4)
≥ 425	
≥ 300	
≥ 250	
≥ 212	
≥ 180	
≥ 150	
≥ 125	
≥ 106	
≥ 75	
< 75	
D50 (mean size)	



Physical characteristics

density	(b) (4)
bulk density	
loss on ignition	
pH	
hardness	
H_2O	

Other information

Nomenclature	silica sand
Product characteristics	naturally occurring
Chemical notation	SiO_2
CAS Number	14808-60-7
EINECS Number	238-878-4

Technical Data

ANALYSE GRANULOMETRIQUE ET CARACTERISTIQUES PHYSIQUES
Méthode: ISO-tamisage

	(b) (4)	
D50		µm
> 355 µm		%
> 250 µm		%
> 180 µm		%
> 125 µm		%
< 63 µm		
densité réelle		kg/dm³
densité apparente		kg/dm³
dureté		Mohs
pH		
perte au feu		%
OD436		%T

ANALYSE CHIMIQUE (XRF) %

	(b) (4)
SiO_2	
Fe_2O_3	
Al_2O_3	
TiO_2	
K_2O	
CaO	

ALUMINA SPECIFICATIONS

Chemical formula and structure / Formule chimique et structure/ Fórmula química y estructura			
$Al_2O_3 \cdot 3H_2O$			Gibbsite (Hydrargillite)
Chem. analysis/ Analyse chimique/ Análisis químico Analytical method following/ Méthode d'analyse utilisée/ Método de análisis empleado		Typical	Specification
Wet basis analysis			
Moisture	Thermogravimetry	(b) (4)	< 5.0 %
Al_2O_3	Calculation		> 61.5
Dry basis analysis			
Fe_2O_3	XRF[1]/ ISO 1268		< 0.020 %
Na_2O	XRF/ ISO 1268		< 0.30 %
SiO_2	XRF/ ISO 1268		< 0.015 %
Phys. Properties / Propriété phys. / Propiedades físicas			
Median particle size (d50)	Laser diffraction		---
Q1 (d25)	Laser diffraction		---
Q3 (d75)	Laser diffraction		---
< 44 micras	Laser diffraction		---
< 74 micras	Laser diffraction		---
Loss of ignition (LOI)	ISO 806		---

Product Specification: Aluminium Hydroxide

Chemical and Physical Analysis		Value	Method
		(b) (4)	
Moisture (20° - 105° C)	%		ISO 806
Al_2O_3	%		Dif
SiO_2	%		XRF*
Fe_2O_3	%		XRF*
TiO_2	%		XRF*
CaO	%		XRF*
Na_2O	%		XRF*
L.O.I. (105° - 1000° C)	%		ISO 806*
Granulometry < 45 µm	%		ISO 13320-1*
			* Data of dried material (105° C)

General Information

Appearance
White powder

Packaging
BigBags or bulk delivery

Safety Information
This product is non-hazardous, non-toxic and not inflammable. Since legislation may vary from country to country the specific instructions have to be adhered to.

REACH reference number
01-2119529246-39-0009

NaOH 50% SPECIFICATIONS

Product Specification Sheet
Sodium Hydroxide Solution (NaOH 50%) Membrane Process
CAS-No. 1310-73-2 (NaOH)

Caustic Soda Solution — Molecular Weight: 39,997 g/mol

General Properties
Sodium hydroxide solution is a clear, colour- and odourless solution of sodium hydroxide in water.
The solution is corrosive to various materials, e.g. aluminium, magnesium and zinc compounds, glass, enamel and many plastic materials.

Delivery - Form
Sodium hydroxide solution; 50%
Transport: road tank car, rail tank car, barge/vessel, ocean vessel

Material Properties	Test method	Unit	Typical value
Total alkalinity as % NaOH	DIN EN 896	% wt	49,0 – 51,0
Sodium carbonate (Na_2CO_3)	DIN EN ISO 9963-2	% wt	max. 0,1
Chloride (Cl^-)	DIN EN ISO 10 304	mg/kg	max. 50
Sulphate (SO_4^{2-})	DIN EN ISO 10 304	mg/kg	max. 50
Chlorate (ClO_3^-)	DIN EN ISO 10 304	mg/kg	max. 10
Iron (Fe)	ISO 6685	mg/kg	max. 5
Sillicon (Si)	DIN EN ISO 11 885	mg/kg	max. 10
Calcium (Ca)	DIN EN ISO 11 885	mg/kg	max. 10
Heavy metals as lead (Pb)	DIN EN ISO 11 885	mg/kg	max. 10
Aluminium (Al)	DIN EN ISO 11 885	mg/kg	max. 1

*) The values given above are typical test results which should be use as a guide only.

Our caustic soda solution comply with the requirements of DIN EN 896 (caustic soda solution for water purification), the additive traffic regulation of the German food and consumer law (LMBG) and the Food Chemicals Codex (FCC IV) of the US Food and Drug Administration (FDA).

Caustic soda solution is certified in the European Union as food additive registered under E-Nr.524.

Physical Data (bibliographical references)

Properties	50%	Value
Density at 20 °C	1525	kg/m³
Dynamic Viscosity		
at 20 °C at 50 °C	85 14	mPa*s mPa*s
Freezing Point (solidification point)	11,6	°C
Boiling Temperature	143	°C
Specific Heat		
at 20 °C at 50 °C	3240 3220	J/(kg K) J/(kg K)

Safety and transport information and toxicological data are included in our actual material safety data sheet (MSDS)

LIQUID CAUSTIC SODA 50% -

CHEMICAL GRADE – Membrane Cell Technology

PDS-1110-0080

Some applications of this product may be regulated or restricted by national or international standards (e.g. for food additives, water treatment, the pharmaceutical industry, etc.). The buyer and the eventual user, in his sole and entire liability, shall respect those standards, orders of any relevant authority, and all existing patents and intellectual properties rights; and shall comply with the laws and the regulations applicable to our products and/or to his activity. The buyer and the eventual user must independently determine the suitability of this product for any particular purpose and its manner of use. Please contact us for further information on grades developed for a specific end-use.

Product Identification:

Liquid Caustic Soda is a clear, colourless, odourless and viscous liquid.

Sodium Hydroxide	NaOH	ID Number	011-002-00-6
Molecular Weight	40,01	EC Number (EINECS)	215-185-5
CAS Number	1310-73-2	UN Number	1824

For further information on product handling, transport, storage and product properties please consult our us.

Product Specifications:

Characteristic	Unit	Value[1] (b) (4)	Method of analysis[2]
Total Alkalinity (NaOH)	g/kg		Titrimetry (ISO 979)
Sodium Carbonate (Na_2CO_3)	g/kg		Titrimetry (ISO 3196)
Sodium Sulphate (Na_2SO_4)	mg/kg		Ion chromatography (ASTM E1787)
Sodium Chloride (NaCl)	mg/kg		Ion chromatography (ASTM E1787)
Iron (Fe)	mg/kg		Photometry (ISO 6685) and/or ICP-AES[3]* (ISO 11885) *(*) Inductively coupled plasma atomic emission spectroscopy*
Mercury (Hg)	mg/kg	≤ 0,1	Flameless atomic absorption spectrometry (ISO 5993)
Sodium Chlorate ($NaClO_3$)	g/kg	≤ 7	Titrimetry
Arsenic (As)	mg/kg	≤ 2	
Cadmium (Cd)	mg/kg	≤ 1	
Chromium (Cr)	mg/kg	≤ 1	ICP-AES[3]* (ISO 11885) *(*) Inductively coupled plasma atomic emission spectroscopy*
Nickel (Ni)	mg/kg	≤ 2	
Lead (Pb)	mg/kg	≤ 0,5	
Antimony (Sb)	mg/kg	≤ 5	
Selenium (Se)	mg/kg	≤ 5	
Insoluble substances	-	conform	Visual test
Organic compounds	-	conform	

(1) The values are expressed per 100% solids, except for the concentration.

(2) The product is analysed with the mentioned methods or using local methods depending on laboratory equipment.

(3) As Heavy Metals following elements are analysed: As, Cd, Cr, Hg, Ni, Pb, Sb and Se.

(b) (4)	***Data sheet for straight feeding stuffs of the positive list***	Version:	1
		Redigeret dato:	04-12-2019
		Udskrevet den:	04-12-2019

Wheat

Manufacturer / distributor	(b) (4)
Country of origin	Denmark
Feeding stuff / product (designation according to the positive list / trade and brand name; supplemented with the number acc. to the positive list)	Wheat (b) (4)
Product description (Characterization of the product and indication of the production procedure)	(b) (4)
Information about the production process Details about the constituents of the basic raw material and about further components, if applicable (flow chart about the processing steps and material flows)	N/A
Processing aids (including all other added substances)	Natural gas/mineral oil for drying.
Information about the composition Average analytical data about the most important valuable constituents	Moisture: (b) (4)
Information about relevant undesirable substances for the risk-oriented self-control (e.g. HACCP)	Monitoring for undesirable substances are performed according to risk-based monitoring plan in accordance with GMP+ BA4
Details about shelf life, storage and transport (spoilage)	Storage: Cool and dry. To avoid condensation ventilated storage area is recommended. Transport: Vehicles needs to be clean, dry and free of smell.
Safety information (flammable, explosive, caustic etc.)	Dust may burn if suspended in air and may create a flash fire/explosion hazard. Avoid ignition sources. Avoid breathing dust. Monitor and avoid heat generation during storage.
Information about specific analytical problems	

Datasheet crude degummed rapeseed oil

Manufacturer / Distributor	(b) (4)
Product discription	Rapeseed oil optained from 00-rapeseed (b) (4)
Information about the production process	Rapeseed oil is extracted from whole (b) (4)
Processing aids	./.
Information about the composition	Rapeseed oil consist of (b) (4) crude fat. Rapeseed oil also contains natural vitamin E as well as dyes, free fatty acids and lecithins
Information about relevant undesirable substances during the the risk-oriented self control (e.g. HACCP)	Control analyzes acc. QS Guide Feed Monitoring
Details about shelf life, storage and transport (spoiling)	Vegetable oils may oxidize (become rancid) on prolongend and improper storage. Storage: cool, dark, if possible under exclusion of air. Shelf live under above storage conditions: 6 months
Safety information (flammable, explosive, caustic etc.)	Rapeseed oil is non toxic, biodegradable and requires no safety precautions during storage and transport.
Indication of critical constituents (endogenous origin or contamination) CCP from HACCP evalua ion	./. The review under the HACCP concept did not result in the definition of critical control points
Informations about specific analytical problems	Free fatty acids, phosphatide-P (Lecithin)
GMO	Not subject to labelling in the sence of the regulations (EU) 1829/2003 and 1830/2003)

September 2019

(b) (4)

Process Certificate

GMP+ Feed Certification scheme – module Feed Safety Assurance issued to:

(b) (4)

Standard(s)

B3 Trade, collection and storage & transhipment.

The certificate relates to the process(es)

TR Trade in animal feed.

(free section)

- ☐ TCF The trade in compound feed.
- ☒ TPR The trade in premixtures.
- ☒ TFM The trade in feed materials.
- ☒ TFA The trade in feed additives.
- ☐ TPCF The trade in pet foods (compound feed).
- ☐ TPFM The trade in pet foods (feed materials).

(b) (4)

(b) (4)

OVERVIEW ANALYZE PROGRAMM E554 ZEOLITE 4AMA

No.	Process phase	Description of hazards	Type of measure	Reference (internal documents producer)
1.a.				
1.b.				
1.c.				
1.d.				
2.a.				
2.b.				
2.c.				
3.				
4.				
5.				
6.				
7.				
8.				

(b) (4)

(b) (4)

Synthetic Sodium Aluminosilicate

Composition: Theoretical Calculations

	Stoichiometry	MW (g/mol)	Content in Additive (g/mol)	Content in Additive (%)	Specification (%)
Na_2O					(b) (4)
Al_2O_3					
SiO_2					
H_2O (Bound)					
Molecular Weight of $Na_{12}Al_{12}Si_{12}O_{48}\cdot 27H_2O$ = 2190 (g/mol)					

BS EN ISO 17294-2:2016

BSI Standards Publication

Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS)

Part 2: Determination of selected elements including uranium isotopes (ISO 17294-2:2016)

bsi.

National foreword

This British Standard is the UK implementation of EN ISO 17294-2:2016. It supersedes BS EN ISO 17294-2:2004 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee EH/3/2, Physical chemical and biochemical methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

Published by BSI Standards Limited 2016

ISBN 978 0 580 82291 9

ICS 13.060.50

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 31 August 2016.

Amendments/corrigenda issued since publication

Date	Text affected

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN ISO 17294-2

August 2016

ICS 13.060.50

Supersedes EN ISO 17294-2:2004

English Version

Water quality - Application of inductively coupled plasma mass spectrometry (ICP-MS) - Part 2: Determination of selected elements including uranium isotopes (ISO 17294-2:2016)

Qualité de l'eau - Application de la spectrométrie de masse avec plasma à couplage inductif (ICP-MS) - Partie 2: Dosage des éléments sélectionnés y compris les isotopes d'uranium (ISO 17294-2:2016)

Wasserbeschaffenheit - Anwendung der induktiv gekoppelten Plasma-Massenspektrometrie (ICP-MS) - Teil 2: Bestimmung von ausgewählten Elementen einschließlich Uran-Isotope (ISO 17294-2:2016)

This European Standard was approved by CEN on 28 February 2016.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Ref. No. EN ISO 17294-2:2016 E

European foreword

This document (EN ISO 17294-2:2016) has been prepared by Technical Committee ISO/TC 147 "Water quality" in collaboration with Technical Committee CEN/TC 230 "Water analysis" the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2017, and conflicting national standards shall be withdrawn at the latest by February 2017.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN ISO 17294-2:2004.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Endorsement notice

The text of ISO 17294-2:2016 has been approved by CEN as EN ISO 17294-2:2016 without any modification.

Contents

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html

The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 2, *Physical, chemical and biochemical methods*.

This second edition cancels and replaces the first edition (ISO 17294-2:2003), which has been technically revised.

ISO 17294 consists of the following parts, under the general title *Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS)*:

— *Part 1: General guidelines*

— *Part 2: Determination of selected elements including uranium isotopes*

Introduction

When applying this part of ISO 17294, it is necessary in each case, depending on the range to be tested, to determine if and to what extent additional conditions are to be established.

INTERNATIONAL STANDARD ISO 17294-2:2016(E)

Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS) —

Part 2: Determination of selected elements including uranium isotopes

WARNING — Persons using this part of ISO 17294 should be familiar with normal laboratory practice. This part of ISO 17294 does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests, conducted in accordance with this part of ISO 17294, be carried out by suitably qualified staff.

1 Scope

This part of ISO 17294 specifies a method for the determination of the elements aluminium, antimony, arsenic, barium, beryllium, bismuth, boron, cadmium, caesium, calcium, cerium, chromium, cobalt, copper, dysprosium, erbium, gadolinium, gallium, germanium, gold, hafnium, holmium, indium, iridium, iron, lanthanum, lead, lithium, lutetium, magnesium, manganese, mercury, molybdenum, neodymium, nickel, palladium, phosphorus, platinum, potassium, praseodymium, rubidium, rhenium, rhodium, ruthenium, samarium, scandium, selenium, silver, sodium, strontium, terbium, tellurium, thorium, thallium, thulium, tin, tungsten, uranium and its isotopes, vanadium, yttrium, ytterbium, zinc and zirconium in water (for example, drinking water, surface water, ground water, waste water and eluates).

Taking into account the specific and additionally occurring interferences, these elements can also be determined in digests of water, sludges and sediments (for example, digests of water as described in ISO 15587-1 or ISO 15587-2).

The working range depends on the matrix and the interferences encountered. In drinking water and relatively unpolluted waters, the limit of quantification (xLQ) lies between 0,002 µg/l and 1,0 µg/l for most elements (see Table 1). The working range typically covers concentrations between several pg/l and mg/l depending on the element and pre-defined requirements.

The quantification limits of most elements are affected by blank contamination and depend predominantly on the laboratory air-handling facilities available on the purity of reagents and the cleanliness of glassware.

The lower limit of quantification is higher in cases where the determination suffers from interferences (see Clause 5) or memory effects (see ISO 17294-1:2004, 8.2).

Table 1 — Lower limits of quantification (*x*LQ) for unpolluted water

Element	Isotope often used	Limit of quantification[a] µg/l	Element	Isotope often used	Limit of quantification[a] µg/l	Element	Isotope often used	Limit of quantification[a] µg/l
Ag	^{107}Ag	0,5	Hf	^{178}Hf	0,1	Ru	^{102}Ru	0,1
	^{109}Ag	0,5	Hg	^{202}Hg	0,05	Sb	^{121}Sb	0,2
Al	^{27}Al	1	Ho	^{165}Ho	0,1		^{123}Sb	0,2
As	^{75}As[c]	0,1	In	^{115}In	0,1	Sc	^{45}Sc	5
Au	^{197}Au	0,5	Ir	^{193}Ir	0,1	Se	^{77}Se[c]	1
B	^{10}B	1	K	^{39}K[c]	5		^{78}Se[c]	0,1
	^{11}B	1	La	^{139}La	0,1		^{82}Se	1
Ba	^{137}Ba	3	Li	^{6}Li	10	Sm	^{147}Sm	0,1
	^{138}Ba	0,5		^{7}Li	1	Sn	^{118}Sn	1
Be	^{9}Be	0,1	Lu	^{175}Lu	0,1		^{120}Sn	1
Bi	^{209}Bi	0,5	Mg	^{24}Mg	1	Sr	^{86}Sr	0,5
Ca	^{43}Ca	100		^{25}Mg	10		^{88}Sr	0,3
	^{44}Ca	50	Mn	^{55}Mn	0,1	Tb	^{159}Tb	0,1
	^{40}Ca	10	Mo	^{95}Mo	0,5	Te	^{126}Te	2
Cd	^{111}Cd	0,1		^{98}Mo	0,3	Th	^{232}Th	0,1
	^{114}Cd	0,5	Na	^{23}Na	10	Tl	^{203}Tl	0,2
Ce	^{140}Ce	0,1	Nd	^{146}Nd	0,1		^{205}Tl	0,1
Co	^{59}Co	0,2	Ni	^{58}Ni[c]	0,1	Tm	^{169}Tm	0,1
Cr	^{52}Cr[c]	0,1		^{60}Ni[c]	0,1	U	^{238}U	0,1
	^{53}Cr	5	P	^{31}P	5		^{235}U	10^{-4}
Cs	^{133}Cs	0,1	Pb	^{206}Pb[b]	0,2		^{234}U	10^{-5}
Cu	^{63}Cu	0,1		^{207}Pb[b]	0,2	V	^{51}V[c]	0,1
	^{65}Cu	0,1		^{208}Pb[b]	0,1	W	^{182}W	0,3
Dy	^{163}Dy	0,1	Pd	^{108}Pd	0,5		^{184}W	0,3
Er	^{166}Er	0,1	Pr	^{141}Pr	0,1	Y	^{89}Y	0,1
Fe	^{56}Fe[c]	5	Pt	^{195}Pt	0,5	Yb	^{172}Yb	0,2
Ga	^{69}Ga	0,3	Rb	^{85}Rb	0,1		^{174}Yb	0,2
	^{71}Ga	0,3	Re	^{185}Re	0,1	Zn	^{64}Zn	1
Gd	^{157}Gd	0,1		^{187}Re	0,1		^{66}Zn	1
	^{158}Gd	0,1	Rh	^{103}Rh	0,1		^{68}Zn	1
Ge	^{74}Ge	0,3	Ru	^{101}Ru	0,2	Zr	^{90}Zr	0,2

[a] Depending on the instrumentation, significantly lower limits can be achieved.

[b] In order to avoid incorrect results due to the varying isotop ratios in the environment, the signal intensities of ^{206}Pb, ^{207}Pb and ^{208}Pb shall be added.

[c] In order to reach these limits, depending on interferences, the use of a collision/reaction cell is recommended

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 5667-1, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes and sampling techniques*

ISO 5667-3, *Water quality — Sampling — Part 3: Preservation and handling of water samples*

ISO 8466-1, *Water quality — Calibration and evaluation of analytical methods and estimation of performance characteristics — Part 1: Statistical evaluation of the linear calibration function*

ISO 15587-1, *Water quality — Digestion for the determination of selected elements in water — Part 1: Aqua regia digestion*

ISO 15587-2, *Water quality — Digestion for the determination of selected elements in water — Part 2: Nitric acid digestion*

ISO 17294-1:2004, *Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS) — Part 1: General guidelines*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 17294-1 and the following apply.

3.1
limit of application
lowest concentration of an analyte that can be determined with a defined level of accuracy and precision

4 Principle

Multi-element determination of selected elements, including uranium isotopes, by inductively coupled plasma mass spectrometry (ICP-MS) consists of the following steps:

— introduction of a measuring solution into a radiofrequency plasma (for example, by pneumatic nebulization) where energy transfer processes from the plasma cause desolvation, decomposition, atomization and ionization of elements;

— as an additional option, collision and reaction cell technology may be to overcome several interferences (see 5.1);

— extraction of the ions from plasma through a differentially pumped vacuum interface with integrated ion optics and separation on the basis of their mass-to-charge ratio by a mass spectrometer (for instance a quadrupole MS);

— transmission of the ions through the mass separation unit (for instance, a quadrupole) and detection, usually by a continuous dynode electron multiplier assembly, and ion information processing by a data handling system;

— quantitative determination after calibration with suitable calibration solutions.

The relationship between signal intensity and mass concentration is usually a linear one over a broad range (usually over more than several orders of magnitude).

The method to be used for determination of uranium isotopes is described in Annex A. With instruments equipped with a magnetic sector field, higher mass resolution spectra can be obtained. This can help to separate isotopes of interest from interfering species.

5 Interferences

5.1 General

In certain cases, isobaric and non-isobaric interferences can occur. The most important interferences in this respect are coinciding masses and physical interferences from the sample matrix. For more detailed information, see ISO 17294-1.

Common isobaric interferences are given in Table 2 (for additional information, see ISO 17294-1). It is recommended that different isotopes of an element be determined in order to select an isotope

that does not suffer from interference. If there are none that meet this requirement, a mathematical correction has to be applied. For the determination of uranium isotopes, the specific procedure detailed in Annex A has to be followed.

Small drifts or variations in intensities should be corrected by the application of the internal standard correction. In general, in order to avoid physical and spectral interferences, the mass concentration of dissolved matter (salt content) should not exceed 2 g/l (corresponding to a conductivity of less than 2 700 µS/cm).

NOTE With the use of collision and reaction cell technology, it is possible to overcome several interferences. As the various options and parameters of those techniques cannot be described in detail in this part of ISO 17294, the user is responsible for demonstrating that the chosen approach is fit for purpose and achieves the necessary performance.

5.2 Spectral interferences

5.2.1 General

For more detailed information on spectral interferences, see ISO 17294-1:2004, 6.2.

5.2.2 Isobaric elemental

Isobaric elemental interferences are caused by isotopes of different elements of the same nominal mass-to-charge ratio and which cannot be separated due to an insufficient resolution of the mass spectrometer in use (for example, ^{114}Cd and ^{114}Sn).

Element interferences from isobars may be corrected for taking into account the influence from the interfering element (see Table 3). In this case, the isotopes used for correction shall be determinable without any interference and with sufficient precision. Possible proposals for correction are often included in the instrument software.

Table 2 — Important isobaric and polyatomic interferences

Element	Isotope	Inter-element interferences caused by isobars and doubly charged ions	Interferences caused by polyatomic ions
Ag	^{107}Ag ^{109}Ag	—	ZrO NbO, ZrOH
As	^{75}As	—	ArCl, CaCl
Au	^{197}Au	—	TaO
B	^{10}B		
	^{11}B	—	BH
Ba	^{138}Ba	La^+, Ce^+	—
Be	^{9}Be	—	$^{18}O_2$
Ca	^{43}Ca	—	CNO
	^{44}Ca	—	COO
Cd	^{111}Cd	—	MoO, MoOH, ZrOH
	^{114}Cd	Sn^+	MoO, MoOH
Co	^{59}Co	—	CaO, CaOH, MgCl
Cr	^{52}Cr	—	ArO, ArC, ClOH
	^{53}Cr	Fe^+	ClO, ArOH,
Cu	^{63}Cu	—	ArNa, POO, MgCl
	^{65}Cu	—	SOOH

NOTE In the presence of elements in high mass concentrations, interferences can be caused by the formation of polyatoms or doubly charged ions which are not listed above.

Table 2 *(continued)*

Element	Isotope	Inter-element interferences caused by isobars and doubly charged ions	Interferences caused by polyatomic ions
Eu	^{151}Eu	—	BaO
	^{153}Eu	—	BaO
Fe	^{54}Fe	—	^{37}Cl^{16}O^{1}H+ ^{40}Ar^{14}N
	^{56}Fe	—	^{40}Ar^{16}O+ ^{40}Ca^{16}O+
	^{57}Fe	—	^{40}Ar^{16}O^{1}H+ ^{40}Ca^{16}O^{1}H+ ^{40}Ar^{17}O+
Ga	^{69}Ga	Ba^{++}	CrO, ArP, ClOO
Ge	^{74}Ge	Se^{+}	ArS, ClCl
In	^{115}In	Sn^{+}	—
Ir	^{193}Ir	—	HfO
Mg	^{24}Mg	—	CC
	^{25}Mg	—	CC
Mn	^{55}Mn	—	NaS, ArOH, ArNH
Mo	^{98}Mo	Ru^{+}	—
Ni	^{58}Ni	Fe^{+}	CaO, CaN, NaCl, MgS
	^{60}Ni	—	CaO, CaOH, MgCl, NaCl
Pd	^{108}Pd	Cd^{+}	MoO, ZrO
Pt	^{195}Pt	—	HfO
Re	^{187}Re	Os^{+}	—
Ru	^{102}Ru	Pd^{+}	—
Sb	^{123}Sb	Te^{+}	—
Sc	^{45}Sc	—	COO, COOH
Se	^{77}Se	—	CaCl, ArCl, ArArH
	^{78}Se	Kr^{+}	ArAr, CaCl
	^{82}Se	Kr^{+}	HBr
Sn	^{120}Sn	Te^{+}	—
V	^{51}V	—	ClO, SOH, ClN, ArNH
W	^{184}W	Os^{+}	—
Zn	^{64}Zn	Ni^{+}	AlCl, SS, SOO, CaO
	^{66}Zn	Ba^{++}	PCl, SS, FeC, SOO
	^{68}Zn	Ba^{++}, Ce^{++}	FeN, PCl, ArS, FeC, SS, ArNN, SOO

NOTE In the presence of elements in high mass concentrations, interferences can be caused by the formation of polyatoms or doubly charged ions which are not listed above.

Table 3 — Examples for suitable isotopes with their relative atomic masses and formulae for correction

Element	Recommended isotope and inter-element correction	
As	^{75}As	−3,127 (^{77}Se − 0,815 ^{82}Se) or
	^{75}As	−3,127 (^{77}Se + 0,322 0 ^{78}Se)
Ba	^{138}Ba	−0,000 900 8 ^{139}La − 0,002 825 ^{140}Ce
Cd	^{114}Cd	−0,026 84 ^{118}Sn
Ge	^{74}Ge	−0,138 5 ^{82}Se
In	^{115}In	−0,014 86 ^{118}Sn
Mo	^{98}Mo	−0,110 6 ^{101}Ru

Table 3 *(continued)*

Element	Recommended isotope and inter-element correction	
Ni	^{58}Ni	−0,048 25 ^{54}Fe
Pb	^{208}Pb	+^{207}Pb + ^{206}Pb
Se	^{82}Se	−1,009 ^{83}Kr
Sn	^{120}Sn	−0,013 44 ^{125}Te
V	^{51}V	^{51}V −3,127 (^{53}Cr −0,113 4 ^{52}Cr)
W	^{184}W	−0,001 242 ^{189}Os

5.2.3 Polyatomic interferences

Polyatomic ions are formed by coincidence of plasma gas components, reagents and sample matrix (for example, interference of the relative mass ^{75}As by ^{40}Ar^{35}Cl and ^{40}Ca^{35}Cl). Examples for correction formulae are given in Table 3 and information on the magnitude of interferences are stated in Table 4. This interference is of particular relevance for several elements (for example, As, Cr, Se, V).

It is recommended that the analyst checks the magnitude of this interference regularly for the particular instrument.

In the case of mathematical corrections, it shall be taken into account that the magnitude of interference depends both on the plasma adjustment (for example, oxide formation rate) and on the mass concentration of the interfering element, which will usually be a variable component of the sample solution.

5.3 Non-spectral interferences

For detailed information on non-spectral interferences, see ISO 17294-1:2004, 6.3.

Table 4 — Important interferences by solutions of Na, K, Ca, Mg, Cl, S, P (ρ = 100 mg/l) and Ba (ρ = 1 000 µg/l)

Element	Isotope	Simulated mass concentration[a] µg/l	Type of interference
As	^{75}As	1,0	ArCl
Co	^{59}Co	0,2 to 0,8	CaO, CaOH
Cr	^{52}Cr	1,0	ClOH
		1,0	ArC
	^{53}Cr	5,0	ClO
Cu	^{63}Cu	1,0 to 3,0	ArNa
		1,0 to 1,6	POO
	^{65}Cu	2,0	ArMg
		2,0	POO
		2,0	SOOH
Ga	^{69}Ga	1,0 to 25	Ba^{++}
		0,3	ArP
		1,0	ClOO
	^{71}Ga	0,2 to 0,6	ArP

[a] Indicates the magnitude of interference without corrective measures. User should check the interferences and decide how to reduce or eliminate them.

Table 4 *(continued)*

Element	Isotope	Simulated mass concentration[a] µg/l	Type of interference
Ge	^{74}Ge	0,3	ClCl
		0,3	ArS
Mn	^{55}Mn	3,0	KO
		3,0	NaS
		3,0	NaS
Ni	^{58}Ni	2,5	CaO, CaN
	^{60}Ni	3 to 12	CaO, CaOH
Se	^{77}Se	10	ArCl
V	^{51}V	1 to 5	ClO, ClN
		1,0	SOH
Zn	^{64}Zn	7	ArMg
		3	CaO
		8	SS, SOO
		1	POOH
	^{66}Zn	2,0	ArMgBa^{++}
		5	SS, SOO
		4	PCl
		2	Ba^{++}
	^{68}Zn	50	ArS, SS, SOO
		4	Ba^{++}

[a] Indicates the magnitude of interference without corrective measures. User should check the interferences and decide how to reduce or eliminate them.

6 Reagents

For the determination of elements at trace and ultratrace level, the reagents shall be of adequate purity. The concentration of the analyte or interfering substances in the reagents and the water should be negligible compared to the lowest concentration to be determined.

For preservation and digestion, nitric acid should be used to minimize interferences by polyatoms.

For uranium isotopes concentration determination, see Annex A.

6.1 Water, grade 1 as specified in ISO 3696, for all sample preparation and dilutions.

6.2 Nitric acid, $\rho(HNO_3) = 1,4$ g/ml.

NOTE Nitric acid is available both as $\rho(HNO_3) = 1,40$ g/ml [$w(HNO_3) = 650$ g/kg] and $\rho(HNO_3) = 1,42$ g/ml [$w(HNO_3) = 690$ g/kg]. Both are suitable for use in this method provided that there is minimal content of the analytes of interest.

6.3 Hydrochloric acid, $\rho(HCl) = 1,16$ g/ml.

6.4 Hydrochloric acid, $c(HCl) = 0,2$ mol/l.

6.5 Sulfuric acid, $\rho(H_2SO_4) = 1,84$ g/ml.

6.6 Hydrogen peroxide, $w(H_2O_2) = 30\ \%$.

NOTE Hydrogen peroxide is often stabilized with phosphoric acid.

6.7 Element stock solutions, $\rho = 1\ 000$ mg/l each of Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Hg, Ho, In, Ir, K, La, Li, Lu, Mg, Mn, Mo, Na, Nd, Ni, P, Pb, Pd, Pr, Pt, Rb, Re, Rh, Ru, Sb, Sc, Se, Sm, Sn, Sr, Tb, Te, Th, Tl, Tm, U, V, W, Y, Yb, Zn and Zr.

Both single-element stock solutions and multi-element stock solutions with adequate specification stating the acid used and the preparation technique are commercially available. Element stock solutions with different concentrations of the analytes (for example, 2 000 mg/l or 10 000 mg/l) are also allowed.

These solutions are considered to be stable for more than one year, but in reference to guaranteed stability, the recommendations of the manufacturer should be considered.

6.8 Anion stock solutions, $\rho = 1\ 000$ mg/l each of Cl^-, PO_4^{3-}, SO_4^{2-}.

Prepare these solutions from the respective acids. The solutions are also commercially available. Anion stock solutions with different concentrations of the analytes (for example, 100 mg/l) are also allowed.

These solutions are considered to be stable for more than one year, but in reference to guaranteed stability, the recommendations of the manufacturer should be considered.

6.9 Multi-element standard solutions.

Depending on the scope, different multi-element standard solutions can be necessary. In general, when combining multi-element standard solutions, their chemical compatibility and the possible hydrolysis of the components shall be regarded. Care shall be taken to prevent chemical reactions (for example, precipitation).

The examples given below also consider the different sensitivities of various mass spectrometers.

The multi-element standard solutions are considered to be stable for several months, if stored in the dark.

This does not apply to multi-element standard solutions that are prone to hydrolysis, in particular, solutions of Bi, Mo, Sn, Sb, Te, W, Hf and Zr.

In reference to guaranteed stability of all standard solutions, see the recommendations of the manufacturer.

6.9.1 Multi-element standard solution A, for example, consisting of the following:

— ρ(As, Se) = 20 mg/l;

— ρ(Ag, Al, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, La, Li, Mg, Mn, Ni, Pb, Rb, Sr, Th, Tl, U, V and Zn) = 10 mg/l.

Pipette 20 ml of each element stock solution (As, Se) (6.7) and 10 ml of each element stock solution (Ag, Al, B, Ba, Be, Bi, Cd, Ce, Co, Cr, Cs, Cu, Fe, La, Li, Mn, Ni, Pb, Rb, Sr, Th, Tl, U, V and Zn) (6.7) into a 1 000 ml volumetric flask.

Add 10 ml of nitric acid (6.2).

Bring to volume with water (6.1) and transfer to a suitable storage bottle.

Multi-element standard solutions with more elements may be used provided that it is verified that these solutions are stable and no chemical reactions occur. This shall be checked again a few days after the first use (sometimes precipitation can occur after preparation).

6.9.2 Multi-element standard solution B, for example, consisting of the following:

— ρ(Au, Hg, Mo, Sb, Sn, W, Zr) = 5 mg/l.

Pipette 2,5 ml of each element stock solution (Au, Hg, Mo, Sb, Sn, W, Zr) (6.7) into a 500 ml volumetric flask.

Add 40 ml of hydrochloric acid (6.3).

Bring to volume with water (6.1) and transfer to a suitable storage bottle.

6.9.3 **Reference-element solution** (internal standard solution).

The choice of elements for the reference-element solution depends on the analytical problem. Solutions of these elements should cover the mass range of interest. The concentrations of these elements in the sample should be negligibly low. The elements In, Lu, Re, Rh and Y have been found suitable for this purpose. Other elements can also be used, depending on the purpose of the analysis, such as stable Bi and Tl for example.

For example, ρ(Y, Re) = 5 mg/l reference-element solution may be used.

Pipette 5 ml of each element stock solution (Y, Re) (6.7) into a 1 000 ml volumetric flask.

Add 10 ml of nitric acid (6.2).

Bring to volume with water (6.1) and transfer to a suitable storage bottle.

NOTE For the determination of mercury (Hg), it can be helpful to add gold (Au) in order to avoid interferences to the reference-element solution to allow a final concentration of 50 µg/l in the solution to be measured [ρ(Au) = 50 µg/l].

6.10 **Multi-element calibration solutions.**

Choose the mass concentrations of the calibration solutions to allow for a sufficient precision and reproducibility and ensure that the working range is covered.

The stability of the calibration solutions should be checked regularly. Due to their rather low respective mass concentrations, they should be replaced by freshly prepared solutions at least every month or more frequently for elements which are prone to hydrolysis. In special cases, daily preparation is necessary. The user has to determine the maximum stability period of the calibration solutions.

Transfer the calibration solution(s) A (6.10.1) and B (6.10.2) to suitable storage bottles.

If the determination is carried out after previous digestion (9.2), the matrix of the calibration solution(s) A (6.10.1) and B (6.10.2) below shall be adjusted to that of the digests after dilution, where appropriate.

The working range in general may cover the range of 0,1 µg/l to 50 µg/l or a part of this.

6.10.1 **Multi-element calibration solution(s) A.**

Prepare the calibration solution(s) A that cover the required working range by diluting the multi-element standard solution A (see 6.9.1). Add sufficient nitric acid (6.2) and other acids, if required, per litre to matrix match with prepared sample solutions and bring up to volume with water (6.1). If necessary, add reference-element solution (6.9.3) to a concentration of, for example, 50 µg/l of the reference-element before bringing up to volume.

6.10.2 **Multi-element calibration solution(s) B.**

Prepare the calibration solution(s) B that cover the required working range by diluting the multi-element standard solution B (6.9.2). Add sufficient hydrochloric acid (6.3) and other acids, if required, per litre to matrix match with prepared sample solutions and bring up to volume with water (6.1). If necessary, add reference-element solution (6.9.3) to a concentration of, for example, 50 µg/l of the reference-element before bringing up to volume.

6.11 Blank calibration solutions.

High demands shall be set concerning the purity. The user should ensure that the background levels of the analytes are not significant to the results of the analysis.

6.11.1 Blank calibration solution A.

Pipette sufficient volume of nitric acid (6.2) and other acids, if required to matrix match with prepared sample solutions, to a volumetric flask made, for example, from perfluoroalkoxy (PFA) or hexafluoroethene propene (FEP) and bring to volume with water (6.1). If necessary, add reference-element solution (6.9.3) to a concentration of, for example, 50 µg/l of the reference-element before bringing up to volume.

If the determination is carried out after previous digestion (9.2), the matrix of the blank calibration solution A shall be adjusted to that of the digests.

6.11.2 Blank calibration solution B.

Pipette sufficient volume of hydrochloric acid (6.3) and other acids, if required to matrix match with prepared sample solutions, to a volumetric flask made, for example, from perfluoroalkoxy (PFA) or hexafluoroethene propene (FEP) and bring to volume with water (6.1). If necessary, add reference-element solution (6.9.3) to a concentration of, for example, 50 µg/l of the reference-element before bringing up to volume.

If the determination is carried out after previous digestion (9.2), the matrix of the blank calibration solution B shall be adjusted to that of the digests.

6.12 Optimization solution.

The optimization solution serves for mass calibration and for optimization of the apparatus conditions, for example, adjustment of maximal sensitivity with respect to minimal oxide formation rate and minimal formation of doubly charged ions.

It should contain elements covering the entire mass range, as well as elements prone to a high oxide formation rate or to the formation of doubly charged ions. For example, an optimization solution containing Mg, Cu, Rh, In, Ba, La, Ce, U and Pb is suitable. Li, Be and Bi are less suitable because they tend to cause memory effects.

The mass concentrations of the elements used for optimization should be chosen to allow count rates of more than 10 000 counts/s.

For further information, see general remarks in ISO 17294-1.

6.13 Matrix solution.

The matrix solutions serve to determine the correction factors for the corresponding formulae. High demands are made concerning the purity of the basic reagents due to the high mass concentrations. The user should ensure that the background levels of the analytes in the matrix solution are not significant to the results of the analysis. The composition may be as follows:

— $\rho(Ca) = 200$ mg/l;

— $\rho(Cl^-) = 300$ mg/l;

— $\rho(PO_4^{3-}) = 25$ mg/l;

— $\rho(SO_4^{2-}) = 100$ mg/l.

Pipette 200 ml of element stock solution (Ca) (6.7), 300 ml of anion stock solution (Cl^-) (6.8), 25 ml of anion stock solution (PO_4^{3-}) (6.8) and 100 ml of anion stock solution (SO_4^{2-}) (6.8) to a 1 000 ml volumetric flask.

Add 10 ml of nitric acid (6.2).

Bring to volume with water (6.1) and transfer to a suitable storage bottle.

The reaction or collision cell technology might replace the use of the matrix solution. This has to be validated accordingly by the user of this part of ISO 17294.

7 Apparatus

The stability of samples and measuring and calibration solutions depends to a high degree on the container material. The material shall be checked according to the specific purpose. For the determination of elements in a very low concentration range, glass or polyvinyl chloride (PVC) should not be used. Instead, it is recommended to use perfluoroalkoxy (PFA), hexafluoroethene propene (FEP) or quartz containers, cleaned with hot, concentrated nitric acid in a closed system. For the determination of elements in a higher concentration range, high density polyethene (HDPE) or polytetrafluoroethene (PTFE) containers are also allowed for the collection of samples.

Immediately before use, all glassware should be washed thoroughly with diluted nitric acid [for example, $w(HNO_3) = 10\ \%$], and then rinsed several times with water (6.1).

The use of piston pipettes is permitted and also enables the preparation of lower volumes of calibration solutions. The application of dilutors is also allowed. Mind that contaminated consumables like pipette tips, disposable vessels and filters might lead to increased blank levels and increase the uncertainty of the analytical result.

For more detailed information on the instrumentation, see ISO 17294-1:2004, Clause 5.

7.1 Mass spectrometer.

A mass spectrometer with inductively coupled plasma (ICP) suitable for multi-element and isotope analysis is required. The spectrometer should be capable of scanning a mass range from 5 m/z (AMU) to 240 m/z (AMU) with a resolution of at least 1 m_r/z peak width at 5 % of peak height (m_r = relative mass of an atom species; z = charge number). The instrument may be fitted with a conventional or extended dynamic range detection system.

7.2 Mass-flow controller.

A mass-flow controller on the nebulizer gas supply is required. Mass-flow controllers for the plasma gas and the auxiliary gas are also useful. A water cooled spray chamber may be of benefit in reducing some types of interferences (for example, from polyatomic oxide species).

NOTE The plasma is very sensitive to variations in the gas flow rate.

7.3 Nebulizer with variable speed peristaltic pump, for which information on different types of nebulizers is given in ISO 17294-1:2004, 5.1.2.

7.4 Argon gas supply, of high purity grade, for instance >99,99 %.

7.5 Glassware, consisting of the following:

7.5.1 Volumetric flasks, for example, 50 ml, 100 ml, 500 ml and 1 000 ml.

7.5.2 Conical (Erlenmeyer) flasks, for example, 100 ml.

7.5.3 Pipettes, for example, 1 ml, 2,5 ml, 10 ml, 20 ml and 25 ml.

7.6 Storage bottles, for the stock, standard, calibration and sample solutions.

For the determination of elements in a normal concentration range, high density polyethene (HDPE) or polytetrafluoroethene (PTFE) bottles are sufficient for the storage of samples. For the determination of elements in an ultratrace level, bottles made from perfluoroalkoxy (PFA) or hexafluoroethene propene (FEP) should be preferred. In any case, the user has to check the suitability of the chosen containers.

8 Sampling

Carry out the sampling in accordance with ISO 5667-1 and ISO 5667-3. Due to the extremely high requirements concerning purity in trace and ultratrace analysis, any impurity shall be avoided.

The mass concentrations of the elements can change rather rapidly after sampling due to adsorption or desorption effects. This is of special importance, for example, in the case of Ag, As, B, Se and Sn. The choice of the container material depends on the mass concentration of the elements to be determined.

For the determination of the dissolved fraction of the elements, filter the sample through a membrane filter, nominal pore size 0,45 µm. Membrane filters shall be tested for impurities by appropriate blank tests. Use several portions of the sample to rinse the filter assembly, discard and then collect the required volume of filtrate.

Add 0,5 ml of nitric acid (6.2) per 100 ml of sample. Ensure that the pH is less than 2; otherwise, add nitric acid as required.

In the case of determination of elements forming compounds that tend to be hydrolyzed, for example, Hg, Sb, Sn, W or Zr, add to an additional sample 1,0 ml of hydrochloric acid (6.3) per 100 ml of water. Ensure that the pH is less than 1; otherwise, add more hydrochloric acid as required.

9 Sample pre-treatment

9.1 Determination of the mass concentration of dissolved elements without digestion

Continue according to Clause 10, using the acidified filtrate specified in Clause 8. If experience has shown that no significant amounts of particles occur, the filtration may be omitted. Those samples shall be colourless and shall have a turbidity of <1,5 FNU (formazin nephelometric unit, see ISO 7027-1).

9.2 Determination of the total mass concentration after digestion

The mass concentration determined according to this subclause does not in all cases represent the total mass concentration. Instead, only the portion that is determinable according to the distinct digestion for a given element composition will be analysed.

A nitric acid digestion shall be carried out in accordance with ISO 15587-2. If aqua regia is chosen, the procedure shall be carried out in accordance with ISO 15587-1, in which case, possible interferences caused by the high content of chloride have to be considered accordingly.

Some elements and their respective compounds (for example, silicates and aluminium oxide) will be dissolved incompletely using this procedure.

For the determination of tin, the following digestion may be used:

a) add 0,5 ml of sulfuric acid (6.5) and 0,5 ml of hydrogen peroxide (6.6) to 50 ml of the homogenized water sample;

b) evaporate the mixture until SO_3 vapour is formed;

c) in case of incomplete digestion, add a small portion of water (6.1) after cooling, add hydrogen peroxide (6.6) once more and repeat the treatment;

d) dissolve the residue in diluted hydrochloric acid (6.4) and adjust the volume to 50 ml with water;

e) treat a blank in the same way.

Special digestion methods might be necessary if Sb, W or Zr is to be determined.

If experience has shown that the elements will be recovered quantitatively without decomposition, the digestion may be omitted.

10 Procedure

10.1 General

In ICP-MS methods, the relationship between measured count rates and mass concentrations of an element is known to be linear over several orders of magnitude. Therefore, linear calibration curves may be used for quantification. In routine measurements, check the linearity of the calibration curves at regular intervals. This check shall be carried out in accordance with ISO 8466-1.

Adjust the instrumental parameters of the ICP-MS system in accordance with the manufacturer's manual.

About 30 min prior to measurement, adjust the instrument to working condition.

Before each series of measurement, the sensitivity and the stability of the system should be checked using the optimization solution (6.12). Check the resolution and the mass calibration as often as required by the manufacturer.

Adjust the instrument with the aid of the optimization solution (6.12) to minimize interfering effects (for example, oxide formation, formation of doubly charged ions) allowing sufficient sensitivity.

According to Table 3, define the relative atomic masses and the corresponding corrections.

Define the rinsing times depending on the length of the flow path; in the case of large variations in mass concentrations in the measuring solutions, allow for longer rinsing periods.

The use of a reference-element solution is recommended. Add the reference-element solution (6.9.3) to the matrix solution (see 6.13), to all multi-element calibration solutions (6.10), to the blank calibration solutions (6.11), and to all measuring solutions. The mass concentration of the reference-elements shall be the same in all solutions.

A mass concentration of ρ(Y, Re) = 50 µg/l is often suitable.

NOTE ICP-MS has excellent multi-element capability. The sensitivity of determination depends on a number of parameters (nebulizer flow, radiofrequency power, lens voltage, lens voltage mode, etc.). The optimal instrument settings cannot be achieved for all elements simultaneously.

10.2 Calibration of the ICP-MS system

When the analytical system is first evaluated, and at intervals afterwards, establish a calibration curve for each element to be determined, e.g. using four to five measuring points (for example, the blank calibration solution and three or four calibration solutions).

For work on a daily basis, one blank solution and one to two calibration solutions are enough but check the validity of the calibration curve with a certified reference sample, a standard sample, or a suitable internal control sample (consider also comments in ISO 17294-1:2004, 9.1).

Typically proceed as follows.

Prepare and measure the blank calibration solutions (6.11) and the multi-element calibration solutions (6.10). Set up a calibration graph according to the manufacturer's instruction and in line with ISO 8466-1. Each reference point should be the mean of at least two replicates.

Take into account possible discrepancies in the isotope composition between the calibration solutions and the measuring solutions (for example, relevant for Li, Pb, U).

10.3 Measurement of the matrix solution for evaluation of the correction factors

In order to evaluate and to update the correction factors, measure the matrix solutions (6.13) or extracts of matrix matched CRM's at regular intervals within a measuring cycle.

10.4 Measurement of the samples

After establishing the calibration curves, measure the blanks and the samples.

Within sufficient small intervals (for example, every 10 samples), check the accuracy of at least one certified reference sample or one standard sample or one internal control sample. If necessary, re-calibrate.

Some elements (for example, Ag, B, Be, Bi, Li, Th and Sb) are rinsed very slowly from the sample inlet system. After high count rates, these memory effects shall be checked by measuring a blank calibration solution (6.11).

11 Calculation

The mass concentrations for each element may be determined with the aid of the instrument software. Carry out the following single steps for each element.

a) Correct the count rates according to the respective formulae (see Table 3).

b) For uranium isotopes, the calculation of the concentration is described in Annex A.

c) Make allowance for the count rates from the blank calibration, calibration and measuring solutions, and relate to the count rates of the reference-elements. Determine the slope and the intercept on the ordinate.

d) Determine the mass concentrations of samples with the aid of the count rates and the calibration graphs.

e) Correct the results taking into account the mass concentrations from the blank calibration solutions and incorporate all dilution steps in the calculation. If the sample is digested (see 9.2), a correction for the procedure blank shall be used if appropriate (digestion blank solution).

According to the requirements set by the analytical quality control, the determination of the mass concentrations using the software of the apparatus shall be verifiable and shall be documented. In all cases, it shall be clear which corrections have been carried out with the aid of the software.

Alternatively, it is also allowed to process the raw data (count rates) by validated in-house software applications.

Report the results to as many significant figures as are acceptable according to the precision of the measuring values.

EXAMPLES Copper (Cu) 0,142 mg/l

Cadmium (Cd) 0,50 µg/l

12 Test report

The test report shall contain at least the following information:

a) test method used, together with a reference to this part of ISO 17294, i.e. ISO 17294-2;

b) complete identification of the sample;

c) expression of results as indicated in Clause 11;

d) sample pre-treatment, if appropriate;

e) any deviations from this part of ISO 17294, and details of all circumstances which could have affected the result.

For the determination of uranium isotopes, see Annex A.

Annex A
(normative)

Determination of the mass concentration of uranium isotopes

A.1 General

This Annex describes methods for measuring the concentration of uranium isotopes in water (for example, drinking water, surface water, ground water, waste water and eluates) by inductively coupled plasma mass spectrometry. On the basis of the concentrations obtained, the activity concentrations of the different isotopes can be calculated.

For the most chemical environmental monitoring purposes, it is important and sufficient do determine the mass concentration of uranium.

For nuclear environmental monitoring purposes, it is important to determine the activity concentrations of each isotope of uranium in order to be able to perform the right dosis estimation (see References [13], [15] and [16]).

This method is applicable to all types of water having a saline load less than 1 g/l. Dilution is possible to obtain a solution having a saline load and activity compatible with preparation and the measurement assembly.

Filtration at 0,45 µm and acidification are recommended.

A.2 Symbols and abbreviated terms

Quantity	Symbol/ abbreviated term	Unit symbol	Term
Mass concentration	C	µg/l	Mass of analyte per unit volume of the sample
Mass concentration of the internal standard solution	C_T	µg/l	Mass of internal standard element per unit volume of the internal standard solution
Internal standard mass	m_T	µg	Mass of the isotope dilution tracer added
Standard uncertainty	$u(C)$	unit of the quantity C	Standard uncertainty associated with the measurement result
Expanded uncertainty	U	unit of the quantity C	Product of the standard uncertainty and the coverage factor k with $k = 1, 2,..., U = k \cdot u(C)$
Standard uncertainty	U	unit of the quantity C	
Instrumental detection limit	IDL	µg/l	IDL is the lowest value that can be measured by the instrument in the most optimal set up and is determined by three times the standard deviation obtained with ten replicates of the blank
Limit of quantification	xLQ	µg/l	xLQ can be determined by ten times the standard deviation obtained with ten replicates of the blank
Limit of quantification	$x\text{LQ}_{ins}$	µg/l	$x\text{LQ}_{ins}$ is the xLQ expressed in counts for the chosen m/z, due to the blank and the instrument

Quantity	Symbol/ abbrevi-ated term	Unit symbol	Term
Limit of application	LOA	µg/l	Lowest or highest concentration of an analyte that can be determined with a defined level of accuracy and precision
Blank standard deviation	s_{No}		
Volume of the sample	V	l	
Background	N_0	Counts	Counts for a given mass in the blank solution
Counts	N	Counts	Raw counts
Net counts	N_{net}	Counts	$N-N_0$ Define N_{netT} in case of the internal standard
Counts calculated when using isotopic dilution	N_{dl}	Counts	
Regression line slope	a	Counts.µg^{-1}.l	$N_{net} = a \cdot C + b$
Coordinate at the origin of the regression line	b	Counts	
Internal standard correction factor	c_{int}		Sample matrix effect correction when an in-ternal standard is added to the sample
Bias per unit mass	α		
Fractionation coefficient deviation	β		
Isotope distribution in the standard solution of ^{233}U	T		Used for isotopic dilution
Measured isotopic ratio	r		
True isotopic ratio	R		

A.3 Principle

ICP-MS can be used to measure the mass concentrations of the different uranium isotopes in a water test portion.

If necessary, the results can be converted in activity concentrations using conversion factors given in Table A.1.

Table A.1 — Uranium isotopes half-lives and specific activities

Uranium isotope	Half-life y	Specific activity Bq/g
234	2,455 (±0,006) × 10^5	2,312 × 10^8
235	704 (±1) × 10^6	7,997 × 10^4
236	23,43 (±0,006) × 10^6	2,392 × 10^6
238	4,468 (±0,005) × 10^9	1,244 × 10^4

Abundances of 99,274 5 % for ^{238}U, 0,720 0 % for ^{235}U and 0,005 5 % for ^{234}U would usually be observed in natural waters.

^{236}U has a low natural abundance and is not usually measured in water samples. But as ^{236}U is a good fingerprint to demonstrate the presence of uranium isotopes in water from anthropogenic sources, the measurement of this isotope may be sometimes required.

The water sample can be directly measured after filtration (at 0,45 µm porosity) without specific chemical separation.

As the water sample is directly measured, matrix effect correction with an internal standard is needed (as described in ISO 17294-1:2004, 6.3.5). For isotopic determination of uranium in water, 233-Uranium is commonly used but 204-Thallium can also be chosen.

It is also important to evaluate the mass bias and to correct it, if necessary.

The following different possibilities are offered:

— external calibrations with ^{238}U, ^{235}U and ^{234}U certified standard solutions can provide ^{238}U, ^{235}U and ^{234}U mass concentrations in the sample test portion;

— external calibrations with ^{238}U certified standard solution can provide ^{238}U concentration in the sample test portion; then the isotopic ratios measured in the sample test portion without internal standard addition lead to the ^{235}U and ^{234}U concentrations in the sample test portion;

— isotopic dilution using ^{233}U (^{236}U is also possible, provided that this isotope is not present in the sample) is another possibility; a known amount of pure ^{233}U certified standard solution is added to the sample test portion and the concentration of the uranium isotopes is based on the isotopic ratios.

In the first case, it can be difficult for the laboratory to get the three different pure and certified standard solutions and three external standard calibration curves have to be prepared.

In the second case, only one certified standard solution is needed, but the test portion shall be measured twice; first with internal standard to get ^{238}U mass concentration and then without internal standard addition to evaluate isotopic ratios

In the last case, the method is more convenient, but requires corrections for the interference of natural uranium isotopes (^{235}U and ^{234}U) in the internal standard solution, the mass bias and spectral interferences like hydrides.

Examples of limits of quantification that can be obtained with a quadrupole ICP-MS are given in Table A.2.

Table A.2 — Examples of limits of quantification

Isotope	*x*LQ $\mu g \cdot l^{-1}$	*x*LQ $mBq \cdot l^{-1}$
234-Uranium	<0,000 1	<20
235-Uranium	<0,001	<0,1
238-Uranium	0,1	<1

A.4 Reagents

Use only reagents of recognized analytical grade.

A.4.1 Laboratory water.

A.4.2 Blank.

Diluted acid solution used to determine the background spectra for the various masses.

A.4.3 Certified standard solutions of isotopes.

Certified standard solution with known isotopic ratios to evaluate the mass bias or reference solution with known isotopic ratios are used for standard bracketing (measure at least twice, before and after sampling; several repetitions are recommended).

A.4.4 Calibration solutions.

Prepare these solutions by successive dilutions of the certified standard solution in 1 % to 2 % nitric acid (volume). Adjust concentrations in line with the measuring range to be calibrated. The calibration curve is established with at least five measuring points (for example, the blank calibration solution and four calibration solutions).

A.4.5 Internal standard solution (for example, of uranium 233 as an internal standard).

Prepare this solution by successive dilutions of the certified standard solution in 1 % to 2 % nitric acid (volume). Adjust the concentration is in line with the validation method chosen. Spike samples with a known amount of this solution before measurement.

A.4.6 Quality control solution.

Solution of certified uranium concentration, different than the one used for calibration.

A.4.7 Argon gas, at least 99,995 % pure.

A.4.8 Diluted nitric acid, 2 % volume, for example.

A.5 Apparatus

Usual laboratory apparatus and, in particular, the following.

A.5.1 Analytical balance, accurate to within 1/10 mg or 1/100 mg.

A.5.2 Argon supply, equipped with low pressure control.

A.5.3 ICP-MS apparatus with associated software, installed in an air-conditioned room.

A.5.4 Auto-sampler device.

A.6 Sampling

A.6.1 General

For the determination of trace amounts of uranium, the prevention of all contamination or losses shall be of primary concern. Dust in the laboratory, impurities in the reagents and on the laboratory equipment which is in contact with the sample are all potential sources of contamination. The sample containers can lead to positive or negative errors in the determination of trace elements by superficial desorption or adsorption.

Perform the following conservation and pre-treatment steps (filtration and acidification) when sampling or immediately afterwards.

The sample collection conditions shall comply with ISO 5667-1.

It is important that the laboratory receive a sample that is truly representative and has not been damaged or modified during transport or storage.

A.6.2 Sample preparation for the determination of dissolved uranium

Filter the sample on a 0,45 µm membrane filter as soon as possible, using a glass or single-use filtration apparatus.

Acidify with nitric acid to ensure that the pH of the sample is less than 2.

A.6.3 Storage

See ISO 5667-3. Perform the analysis as soon as possible.

A.7 Procedure

A.7.1 General

Follow the instructions provided by the instrument manufacturer and the steps described in this part of ISO 17294.

For each analysis performed on this instrument, the sensitivity, the instrumental detection limit, precision, and the interferences should be established for the masses of interest.

Before analysing a sample or prior to a series of samples, analyse the quality control solution as if it was a sample. Ensure that the measured value of the concentration does not deviate from the expected value (within measurement limits). If the deviation exceeds the established measurement limits (optimum sensitivity, optimum stability), follow the recommendations of the instrument manufacturer and perform the adjustment again.

The value of the blank solution is measured using the same procedure as for the samples. This value shall be subtracted from those measured in the samples.

Begin sample analysis by cleaning the system with the blank solution. Repeat this operation after each sample.

A.7.2 Quantification

Uranium isotopes can be quantified in the following three different ways:

— using an external calibration solution for each isotope;

— using an external calibration solution for one of the isotopes (for example, for ^{238}U) with an internal standard and isotopic ratios to determine the other isotopes of uranium;

— using an internal standard solution and corrections (isotopic dilution).

A.7.3 External calibration

Prepare the calibration solutions by adding an internal standard (^{233}U or stable thallium can be used).

The formula of the calibration curves are determined by linear regression using the least square method.

Analyse the quality control solutions to validate the external calibrations.

Perform samples measurements.

Total uranium concentration is sometimes quantified using the calibration of ^{238}U. Then it is recommended to check that isotopic compositions of the sample and the working solution are identical, otherwise it is necessary to perform corrections.

A.7.4 External calibration for ^{238}U and isotopic ratios

Prepare the calibration solutions for ^{238}U by adding ^{233}U as an internal standard, for example. It is also possible to choose stable elements such as Tl.

The formula of the ^{238}U calibration curve is determined by linear regression using the least square method.

Analyse the standard solution to validate the external calibration.

Sample test portion is measured twice: first time with ^{233}U, as an internal tracer to correct matrix effects, to determine ^{238}U concentration, second time without ^{233}U to determine isotopic ratios.

A.7.5 Internal calibration by isotopic dilution

The uranium concentration will be quantified in relation to the ^{233}U standard solution isotopic composition and concentration introduced in the samples.

Isotopic ratios in the standard tracer solution shall be different than the one in the sample test portion.

The mass of tracer ^{233}U added shall be closed to the one of the uranium isotopes in the sample test portion.

Perform samples measurements.

As the ^{233}U certified standard solution is not pure, impurities of uranium natural isotopes shall be quantified with precision and corrections shall be made as well as for mass bias.

A.8 Expression of results

A.8.1 General

The results are expressed in mass concentration with their associated uncertainty. The coverage factor is specified in the presentation of the results.

If dilutions were carried out, apply the appropriate factor to the values of the sample.

The result is expressed as an estimate of the "true" value, to which an uncertainty is associated, itself a combination of elementary uncertainties.

A.8.2 Mass bias evaluation

The mass bias is a fundamental notion in mass spectrometry and the user shall take it into account during calculations to obtain good accuracy of measurement. This is a systematic error produced by the instrument. However, the mass bias may be small for heavy ions as they are better focused through the skimmer cone after the sampling cone.

This fractionation coefficient deviation, β, can be defined as a function of the different masses studied. The true ratio of isotopes A and B (R) can be expressed from the ratio measured (r) by different relations called linear law, power law, kinetic law, equilibrium law or generalized power law.

The bias per unit mass, α, determined measuring a certified solution or a reference solution when using standard bracketing. Two examples are given:

Linear law, given in Formula (A.1):

$$\frac{r}{R} = 1 + \alpha \Delta m \tag{A.1}$$

Exponential (kinetic) law, given in Formula (A.2):

$$\beta = \mathrm{In}\left(\frac{R}{r}\right) \Big/ \mathrm{In}\left(m_A / m_B\right) \tag{A.2}$$

And power law, given in Formula (A.3):

$$R = r\left(\frac{m_A}{m_B}\right)\beta \tag{A.3}$$

The linear law is commonly used for determination of uranium isotopes concentration in water.

In the following, the raw counts will be corrected of the mass bias, if necessary.

The associated uncertainty shall be determined, depending on the law chosen (see Reference [6]).

A.8.3 External calibration

The calibration curve is established from a series of working solutions of known and increasing concentrations, including the expected concentrations (at least five points that can be four different mass concentrations and the blank) and is expressed with Formula (A.4):

$$N_{\mathrm{net}} = a \cdot C + b \tag{A.4}$$

It is recommended to have the mass concentration in the sample very close to the centroid of the curve in order to minimize the standard uncertainty linked to the calibration curve.

The formula of the calibration curve is determined by the least squares method. Construct a calibration curve by isotope that can be measured in the standard.

Additionally, the use of an internal standard (Uranium 233, for example) is useful to correct the signal fluctuations. In cases where an internal standard is used, a correction factor α is introduced. This correction is applied by the software.

The mass concentration of the uranium isotopes, expressed in $\mu g \cdot l^{-1}$, is equal to Formula (A.5) and Formula (A.6):

$$\rho\left({}^{23i}\mathrm{U}\right) = \frac{N_{\mathrm{net}}\left({}^{23i}\mathrm{U}\right) - b}{a} \tag{A.5}$$

$$N_{\mathrm{net}}\left({}^{23i}\mathrm{U}\right) = c_{\mathrm{int}} \times N\left({}^{23i}\mathrm{U}\right) - N_0\left({}^{23i}\mathrm{U}\right) \tag{A.6}$$

with i = 4, 5, 6 or 8 depending on the uranium isotope quantified.

A.8.4 External calibration and isotopic ratios

The ^{238}U mass concentration is determined as explained in Clause 8.

The concentration of the ^{238}U, expressed in µg·l^{-1}, is equal to Formula (A.7) and Formula (A.8):

$$\rho\left(^{238}\mathrm{U}\right) = \frac{N_{\mathrm{net}}\left(^{238}\mathrm{U}\right) - b}{a} \tag{A.7}$$

$$N_{\mathrm{net}}\left(^{238}\mathrm{U}\right) = c_{\mathrm{int}} \times N\left(^{238}\mathrm{U}\right) - N_0\left(^{238}\mathrm{U}\right) \tag{A.8}$$

Then, the isotopic ratios are used to determine the concentration of the other isotopes based on the counts obtained in the sample test portion measured without internal standard, as given in Formula (A.9):

$$\rho\left(^{23i}\mathrm{U}\right) = \rho\left(^{238}\mathrm{U}\right) \times \frac{N_{\mathrm{net}}\left(^{23i}\mathrm{U}\right)}{N_{\mathrm{net}}\left(^{238}\mathrm{U}\right)} \times \frac{1}{\left(1 + \alpha_{(i-8)}\right)} \tag{A.9}$$

with i = 4, 5, or 6 on the basis of the uranium isotope quantified.

A.8.5 Internal calibration by isotopic dilution

Uranium 233 is generally used as isotope dilution tracer: a known quantity of ^{233}U, generally determined by weighing, is added to each sample and is thus used to calculate the concentration of other uranium isotopes present in the sample.

The mass concentration of the uranium isotopes, expressed in µg.l^{-1}, is equal to Formula (A.10):

$$\rho\left(^{23i}\mathrm{U}\right) = \frac{m\left(^{23i}\mathrm{U}\right)}{V} \tag{A.10}$$

with Formula (A.11):

$$m\left(^{23i}\mathrm{U}\right) = m_T \times \frac{N_{\mathrm{net}}\left(^{23i}\mathrm{U}\right)}{N_{\mathrm{net}}\left(^{233}\mathrm{U}\right)} \times \frac{1}{\left(1 + \alpha_{(i-3)}\right)} \tag{A.11}$$

and the value of N_{net}, which is expressed as given in Formula (A.12) for the various isotopes to be analysed and the isotope dilution tracer:

$$N_{\mathrm{net}} = N_{\mathrm{dl}} - N_0 \tag{A.12}$$

with Formula (A.13):

$$N_{\mathrm{dl}}\left(^{23i}\mathrm{U}\right) = N\left(^{23i}\mathrm{U}\right) - N_{\mathrm{net}}\left(^{233}\mathrm{U}\right) \times T\left(^{23i}\mathrm{U}\right) \tag{A.13}$$

where T (^{23i}U) is the rate of the isotope 23i (i = 4, 5, 6 or 8) present as impurity in the tracer used.

This correction is particularly significant for the measurement of Uranium 234 by isotopic dilution.

A.9 Uncertainties

A.9.1 Uncertainties associated with external calibration

The measurement uncertainty is expressed in relation to the error associated with the calibration and the error associated with the measurement, as given in Formula (A.14):

$$u\left[C\left(^{23i}\mathrm{U}\right)\right] = \left[u^2\left(\mathrm{cal}\right) + u^2\left(N_{\mathrm{net}}\left(^{23i}\mathrm{U}\right)\right)\right]^{\frac{1}{2}} \tag{A.14}$$

with Formula (A.15):

$$u\left(N_{\mathrm{net}}\left(^{23i}\mathrm{U}\right)\right) = \left[\left(N\left(^{23i}\mathrm{U}\right)\right)^2 \times u^2\left(c_{\mathrm{int}}\right) + {c_{\mathrm{int}}}^2 \times u^2\left(N\left(^{23i}\mathrm{U}\right)\right) + u^2\left(N_0\left(^{23i}\mathrm{U}\right)\right)\right]^{\frac{1}{2}} \tag{A.15}$$

A.9.2 External calibration and isotopic ratios

For ^{238}U mass concentration, the uncertainty is expressed as in A.9.1.

For the other isotopes, the uncertainty is expressed as given in Formula (A.16):

$$u\left[\rho\left(^{23i}\mathrm{U}\right)\right] = \left[u_{\mathrm{rel}}^2\left(\rho\left(^{238}\mathrm{U}\right)\right) + u_{\mathrm{rel}}^2\left(N_{\mathrm{net}}\left(^{23i}\mathrm{U}\right)\Big/N_{\mathrm{net}}\left(^{238}\mathrm{U}\right)\right) + u_{\mathrm{rel}}^2\left(1\Big/\left(1+\alpha_{(i-8)}\right)\right)\right]^{\frac{1}{2}} \tag{A.16}$$

A.9.3 Isotope dilution

The measurement uncertainty is expressed by Formula (A.17):

$$u\left(C\left(^{23i}\mathrm{U}\right)\right) = \left[u_{\mathrm{rel}}^2\left(m\left(^{23i}\mathrm{U}\right)\right) + u_{\mathrm{rel}}^2\left(V\right)\right]^{\frac{1}{2}} \tag{A.17}$$

with Formula (A.18):

$$u\left[C\left(^{238}U\right)\right] = \left[u_{\mathrm{rel}}^2\left(C\left(m_{\mathrm{T}}\right)\right) + u_{\mathrm{rel}}^2\left(N_{\mathrm{net}}\left(^{238}U\right)\Big/N_{\mathrm{net}}\left(^{233}U\right)\right) + u_{\mathrm{rel}}^2\left(1\Big/\left(1+\alpha_{(i-3)}\right)\right)\right]^{\frac{1}{2}} \tag{A.18}$$

A.9.4 Instrumental detection limit

The instrumental detection limit, for a given mass, is obtained from an extension of the standard deviation associated with the measurements obtained for 10 test portions of the blank.

The detection limit (DL) is expressed in $\mu g \cdot l^{-1}$ dividing IDL (equivalent to three times the background counts deviation) by the slope of the regression line when using external calibration, as given in Formula (A.19):

$$\mathrm{DL}\left(^{23i}\mathrm{U}\right) = \left(3s_{N_0\left(^{23i}\mathrm{U}\right)}\right)\Big/a \tag{A.19}$$

In the case of isotope dilution (with ^{233}U for example), the formula becomes as given in Formula (A.20):

$$DL = \rho_T \times \frac{IDL_{ins}\left(^{23i}U\right)}{N_{netT}} \quad (A.20)$$

A.9.5 Limit of quantification

The limit of quantification, for a given mass, can be evaluated as 10 times the standard deviation associated with the measurements obtained for 10 test portions of the blank, as given in Formula (A.21):

$$xLQ_{ins}\left(^{23i}U\right) = 10s_{N_0\left(^{23i}U\right)} \quad (A.21)$$

The limit of quantification can be expressed in $\mu g.l^{-1}$ dividing xLQ by the slope of the regression line when using external calibration, as given in Formula (A.22):

$$xLQ\left(^{23i}U\right) = 10s_{N_0\left(^{23i}U\right)} \Big/ a \quad (A.22)$$

and in the case of isotope dilution (with ^{233}U for example) by Formula (A.23):

$$xLQ = \rho_T \times \frac{LQ_{ins}\left(^{23i}U\right)}{N_{netT}} \quad (A.23)$$

A.10 Test report

The test report has to conform to the requirements of ISO/IEC 17025 and shall contain at least the following information:

a) reference to the measurement and evaluation procedure used;

b) identification of the sample;

c) test result, $C \pm k{\cdot}u(C)$ or $C \pm U$ with the associated k value and units in which the results are expressed.

The following complementary information can be provided:

a) limit of application;

b) any relevant information likely to affect and/or explaining the results;

c) if necessary, the mass concentrations can be transformed in activity concentrations expressed in $Bq{\cdot}l^{-1}$.

Annex B
(informative)

Description of the matrices of the samples used for the interlaboratory trial

B.1 Surface water

The surface water sample for the interlaboratory trial (see Table C.1) was taken from the little stream "Meitze" which is located in the low, German mountain range, Harz ("Lower Saxony"). The sample was homogenized and filtered through a membrane filter with a pore size of 0,45 µm. Then it was acidified with 1 % (by volume) concentrated nitric acid. The following matrix elements were quantified (see Table B.1):

Table B.1 — Matrix of the surface water used for the interlaboratory trial

Parameter or ion		Unit	Result
pH value			6,2
Electrical conductivity	(25 °C)	µS/cm	310
Calcium	(Ca^{2+})	mg/l	30,0
Magnesium	(Mg^{2+})	mg/l	4,6
Sodium	(Na^{+})	mg/l	41,0
Potassium	(K^{+})	mg/l	5,6
Iron	(Fe^{2+})	mg/l	1,7
Chloride	(Cl^{-})	mg/l	25,0
Sulfate	(SO_4^{2-})	mg/l	28,0
Nitrate	(NO_3^{-})	mg/l	10,5
Hydrogen carbonate	(HCO_3^{-})	mg/l	141

The following elements were spiked using the respective single-element stock solutions (6.7):

— arsenic;

— beryllium;

— bismuth;

— cadmium;

— caesium;

— chromium;

— gallium;

— selenium;

— thallium;

— uranium.

B.2 Synthetic standard

The synthetic standard for the interlaboratory trial (see Table C.3) was prepared by diluting the respective single-element stock solutions (6.7). 10 ml of hydrochloric acid (6.3) per litre was added and the solution was filled up to volume with water (6.1).

Annex C
(informative)

Performance data

An interlaboratory trial was carried out in Germany in 1997. The results are given in Table C.1 to Table C.3. For the description of the sample matrices, see Annex B.

An independent interlaboratory trial for the uranium isotopes was carried out in France in 2013. The trial yielded the results given in Table C.4.

Table C.1 — Performance data for the matrix surface water[a,b]

Element	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
			%	µg/l	µg/l	%	µg/l	%
As	37	145	3,3	6,90	0,954	13,8	0,432	6,3
Ba	38	149	5,7	41,1	2,53	6,1	1,04	2,5
Cd	37	147	5,2	5,75	0,491	8,5	0,234	4,1
Co	38	151	2,6	2,33	0,269	11,6	0,140	6,0
Cr	38	151	0,0	3,39	0,634	18,7	0,294	8,7
Cu	38	151	2,6	26,7	2,02	7,6	0,93	3,5
Mn	39	155	2,5	205,0	13,2	6,4	5,9	2,9
Mo	38	150	2,6	4,45	0,402	9,0	0,187	4,2
Ni	35	137	11,0	5,44	0,786	14,5	0,397	7,3
Pb	39	155	2,5	13,6	1,13	8,3	0,64	4,7
Sn	34	132	3,6	1,19	0,241	20,3	0,157	13,2
Sr	40	158	0,0	117,0	8,1	6,9	3,4	3,0
Tl	31	124	8,1	0,272	0,046 0	16,9	0,029 2	10,7
V	33	129	8,5	1,15	0,311	27,0	0,121	10,5
Zn	36	143	7,7	27,6	2,56	9,3	1,43	5,2

l number of laboratories

n number of values

o percentage of outliers

$\bar{\bar{x}}$ total mean

s_R reproducibility standard deviation

$C_{V,R}$ coefficient of variation of reproducibility

s_r repeatability standard deviation

$C_{V,r}$ coefficient of variation of repeatability

[a] Antimony (total mean 0,33 µg/l) and zirconium (total mean 0,98 µg/l) have been measured as well in the matrix surface water. In both cases, a satisfactory reproducibility coefficient of variation could not be achieved.

[b] All data refer to the determination of the mass concentration of dissolved elements (9.1).

Table C.2 — Performance data for the matrix aqua regia digest (see ISO 15587-1)

Element	l	n	o %	$\bar{\bar{x}}$ µg/l	s_R µg/l	$C_{V,R}$ %	s_r µg/l	$C_{V,r}$ %
As	37	145	3,3	20,1	4,36	21,7	1,44	7,2
Ba	37	147	7,0	437,0	19,6	4,5	11,7	2,7
Cd	37	141	5,4	2,11	0,542	25,7	0,227	10,8
Co	39	154	2,5	145,0	8,4	5,8	5,7	3,9
Cr	38	151	5,0	363,0	24,1	6,6	12,3	3,4
Cu	38	150	5,1	3 334,0	239,6	7,2	117,0	3,5
Mn	39	155	2,5	1 029,0	73,2	7,1	36,4	3,5
Mo	39	154	2,5	15,2	1,14	7,5	0,57	3,7
Ni	37	146	8,2	184,0	17,4	9,4	7,2	3,9
Pb	37	146	7,6	793,0	49,0	6,2	27,9	3,5
Sb	36	143	7,7	170,0	12,5	7,4	5,5	3,2
Sn	38	150	2,6	415,0	37,4	9,0	16,3	3,9
Sr	40	155	1,3	89,9	6,21	6,9	3,37	3,8
Tl	29	112	8,9	0,276	0,076 5	27,7	0,049 4	17,9
V	36	140	5,4	44,0	8,87	20,2	2,06	4,7
Zn	38	150	2,6	711,0	58,1	8,2	32,9	4,6
Zr	31	117	7,1	2,87	0,752	26,2	0,403	14,0

NOTE For the explanation of symbols, see Table C.1.

Table C.3 — Performance and recovery data for the matrix synthetic standard

Element	l	n	o %	X µg/l	$\bar{\bar{x}}$ µg/l	η %	s_R µg/l	$C_{V,R}$ %	s_r µg/l	$C_{V,r}$ %
As	37	146	5,2	192,0	186,0	97,1	14,8	7,9	6,4	3,4
Ba	37	147	7,5	8,0	7,90	98,8	0,580	7,3	0,319	4,0
Cd	34	135	12,9	2,0	1,98	99,1	0,190	9,6	0,135	6,8
Co	40	159	0,0	42,0	41,5	98,8	3,02	7,3	1,55	3,7
Cr	36	142	7,8	9,0	9,35	103,9	1,986	21,2	0,721	7,7
Cu	39	155	0,0	48,0	48,2	100,3	3,83	8,0	1,64	3,4
Mn	39	155	2,5	97,0	95,3	98,2	6,52	6,8	3,05	3,2
Mo	37	146	8,2	7,0	6,85	97,8	0,474	6,9	0,256	3,7
Ni	40	157	1,3	93,0	91,2	98,0	8,55	9,4	3,91	4,3
Pb	36	142	8,4	6,0	6,43	107,2	0,491	7,6	0,287	4,5
Sb	39	154	0,0	114,0	114,0	99,9	11,1	9,8	4,0	3,5
Sn	38	149	3,2	120,0	117,0	97,9	8,4	7,1	4,2	3,5
Sr	40	157	1,3	24,0	23,3	97,1	1,66	7,1	1,07	4,6
Tl	31	121	14,8	0,9	0,892	99,1	0,059 7	6,7	0,041 9	4,7

X assigned value

η recovery rate

NOTE For an explanation of the other symbols, see Table C.1.

Table C.3 *(continued)*

Element	l	n	o %	X µg/l	$\bar{x}$ µg/l	η %	s_R µg/l	$C_{V,R}$ %	s_r µg/l	$C_{V,r}$ %
V	35	138	12,7	245,0	240,0	97,9	26,9	11,2	11,3	4,7
Zn	39	155	0,0	183,0	188,0	102,5	17,2	9,2	7,1	3,8
Zr	31	119	7,8	4,0	4,47	111,8	0,967	21,6	0,334	7,5

X assigned value

η recovery rate

NOTE For an explanation of the other symbols, see Table C.1.

Table C.4 — Performance data for uranium isotopes in the matrix mineral water (natural mineral water, bottled, without carbon dioxide)

Element	l	n	o %	$\bar{x}$ µg/l	s_R µg/l	$C_{V,R}$ %
U	8	8	0	14,6	0,42	2,9
Isotope	l	n	o %	$\bar{x}$ Bq/l	s_R Bq/l	$C_{V,R}$ %
^{234}U	8	8	0	0,255	0,021	8,17
^{235}U	8	8	0	0,008 3	0,000 19	2,26
^{238}U	8	8	0	0,179	0,005 5	3,08

Bibliography

[1] ISO 7027-1, *Water quality — Determination of turbidity — Part 1: Quantitative methods*

[2] ISO/TS 13530, *Water quality — Guidance on analytical quality control for chemical and physicochemical water analysis*

[3] DE BIEVRE P., & BARNES I.L. Int. J. Mass Spectrom. Ion Process. 1985, **65** pp. 211–230

[4] ADAMS F., & GIJBELS R. and Grieken, van R. Chemical analysis. Volume **95**. A series of monographs on analytical chemistry and its applications, inorganic mass spectrometry. John Wiley and Sons, New York (1988)

[5] GRAY A.L. Application of plasma source mass spectrometry II. Grenville-Holland. G, and Eaton. A.N. (Eds.), The Royal Society of Chemistry (RSC) (1993) ISBN 0-85186-465-1

[6] DATE A.R., & GRAY A.L. eds. *Applications of inductively coupled plasma mass spectrometry.* Blackie & Son Ltd, London, 1989

[7] JARVIS K.E., GRAY A.L., HOUK R.S. *Handbook of inductively coupled plasma mass spectrometry.* Blackie, Glasgow, London, 1992

[8] MONTASER A. ed. *Inductively coupled plasma mass spectrometry.* Wiley VCH New York, 1998

[9] THOMPSON M., & WALSH J.N. *Handbook of inductively coupled plasma spectrometry.* Blackie & Son Ltd, London, Second Edition, 1989

[10] BROEKAERT J.A.C. Analytiker-Taschenbuch Volume 9. Günzler, H. and others (Eds.), Springer Verlag, Heidelberg (1990)

[11] HOLLAND G., & TANNER S.D. *Plasma source mass spectrometry. New developments and applications. The Royal Society of Chemistry.* RSC, 1999

[12] WELZ B., & SPERLING M. *Atomic absorption spectroscopy.* Wiley-VCH, Federal Republic of Germany, 1999

[13] WHO, Guidelines for drinking water quality. 2005, Vol.**1**

[14] BUREAU INTERNATIONAL DES POIDS ET MESURES (BIPM). Monographie BIPM-5. http://www.bipm.org/fr/publications/monographie-ri-5.html. Nuclear data base: Decay data evaluation project. http://www.nucleide.org/DDEP_WG/DDEPdata.htmhttp://www.nucleide.org/DDEP_WG/DDEPdata.htm

[15] POURCELOT ET AL. Isotopic evidence of natural uranium and spent fuel uranium releases into the environment. *J. Environ. Monit.* 2011, **13** pp. 355–361

[16] POINTURIER F. ET AL. *Plasma source Mass spectrometry-Current trends and future developments.* RSC Publishing Cambridge, UK, 2005, pp. 130–42.

[17] WOMBACHER F. REHKAMPER M., Investigation of the mass discrimination of multiple collector ICP-MS using neodymium isotopes and the generalised power law. *J. Anal. At. Spectrom.* 2003, **18** pp. 1371–1375

[18] GRANIER G. ET AL. Round robin for uranium isotopic measurements by mass spectrometry. *J. Radioanal. Nucl. Chem.* 2009, **279** (3)

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COMMISSION REGULATION (EC) No 152/2009

of 27 January 2009

laying down the methods of sampling and analysis for the official control of feed

(Text with EEA relevance)

(OJ L 54, 26.2.2009, p. 1)

Amended by:

		Official Journal		
		No	page	date
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►**M2**	Commission Regulation (EU) No 51/2013 of 16 January 2013	L 20	33	23.1.2013
►**M3**	Commission Regulation (EU) No 691/2013 of 19 July 2013	L 197	1	20.7.2013
►**M4**	Commission Regulation (EU) No 709/2014 of 20 June 2014	L 188	1	27.6.2014
►**M5**	Commission Regulation (EU) 2017/645 of 5 April 2017	L 92	35	6.4.2017
►**M6**	Commission Regulation (EU) 2017/771 of 3 May 2017	L 115	22	4.5.2017
►**M7**	Commission Implementing Regulation (EU) 2020/1560 of 26 October 2020	L 357	17	27.10.2020

Corrected by:

►**C1** Corrigendum, OJ L 62, 6.3.2013, p. 36 (51/2013)

▼B

COMMISSION REGULATION (EC) No 152/2009

of 27 January 2009

laying down the methods of sampling and analysis for the official control of feed

(Text with EEA relevance)

▼M3

Article 1

Sampling for the official control of feed, in particular as regards the determination of constituents, including material which contains or consists of or is produced from genetically modified organisms (GMOs), feed additives as defined by Regulation (EC) No 1831/2003 of the European Parliament and of the Council [1], undesirable substances as defined by Directive 2002/32/EC of the European Parliament and of the Council [2] shall be carried out in accordance with the methods set out in Annex I.

The method of sampling set out in Annex I is applicable for the control of feed as regards the determination of pesticide residues as defined in Regulation (EC) No 396/2005 of the European Parliament and of the Council [3] and control of compliance with Regulation (EU) No 619/2011.

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Article 2

Preparation of samples for analysis and expression of results shall be carried out in accordance with the methods set out in Annex II.

Article 3

Analysis for the official control of feed shall be carried out using the methods set out in Annex III (Methods of analysis to control the composition of feed materials and compound feed, Annex IV (Methods of analysis to control the level of authorised additives in feed), Annex V (Methods of analysis to control undesirable substances in feed) and Annex VI (Methods of analysis for the determination of constituents of animal origin for the official control of feed).

Article 4

The energy value of compound poultry feed shall be calculated in accordance with Annex VII.

Article 5

The methods of analysis to control illegal presence of no longer authorised additives in feed set out in Annex VIII shall be used for confirmatory purposes.

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[1] OJ L 268, 18.10.2003, p. 29.
[2] OJ L 140, 30.5.2002, p. 10.
[3] OJ L 70, 16.3.2005, p. 1.

▼B

Article 6

Directives 71/250/EEC, 71/393/EEC, 72/199/EEC, 73/46/EEC, 76/371/EEC, 76/372/EEC, 78/633/EEC, 81/715/EEC, 84/425/EEC, 86/174/EEC, 93/70/EEC, 93/117/EC, 98/64/EC, 1999/27/EC, 1999/76/EC, 2000/45/EC, 2002/70/EC and 2003/126/EC are repealed.

References to the repealed Directives shall be construed as references to this Regulation and shall be read in accordance with the correlation tables in Annex IX.

Article 7

This Regulation shall enter into force on the twentieth day following that of 20th day following its publication in the ***Official Journal of the European Union.***

It shall apply from 26 August 2009.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

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ANNEX I

METHODS OF SAMPLING

1. PURPOSE AND SCOPE

Samples intended for the official control of feed shall be taken according to the methods described below. Samples thus obtained shall be considered as representative of the sampled portions.

The purpose of representative sampling is to obtain a small fraction from a lot in such a way that a determination of any particular characteristic of this fraction will represent the mean value of the characteristic of the lot. The lot shall be sampled by repeatedly taking incremental samples at various single positions in the lot. These incremental samples shall be combined by mixing to form an aggregate sample from which representative final samples shall be prepared by representative dividing.

If by a visual inspection, portions of the feed to be sampled show a difference in quality from the rest of the feed from the same lot, such portions shall be separated from the rest of the feed and treated as a separate sublot. If it is not possible to divide the feed into separate sublots, the feed shall be sampled as one lot. In such cases, mention shall be made of this fact in the sampling report.

Where a feed sampled in accordance with the provisions of this Regulation is identified as not satisfying EU requirements, is part of a lot of feed of the same class or description, it shall be presumed that all of the feed in that lot is so affected, unless following a detailed assessment there is no evidence that the rest of the lot fails to satisfy the EU requirements.

2. DEFINITIONS

— Lot (or batch): an identified quantity of feed determined to have common characteristics, such as origin, variety, type of packaging, packer, consignor or labelling, and in case of a production process, a unit of production from a single plant using uniform production parameters or a number of such units, when produced in continuous order and stored together.

— Sampled portion: A lot or an identified part of the lot or sublot.

— Sealed sample: a sample sealed in such a manner as to prevent any access to the sample without breaking or removing the seal.

— Incremental sample: A quantity taken from one point in the sampled portion.

— Aggregate sample: An aggregate of incremental samples taken from the same sampled portion.

— Reduced sample: A part of the aggregate sample, obtained from the latter by a process of representative reduction.

— Final sample: A part of the reduced sample or of the homogenised aggregate sample.

— Laboratory sample: a sample intended for the laboratory (as received by the laboratory) and can be the final, reduced or aggregate sample.

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3. GENERAL PROVISIONS

— Sampling personnel: the samples shall be taken by persons authorised for that purpose by the competent authority.

— The sample has to be sealed in such a manner as to prevent any access to the sample without breaking or removing the seal. The seal's mark should be clearly identifiable and clearly visible. Alternatively, the sample can be put in a recipient which can be closed in such a manner that it cannot be opened without irreversibly damaging the receptacle or container, avoiding the re-use of the receptacle or container.

— Identification of the sample: the sample has to be indelibly marked and must be identified in such a way that there is an unambiguous link to the sampling report.

— From each aggregate sample at least two final samples are taken: at least one for control (enforcement) and one for the feed business operator (defence). Eventually, one final sample may be taken for reference. In case the complete aggregate sample is homogenized, the final samples are taken from the homogenized aggregate sample, unless such procedure conflicts with Member States' rules as regards the right of the feed business operator.

4. APPARATUS

4.1. The sampling apparatus must be made of materials which cannot contaminate the products to be sampled. Apparatus which is intended to be used multiple times must be easy to clean to avoid any cross-contamination.

4.2. **Apparatus recommended for the sampling of solid feed**

4.2.1. ***Manual sampling***

4.2.1.1. Flat-bottomed shovel with vertical sides

4.2.1.2. Sampling spear with a long split or compartments. The dimensions of the sampling spear must be appropriate to the characteristics of the sampled portion (depth of container, dimensions of sack, etc.) and to the particle size of the feed.

In case the sampling spear has several apertures, in order to ensure that the sample is taken at the different locations alongside the spear, the apertures should be separated by compartments or sequentially staggered apertures.

4.2.2. ***Mechanical sampling***

Appropriate mechanical apparatus may be used for the sampling of moving feed. Appropriate means that at least the whole section of the flow is sampled.

Sampling of feed in motion (at high flow rates) can be performed by automatic samplers.

4.2.3. ***Divider***

If possible and appropriate, apparatus designed to divide the sample into approximately equal parts should be used for the preparation of reduced samples in a representative way.

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5. QUANTITATIVE REQUIREMENTS AS REGARDS NUMBER OF INCREMENTAL SAMPLES

— The quantitative requirements in points 5.1 and 5.2 as regards the number of incremental samples are applicable for sampled portion sizes up to a maximum of 500 tonnes and which can be sampled in a representative way. The sampling procedure described is equally valid for quantities larger than prescribed maximum sampled portion size provided that the maximum number of incremental samples given in the tables below is ignored, the number of incremental samples being determined by the square-root formula given in the appropriate part of the procedure (see point 5.3) and the minimum aggregate sample size increased proportionally. This does not prevent a large lot being divided into smaller sublots and each sublot sampled in accordance with the procedure described in points 5.1 and 5.2.

— The size of the sampled portion must be such that each of its constituent parts can be sampled.

— For very large lots or sublots (> 500 tonnes) and for lots which are transported or stored in such a way that sampling cannot be done in accordance with the sampling procedure provided for in points 5.1 and 5.2 of this chapter, the sampling procedure as provided for in point 5.3 is to be applied.

— In case the feed business operator is required by legislation to comply with this Regulation within the frame of a mandatory monitoring system, the feed business operator may deviate from the quantitative requirements as provided for in this chapter to take into account operational characteristics on the condition that the feed business operator has demonstrated to the satisfaction of the competent authority the equivalence of the sampling procedure as regards representativeness and after authorisation from the competent authority.

— In exceptional cases, if it is not possible to carry out the method of sampling set out as regards the quantitative requirements because of the unacceptable commercial damage to the lot (because of packaging forms, means of transport, way of storage etc.) an alternative method of sampling may be applied provided that it is as representative as possible and is fully described and documented.

5.1. **Quantitative requirements as regards incremental samples in relation to the control of substances or products uniformly distributed throughout the feed**

5.1.1. ***Loose solid feed***

Size of sampled portion	Minimum number of incremental samples
≤ 2,5 tonnes	7
> 2,5 tonnes	√ 20 times the number of tonnes making up the sampled portion (*), up to 40 incremental samples

(*) Where the number obtained is a fraction, it shall be rounded up to the next whole number.

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5.1.2. ***Loose liquid feed***

Size of sampled portion	Minimum number of incremental samples
≤ 2,5 tonnes or ≤ 2 500 litres	4 (*)
> 2,5 tonnes or > 2 500 litres	7 (*)

(*) In case it is not possible to make the liquid homogeneous, the number of incremental samples has to be increased.

5.1.3. ***Packaged feed***

Feed (solid and liquid) can be packaged in bags, sacks, cans, barrels etc. which are referred to in the table as units. Large units (≥ 500 kg or litres) have to be sampled in accordance with the provisions foreseen for loose feed (see points 5.1.1 and 5.1.2).

Size of sampled portion	Minimum number of units from which (at least) one incremental sample has to be taken (*)
1 to 20 units	1 unit (**)
21 to 150 units	3 units (**)
151 to 400 units	5 units (**)
> 400 units	¼ of the √ number of units making up the sampled portion (***), up to 40 units

(*) In the case where opening of an unit might affect the analysis (e.g. perishable wet feeds) an incremental sample shall be the unopened unit.
(**) For units whose contents do not exceed 1 kg or one litre, an incremental sample shall be the contents of one original unit.
(***) Where the number obtained is a fraction, it shall be rounded up to the next whole number.

5.1.4. ***Feed blocks and mineral licks***

Minimum one block or lick to be sampled per sampled portion of 25 units, up to a maximum of four blocks or licks.

For blocks or licks weighing not more than 1 kg each, an incremental sample shall be the contents of one block or one lick.

5.1.5. ***Roughages/forage***

Size of sampled portion	Minimum number of incremental samples (*)
≤ 5 tonnes	5
> 5 tonnes	√ 5 times the number of tonnes making up the sampled portion (**), up to 40 incremental samples

(*) It is acknowledged that in certain situations (e.g. silages) it is not possible to take the required incremental samples, without causing unacceptable damage to the lot. An alternative method of sampling may be applied in such situations and a guidance for sampling such lots will be elaborated before the entry into application of this Regulation.
(**) Where the number obtained is a fraction, it shall be rounded up to the next whole number.

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5.2. **Quantitative requirements as regards incremental samples in relation to the control of constituents or substances likely to be distributed non-uniformly in feed**

These quantitative requirements as regards incremental samples are to be used in the following situations:

— control of aflatoxins, rye ergot, other mycotoxins and harmful botanical impurities in feed materials;

— control of cross contamination by a constituent, including GM material, or substance for which non-uniform distribution is expected in feed materials.

In case the control authority has strong suspicion that such a non-uniform distribution occurs also in case of cross contamination by a constituent or substance in a compound feed, the quantitative requirements as provided for in the table below can be applied.

Size of sampled portion	Minimum number of incremental samples
< 80 tonnes	See quantitative requirements under point 5.1. The number of incremental samples to be taken has to be multiplied by 2,5.
≥ 80 tonnes	100

5.3. **Quantitative requirements as regards the incremental samples in the case of very large lots**

In the case of large sampled portions (sampled portions > 500 tonnes), the number of incremental samples to be taken = 40 incremental samples + √ tonnes in relation to the control of substances or products uniformly distributed throughout the feed or 100 incremental samples + √ tonnes in relation to the control of constituents or substances likely to be distributed non-uniformly in feed materials.

6. QUANTITATIVE REQUIREMENTS AS REGARDS AGGREGATE SAMPLE

A single aggregate sample per sampled portion is required.

	Nature of feed	Minimum size of aggregate sample (*) (**)
6.1.	Loose feed	4 kg
6.2.	Packaged feed:	4 kg (***)

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A single aggregate sample per sampled portion is required.

	Nature of feed	Minimum size of aggregate sample (*) (**)
6.3.	Liquid or semi-liquid feed:	4 litres
6.4.	Feed blocks or mineral licks:	
6.4.1.	each weighing more than 1 kg	4 kg
6.4.2.	each weighing not more than 1 kg	weight of four original blocks or licks
6.5.	Roughage/forage	4 kg (****)

(*) In case the sampled feed is of high value, a smaller quantity of aggregate sample can be taken on the condition this is described and documented in the sampling report.

(**) In accordance with the provisions of Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired (OJ L 166, 25.6.2011, p. 9), the aggregate sample for the control of the presence of genetically modified material must contain at least 35 000 seeds/grains. This means that for maize the size of the aggregate sample must be at least 10,5 kg and for soybean 7 kg. For other seeds and grains such as barley, millet, oat, rice, rye, wheat and rapeseed, the aggregate sample size of 4 kg corresponds to more than 35 000 seeds.

(***) In case of packaged feed, it may also not be possible to achieve the size of 4 kg for the aggregate sample depending of the size of the individual units.

(****) In case it concerns roughage or forage with a low specific gravity (e.g. hay, straw), the aggregate sample should have a minimum size of 1 kg.

7. QUANTITATIVE REQUIREMENTS AS REGARDS FINAL SAMPLES

Final samples

Analysis of at least one final sample is required. The amount in the final sample for analysis shall be not less than the following:

Solid feed	500 g (*) (**) (***)
Liquid or semi-liquid feed	500 ml (*)

(*) In accordance with the provisions of Regulation (EU) No 619/2011, the final sample for the control of the presence of genetically modified material must contain at least 10 000 seeds/grains. This means that for maize the size of the final sample must be at least 3 000 g and for soybean 2 000 g. For other seeds and grains such as barley, millet, oat, rice, rye, wheat and rapeseed, the final sample size of 500 g corresponds to more for 10 000.

(**) In case the size of the aggregate sample is significantly less than 4 kg or litre (see footnotes point (6), also a smaller quantity of final sample can be taken on the condition this is described and documented in the sampling report.

(***) In case of sampling pulses, cereal grains and tree nuts for the determination of pesticide residues, the minimum size of the final sample shall be 1 kg in accordance with the provisions of Commission Directive 2002/63/EC (OJ L 187, 16.7.2002, p. 30).

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8. METHOD OF SAMPLING FOR VERY LARGE LOTS OR LOTS STORED OR TRANSPORTED IN A WAY WHEREBY SAMPLING THROUGHOUT THE LOT IS NOT FEASIBLE

8.1. **General principles**

In case the way of transport or storage of a lot does not enable to take incremental samples throughout the whole lot, sampling of such lots should preferably be done when the lot is in flow.

In the case of large warehouses destined to store feed, operators should be encouraged to install equipment in the warehouse enabling (automatic) sampling across the whole stored lot.

In case of applying the sampling procedures as provided for in this chapter 8, the feed business operator or his representative is informed of the sampling procedure. In case this sampling procedure is questioned by the feed business operator or his representative, the feed business operator or his representative shall enable the competent authority to sample throughout the whole lot at his/her cost.

8.2. **Large lots transported by ship**

8.2.1. ***Dynamic sampling of large lots transported by ship***

The sampling of large lots in ships is preferably carried out while the product is in flow (dynamic sampling).

The sampling is to be done per hold (entity that can physically be separated). Holds are however emptied partly one after the other so that the initial physical separation does no longer exist after transfer into storage facilities. Sampling can therefore be performed in function of the initial physical separation or in function of the separation after transfer into the storage facilities.

The unloading of a ship can last for several days. Normally, sampling has to be performed at regular intervals during the whole duration of unloading. It is however not always feasible or appropriate for an official inspector to be present for sampling during the whole operation of unloading. Therefore sampling is allowed to be undertaken of part (sampled portion) of the whole lot. The number of incremental samples is determined by taking into account the size of the sampled portion.

In the case of sampling a part of a lot of feed of the same class or description and that part of the lot has been identified as not satisfying EU requirements, it shall be presumed that all of the feed in that lot is so affected, unless following a detailed assessment there is no evidence that the rest of the lot fails to satisfy the EU requirements.

Even if the official sample is taken automatically, the presence of an inspector is necessary. However in case the automatic sampling is done with preset parameters which cannot be changed during the sampling and the incremental samples are collected in a sealed receptacle, preventing any possible fraud, then the presence of an inspector is only required at the beginning of the sampling, every time the receptacle of the samples needs to be changed and at the end of the sampling.

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8.2.2. ***Sampling of lots transported by ship by static sampling***

In case the sampling is done in a static way the same procedure as foreseen for storage facilities (silos) accessible from above has to be applied (see point 8.4.1).

The sampling has to be performed on the accessible part (from above) of the lot/hold. The number of incremental samples is determined by taking into account the size of the sampled portion. In the case of sampling a part of a lot of feed of the same class or description and that part of the lot has been identified as not satisfying EU requirements, it shall be presumed that all of the feed in that lot is so affected, unless following a detailed assessment there is no evidence that the rest of the lot fails to satisfy the EU requirements.

8.3. **Sampling of large lots stored in warehouses**

The sampling has to be performed on the accessible part of the lot. The number of incremental samples is determined by taking into account the size of the sampled portion. In the case of sampling a part of a lot of feed of the same class or description and that part of the lot has been identified as not satisfying EU requirements, it shall be presumed that all of the feed in that lot is so affected, unless following a detailed assessment there is no evidence that the rest of the lot fails to satisfy the EU requirements.

8.4. **Sampling of storage facilities (silos)**

8.4.1. ***Sampling of silos (easily) accessible from above***

The sampling has to be performed on the accessible part of the lot. The number of incremental samples is determined by taking into account the size of the sampled portion. In the case of sampling a part of a lot of feed of the same class or description and that part of the lot has been identified as not satisfying EU requirements, it shall be presumed that all of the feed in that lot is so affected, unless following a detailed assessment there is no evidence that the rest of the lot fails to satisfy the EU requirements.

8.4.2. ***Sampling of silos not accessible from above (closed silos)***

8.4.2.1. Silos not accessible from above (closed silos) with size > 100 tonnes

Feed stored in such silos cannot be sampled in a static way. Therefore in case the feed in the silo has to be sampled and there is no possibility to move the consignment, the agreement has to be made with the operator that he or she has to inform the inspector about when the silo will be unloaded in order to enable sampling when the feed is in flow.

8.4.2.2. Silos not accessible from above (closed silos) with size < 100 tonnes

Sampling procedure involves the release into a receptacle of a quantity of 50 to 100 kg and taking the sample from it. The size of the aggregate sample corresponds to the whole lot and the number of incremental samples relate to the quantity of the silo released in a receptacle for sampling. In the case of sampling a part of a lot of feed of the same class or description and that part of the lot has been identified as not satisfying EU requirements, it shall be presumed that all of the feed in that lot is so affected, unless following a detailed assessment there is no evidence that the rest of the lot fails to satisfy the EU requirements.

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8.5. **Sampling of loose feed in large closed containers**

Such lots can often only be sampled when unloaded. It is in certain cases not possible to unload at the point of import or control and therefore the sampling should take place when such containers are unloaded.

9. INSTRUCTIONS FOR TAKING, PREPARING AND PACKAGING THE SAMPLES

9.1. **General**

The samples must be taken and prepared without unnecessary delay bearing in mind the precautions necessary to ensure that the product is neither changed nor contaminated. Instruments and also surfaces and containers intended to receive samples must be clean and dry.

9.2. **Incremental samples**

Incremental samples must be taken at random throughout the whole sampled portion and they must be of approximately equal sizes.

The incremental sample size is at least 100 grams or 25 grams in case of roughage or forage with low specific gravity.

In case that in accordance with the rules for the sampling procedure established in point 8 less than 40 incremental samples have to be taken, the size of the incremental samples shall be determined in function of the required size of the aggregate sample to be achieved (see point (6).

In case of sampling of small lots of packaged feed where according to the quantitative requirements a limited number of incremental samples have to be taken, an incremental sample shall be the contents of one original unit whose contents do not exceed 1 kg or one litre.

In case of sampling of packaged feed composed of small units (e.g. < 250 g), the size of the incremental sample depends on the size of the unit.

9.2.1. ***Loose feed***

Where appropriate, sampling may be carried out when the sampled portion is being moved (loading or unloading).

9.2.2. ***Packaged feed***

Having selected the required number of units for sampling as indicated in chapter 5, part of the contents of each unit shall be removed using a spear or shovel. Where necessary, the samples shall be taken after emptying the units separately.

9.2.3. ***Homogeneous or homogenisable liquid or semi-liquid feed***

Having selected the required number of units for sampling as indicated in chapter 5, the contents shall be homogenised if necessary and an amount taken from each unit.

The incremental samples may be taken when the contents are being discharged.

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9.2.4. ***Non-homogenisable, liquid or semi-liquid feed***

Having selected the required number of units for sampling as indicated in chapter 5, samples shall be taken from different levels.

Samples may also be taken when the contents are being discharged but the first fractions shall be discarded.

In either case the total volume taken must not be less than 10 litres.

9.2.5. ***Feed blocks and mineral licks***

Having selected the required number of blocks or licks for sampling as indicated in chapter 5, a part of each block or lick can be taken. In case of suspicion of a non-homogeneous block or lick, the whole block or lick can be taken as sample.

For blocks or licks weighing not more than 1 kg each, an incremental sample shall be the contents of one block or one lick.

9.3. **Preparation of aggregate samples**

The incremental samples shall be mixed to form a single aggregate sample.

9.4. **Preparation of final samples**

The material in the aggregate sample shall be carefully mixed ([1]).

— Each sample shall be put into an appropriate container/receptacle. All necessary precautions shall be taken to avoid any change of composition of the sample, contamination or adulteration which might arise during transportation or storage.

— In case of the control of constituents or substances uniformly distributed throughout the feed, the aggregate sample can be representatively reduced to at least 2,0 kg or 2,0 litres (reduced sample) ([2]) preferably either by using a mechanical or automatic divider. For the control of the presence of pesticide residues in pulses, cereal grains and tree nuts, the minimum size of the reduced sample shall be 3 kg. In case the nature of the feed does not allow using a divider or the divider is not available, then the sample can be reduced by the quartering method. From the reduced samples the final samples (for control, defence and reference) shall then be prepared of approximately the same amount and conforming to the quantitative requirements of chapter 7. In case of the control of constituents, including genetically modified material, or substances likely to be distributed non-uniformly in feed materials, the aggregate sample shall be:

  — completely homogenized and divided afterwards into final samples or

  — reduced to at least 2 kg or 2 litres ([3]) by using a mechanical or automatic divider. Only in the case that the nature of the feed does not allow for using a divider, the sample can, if necessary, be reduced by quartering method. For the control of the presence of genetically modified material in the frame of Regulation (EU) No 619/2011, the reduced sample must contain at least 35 000 seeds/grains to enable to obtain the final samples for enforcement, defence and reference of at least 10 000 seeds grain (see footnote (**) in chapter 6 and footnote (*) in chapter 7).

([1]) Any lumps shall be broken up (if necessary by separating them out and returning them to the sample).
([2]) Except in the case of roughage or forage with low specific gravity.
([3]) Except in the case of roughage or forage with low specific gravity.

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9.5. **Packaging of samples**

The containers or packages shall be sealed and labelled in such a manner that they cannot be opened without damaging the seal. The total label must be incorporated in the seal.

9.6. **Sending of samples to the laboratory**

The sample shall be sent without unnecessary delay to the designated analytical laboratory, together with the information necessary for the analyst.

10. SAMPLING RECORD

A record must be kept of each sample, permitting each sampled portion and its size to be identified unambiguously.

The record shall also mention any deviation of the sampling procedure as provided for in this Regulation.

Besides making the record available to the official control laboratory, the record shall be made available to the feed business operator and/or the laboratory designated by the feed business operator.

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ANNEX II

GENERAL PROVISIONS ON METHODS OF ANALYSIS FOR FEED

A. PREPARATION OF SAMPLES FOR ANALYSIS

1. **Purpose**

The procedures described below concern the preparation for analysis of samples, sent to the control laboratories after sampling in accordance with the provisions laid down in Annex I.

The laboratory samples must be prepared in such a way that the amounts weighed out, as provided for in the methods of analysis, are homogeneous and representative of the final samples.

2. **Precautions to be taken**

The sample preparation procedure to be followed is dependent on the methods of analysis to be used and the constituents or substances to be controlled. It is therefore of major importance that it is ensured that the followed sample preparation procedure is appropriate for the used method of analysis and for constituents or substances to be controlled.

All the necessary operations must be performed in such a way as to avoid as far as possible contamination of the sample and changes of its composition.

Grinding, mixing and sieving shall be carried out without delay with minimal exposure of the sample to the air and light. Mills and grinders likely to appreciably heat the sample shall not be used.

Manual grinding is recommended for feed which are particularly sensitive to heat. Care shall also be taken to ensure that the apparatus itself is not a source of contamination.

If the preparation cannot be carried out without significant changes in the moisture content of the sample, determine the moisture content before and after preparation according to the method laid down in Part A of Annex III.

3. **Procedure**

3.1. ***General procedure***

The test aliquot is taken from the final sample. Coning and quartering is not recommended because this might provide test aliquots with high splitting error.

3.1.1. Feed which can be ground as such

— Mix the sieved final sample and collect it in a suitable clean, dry container fitted with an air-tight stopper. Mix again in order to ensure full homogenisation, immediately before weighing out the amount for analysis (test aliquot).

3.1.2. Feed which can be ground after drying

— Unless otherwise specified in the methods of analysis, dry the final sample to bring its moisture content down to a level of 8 to 12 %, according to the preliminary drying procedure described under point 4.3 of the method of determination of moisture mentioned in Part A of Annex III). Then proceed as indicated in section 3.1.1.

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3.1.3. Liquid or semi-liquid feed

— Collect the final sample in a suitable clean, dry container, fitted with an air-tight stopper. Mix thoroughly in order to ensure full homogenisation immediately before weighing out the amount for analysis (test aliquot).

3.1.4. Other feed

— Final samples which cannot be prepared according to one of the above procedures shall be treated by any other procedure which ensures that the amounts weighed out for the analysis (test aliquot) are homogeneous and representative of the final samples.

3.2. ***Specific procedure in case of examination by visual inspection or by microscopy or in cases where the whole aggregate sample is homogenised***

— In case of an examination by visual inspection (without making use of microscope), the whole laboratory sample is used for examination.

— In case of a microscopic examination, the laboratory may reduce the aggregate sample, or further reduce the reduced sample. The final samples for defence and eventually reference purposes are taken following a procedure equivalent to the procedure followed for the final sample for enforcement.

— In case the whole aggregate sample is homogenized, the final samples are taken from the homogenized aggregate sample.

4. **Storage of samples**

Samples must be stored at a temperature that will not alter their composition. Samples intended for the analysis of vitamins or substances which are particularly sensitive to light shall be stored in such conditions that the sample is not adversely affected by light.

B. PROVISIONS RELATING TO REAGENTS AND APPARATUS USED IN METHODS OF ANALYSIS

1. Unless otherwise specified in the methods of analysis, all analytical reagents must be analytically pure (a.p.). When trace analysis is carried out, the purity of the reagents must be checked by a blank test. Depending upon the results obtained, further purification of the reagents may be required.

2. Any operation involving preparation of solutions, dilution, rinsing or washing, mentioned in the methods of analysis without indication as to the nature of the solvent or diluent employed, implies that water must be used. As a general rule, water shall be demineralised or distilled. In particular cases, which are indicated in the methods of analysis, it must be submitted to special procedures of purification.

3. In view of the equipment normally found in control laboratories, only those instruments and apparatus which are special or require specific usage are referred to in the methods of analysis. They must be clean, especially when very small amounts of substances have to be determined.

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C. APPLICATION OF METHODS OF ANALYSIS AND EXPRESSION OF THE RESULTS

1. **Extraction procedure**

Several methods determine a specific extraction procedure. As a general rule, other extraction procedures than the procedure referred to in the method can be applied on the condition that the used extraction procedure has been proven to have the equivalent extraction efficiency for the matrix analysed as the procedure mentioned in the method.

2. **Clean-up procedure**

Several methods determine a specific clean-up procedure. As a general rule, other clean-up procedures than the procedure referred to in the method can be applied on the condition that the used clean-up procedure has been proven to result in equivalent analytical results for the matrix analysed as the procedure mentioned in the method.

3. **Number of determinations**

In case of the analysis of undesirable substances, if the result of the first determination is significantly (> 50 %) lower than the specification to be controlled, no additional determinations are necessary, on the condition that the appropriate quality procedures are applied. In other cases a duplicate analysis (second determination) is necessary to exclude the possibility of internal cross-contamination or an accidental mix-up of samples. The mean of the two determinations, taking into account the measurement uncertainty is used for verification of compliance.

In case of the control of the declared content of a substance or ingredient, if the result of the first determination confirms the declared content, i.e. the analytical result falls within the acceptable range of variation of the declared content, no additional determinations are necessary, on the condition that the appropriate quality procedures are applied. In other cases a duplicate analysis (second determination) is necessary to exclude the possibility of internal cross-contamination or an accidental mix-up of samples. The mean of the two determinations, taking into account the measurement uncertainty is used for verification of compliance.

In some cases this acceptable range of variation is defined in legislation such as in Regulation (EC) No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC ([1]).

4. **Reporting of the method of analysis used**

The analysis report shall mention the method of analysis used.

5. **Reporting of the analytical result**

The analytical result shall be expressed in the manner laid down in the method of analysis to an appropriate number of significant figures and shall be corrected, if necessary, to the moisture content of the final sample prior to preparation.

([1]) OJ L 229, 1.9.2009, p. 1.

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6. **Measurement uncertainty and recovery rate in case of analysis of undesirable substances**

As regards undesirable substances within the meaning of Directive 2002/32/EC, a product intended for animal feed shall be considered as non-compliant with the established maximum content, if the analytical result, relative to a feed with a moisture content of 12 %, is deemed to exceed the maximum content taking into account expanded measurement uncertainty and correction for recovery. In order to assess compliance, the analysed concentration is used after being corrected for recovery and after deduction of the expanded measurement uncertainty. This procedure is only applicable in cases where the method of analysis enables the estimation of measurement uncertainty and correction for recovery (e.g. not possible in case of microscopic analysis).

The analytical result shall be reported as follows (in so far the used method of analysis enables to estimate the measurement uncertainty and recovery rate):

(a) corrected for recovery, the level of recovery being indicated. The correction for recovery is not necessary in case the recovery rate is between 90-110 %.

(b) as ‘x +/– U’, whereby x is the analytical result and U is the expanded measurement uncertainty, using a coverage factor of 2 which gives a level of confidence of approximately 95 %.

However, if the result of the analysis is significantly (> 50 %) lower than the specification to be controlled, and on the condition that the appropriate quality procedures are applied and the analysis serves only the purpose of checking compliance with legal provisions, the analytical result might be reported without correction for recovery and the reporting of the recovery rate and measurement uncertainty might be omitted in these cases.

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ANNEX III

METHODS OF ANALYSIS TO CONTROL THE COMPOSITION OF FEED MATERIALS AND COMPOUND FEED

A. DETERMINATION OF MOISTURE

1. **Purpose and Scope**

This method makes it possible to determine the moisture content of feed. In case of feed containing volatile substances, such as organic acids, it is to be observed that also significant amount of volatile substances are determined together with the moisture content.

It does not cover the analysis of milk products as feed materials, the analysis of mineral substances and mixtures composed predominantly of mineral substances, the analysis of animal and vegetable fats and oils or the analysis of the oil seeds and oleaginous fruit.

2. **Principle**

The sample is desiccated under specified conditions which vary according to the nature of the feed. The loss in weight is determined by weighing. It is necessary to carry out preliminary drying when dealing with solid feed which has high moisture content.

3. **Apparatus**

3.1. Crusher of non-moisture-absorbing material which is easy to clean, allows rapid, even crushing without producing any appreciable heating, prevents contact with the outside air as far as possible and meets the requirements laid down in 4.1.1 and 4.1.2 (e.g. hammer or water cooled micro-crushers, collapsible cone mills, slow motion or cog wheeled crushers).

3.2. Analytical balance, accurate to 1 mg.

3.3. Dry containers of non-corrodible metal or of glass with lids ensuring airtight closure; working surface allowing the test sample to be spread at about 0,3 g/cm^2.

3.4. Electrically heated isothermal oven (± 2 °C) properly ventilated and ensuring rapid temperature regulation ([1]).

3.5. Adjustable electrically heated vacuum oven fitted with an oil pump and either a mechanism for introducing hot dried air or a drying agent (e.g. calcium oxide).

3.6. Desiccator with a thick perforated metal or porcelain plate, containing an efficient drying agent.

4. **Procedure**

N.B. The operations described in this section must be carried out immediately after opening the packages of samples. Analysis must be carried out at least in duplicate.

([1]) For the drying of cereals, flour, groats and meal, the oven must have a thermal capacity such that, when pre-set at 131 °C, it will return to that temperature in less than 45 minutes after the maximum number of test samples have been placed inside to dry simultaneously. Ventilation must be such that, when as many samples of common wheat as it can contain are dried for two hours, the results differ from those obtained after four hours of drying by less than 0,15 %.

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4.1. ***Preparation***

4.1.1. Feed other than those coming under 4.1.2 and 4.1.3

Take at least 50 g of the sample. If necessary, crush or divide in such a way as to avoid any variation in moisture content (see 6).

4.1.2. Cereals and groats

Take at least 50 g of the sample. Grind into particles of which at least 50 % will pass through a 0,5 mm mesh sieve and will leave no more than 10 % reject on a 1 mm round-meshed sieve.

4.1.3. Feed in liquid or paste form, feed predominantly composed of oils and fats

Take about 25 g of the sample, weigh to the nearest 10 mg, add an appropriate quantity of anhydrous sand weighed to the nearest 10 mg and mix until a homogeneous product is obtained.

4.2. ***Drying***

4.2.1. Feed other than those coming under 4.2.2 and 4.2.3

Weigh a container (3.3) with its lid to the nearest 1 mg. Weigh into the weighed container, to the nearest 1 mg, about 5 g of the sample and spread evenly. Place the container, without its lid, in the oven preheated to 103 °C. To prevent the oven temperature from falling unduly, introduce the container as rapidly as possible. Leave to dry for four hours reckoned from the time when the oven temperature returns to 103 °C. Replace the lid on the container, remove the latter from the oven, leave to cool for 30 to 45 minutes in the desiccator (3.6) and weigh to the nearest 1 mg.

For feed composed predominantly of oils and fats, dry in the oven for an additional 30 minutes at 130 °C. The difference between the two weighings must not exceed 0,1 % of moisture.

4.2.2. Cereals, flour, groats and meal

Weigh a container (3.3) with its lid to the nearest 0,5 mg. Weigh into the weighed container, to the nearest 1 mg, about 5 g of the crushed sample and spread evenly. Place the container, without its lid, in the oven preheated to 130 °C. To prevent the oven temperature from falling unduly, introduce the container as rapidly as possible. Leave to dry for two hours reckoned from the time when the oven temperature returns to 130 °C. Replace the lid on the container, remove the latter from the oven, leave to cool for 30 to 45 minutes in the desiccator (3.6) and weigh to the nearest 1 mg.

4.2.3. Compound feed containing more than 4 % of sucrose or lactose: feed materials such as locust beans, hydrolysed cereal products, malt seeds, dried beet chips, fish and sugar solubles; compound feed containing more than 25 % of mineral salts including water of crystallisation.

Weigh a container (3.3) with its lid to the nearest 0,5 mg. Weigh into the weighed container, to the nearest 1 mg, about 5 g of the sample and spread evenly. Place the container, without its lid, in the vacuum oven (3.5) preheated to between 80 °C and 85 °C. To prevent the oven temperature from falling unduly, introduce the container as rapidly as possible.

Bring the pressure up to 100 Torr and leave to dry for four hours at this pressure, either in a current of hot, dry air or using a drying agent (about 300 g for 20 samples). In the latter instance, disconnect the vacuum pump when the prescribed pressure has been reached. Reckon drying time from the moment when the oven temperature returns to 80 °C to 85 °C. Carefully bring the oven back to atmospheric pressure. Open the oven, place the lid on the container immediately,

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remove the container from the oven, leave to cool for 30 to 45 minutes in the desiccator (3.6) and weigh to the nearest 1 mg. Dry for an additional 30 minutes in the vacuum oven at 80 °C to 85 °C and reweigh. The difference between the two weighings must not exceed 0,1 % of moisture.

4.3. ***Preliminary drying***

4.3.1. Feed other than those coming under 4.3.2

Solid feed with a high moisture content which makes crushing difficult must be subjected to preliminary drying as follows:

Weigh 50 g of ***uncrushed*** sample to the nearest 10 mg (compressed or agglomerated feed may be roughly divided if necessary) in a suitable container (e.g. a 20 × 12 cm aluminium plate with a 0,5 cm rim). Leave to dry in an oven from 60 °C to 70 °C until the moisture content has been reduced to between 8 % and 12 %. Remove from the oven, leave to cool uncovered in the laboratory for one hour and weigh to the nearest 10 mg. Crush immediately as indicated in 4.1.1 and dry as indicated in 4.2.1 or 4.2.3 according to the nature of the feed.

4.3.2. Cereals

Grain with a moisture content of over 17 % must be subjected to preliminary drying as follows:

Weigh 50 g of unground grain to the nearest 10 mg in a suitable container (e.g. a 20 × 12 cm aluminium plate with a 0,5 cm rim). Leave to dry for 5 to 7 minutes in an oven at 130 °C. Remove from the oven, leave to cool uncovered in the laboratory for two hours and weigh to the nearest 10 mg. Grind immediately as indicated in 4.1.2 and dry as indicated in 4.2.2.

5. **Calculation of results**

The moisture content (X), as a percentage of the sample, is calculated by using the following formulae:

5.1. ***Drying without preliminary drying***

$$X = \frac{(m - m_0)}{m} \times 100$$

where:

m = initial weight, in grammes, of the test sample,
m_0 = weight, in grammes, of the dry test sample.

5.2. ***Drying with preliminary drying***

$$X_p = \left[\frac{(m_2 - m_0) \times m_1}{m_2} + m - m_1\right] \times \frac{100}{m} = 100 \times \left(1 - \frac{m_1 \times m_0}{m \times m_2}\right)$$

where:

m = initial weight, in grammes, of the test sample,
m_1 = weight, in grammes, of the test sample after preliminary drying,
m_2 = weight, in grammes, of the test sample after crushing or grinding,
m_0 = weight, in grammes, of the dry test sample.

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5.3. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample shall not exceed 0,2 % of the absolute value of moisture.

6. **Observation**

If crushing proves necessary and if this is seen to alter the moisture content of the product, the results of the analysis of the components of the feed must be corrected on the basis of the moisture content of the sample in its initial state.

B. DETERMINATION OF MOISTURE IN ANIMAL AND VEGETABLE FATS AND OILS

1. **Purpose and scope**

This method makes it possible to determine the water and volatile substances content of animal and vegetable fats and oils.

2. **Principle**

The sample is dried to constant weight (loss in weight between two successive weighings must be less than or equal to 1 mg) at 103 °C. The loss in weight is determined by weighing.

3. **Apparatus**

3.1. Flat-bottomed dish, of a corrosion-resistant material, 8 to 9 cm in diameter and approximately 3 cm high.

3.2. Thermometer with a strengthened bulb and expansion tube at the top end, graduated from approximately 80 °C to at least 110 °C, and approximately 10 cm in length.

3.3. Sand bath or electric hot-plate.

3.4. Desiccator, containing an efficient drying agent.

3.5. Analytical balance.

4. **Procedure**

Weigh out to the nearest mg approximately 20 g of the homogenised sample into the dry, weighed dish (3.1) containing the thermometer (3.2). Heat on the sand bath or hot-plate (3.3), stirring continuously with the thermometer, so that the temperature reaches 90 °C in about 7 minutes.

Reduce the heat, watching the frequency with which bubbles rise from the bottom of the dish. The temperature must not exceed 105 °C. Continue to stir, scraping the bottom of the dish, until bubbles stop forming.

In order to ensure complete elimination of moisture, reheat several times to 103 °C ± 2 °C, cooling to 93 °C between successive heatings. Then leave to cool to room temperature in the desiccator (3.4) and weigh. Repeat this operation until the loss in weight between two successive weighings no longer exceeds 2 mg.

N.B: An increase in the weight of the sample after repeated heating indicates an oxidation of the fat, in which case calculate the result from the weighing carried out immediately before the weight began to increase.

5. **Calculation of results**

The moisture content (***X***), as a percentage of the sample, is given by the following formula:

$$X = (m_1 - m_2) \times \frac{100}{m}$$

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where:

m = weight, in grammes, of the test sample,
m_1 = weight, in grammes, of the dish with its contents before heating,
m_2 = weight, in grammes, of the dish with its contents after heating.

Results lower than 0,05 % must be recorded as 'lower than 0,05 %'.

Repeatability

The difference in moisture between the results of two parallel determinations carried out on the same sample must not exceed 0,05 %, in absolute value.

C. DETERMINATION OF THE CONTENT OF CRUDE PROTEIN

1. **Purpose and scope**

This method makes it possible to determine the crude protein content of feed on the basis of the nitrogen content, determined according to the Kjeldahl method.

2. **Principle**

The sample is digested by sulphuric acid in the presence of a catalyst. The acid solution is made alkaline with sodium hydroxide solution. The ammonia is distilled and collected in a measured quantity of sulphuric acid, the excess of which is titrated with a standard solution of sodium hydroxide.

Alternatively, the liberated ammonia is distilled into an excess of boric acid solution, followed by titration with hydrochloric acid or sulphuric acid solution.

3. **Reagents**

3.1. Potassium sulphate.

3.2. Catalyst: copper (II) oxide CuO or copper (II) sulphate pentahydrate, $CuSO_4$ $5H_2O$.

3.3. Granulated zinc.

3.4. Sulphuric acid, $\rho 20$ = 1,84 g/ml.

3.5. Sulphuric acid, standard volumetric solution, c(H_2SO_4) = 0,25 mol/l.

3.6. Sulphuric acid, standard volumetric solution, c(H_2SO_4) = 0,10 mol/l.

3.7. Sulphuric acid, standard volumetric solution, c(H_2SO_4) = 0,05 mol/l.

3.8. Methyl red indicator; dissolve 300 mg of methyl red in 100 ml of ethanol, σ = 95 %-96 % (v/v).

3.9. Sodium hydroxide solution (Technical grade may be used) β = 40 g/100 ml (m/v: 40 %).

3.10. Sodium hydroxide, standard volumetric solution c(NaOH) = 0,25 mol/l.

3.11. Sodium hydroxide, standard volumetric solution c(NaOH) = 0,10 mol/l.

3.12. Granulated pumice stone, washed in hydrochloric acid and ignited.

3.13. Acetanilide (m.p. = 114 °C, N-content = 10,36 %).

3.14. Sucrose (nitrogen free).

3.15. Boric acid (H_3BO_3).

3.16. Methyl red indicator solution: dissolve 100 mg methyl red in 100 ml ethanol or methanol.

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3.17. Bromocresol green solution: dissolve 100 mg bromocresol green in 100 ml ethanol or methanol.

3.18. Boric acid solution (10 g/l to 40 g/l depending on the apparatus used).

When colorimetric end-point detection is applied, methyl red and bromocresol indicators must be added to the boric acid solutions. If 1 litre of the boric acid solution is prepared, before adjusting to volume, 7 ml methyl red indicator solution (3.16) and 10 ml bromocresol green solution (3.17) shall be added.

Dependent on the water used, the pH of the boric acid solution might differ from batch to batch. Often an adjustment with a small volume of alkali is necessary to obtain a positive blank.

Note: The addition of about 3 ml to 4 ml of NaOH (3.11) into 1 litre of 10 g/l boric acid usually gives good adjustments. Store the solution at room temperature and protect the solution from light and sources of ammonia fumes during storage.

3.19. Hydrochloric acid standard volumetric solution c(HCl) = 0,10 mol/l.

Note: Other concentrations of volumetric solutions (3.5, 3.6, 3.7, 3.10, 3.11, and 3.19) can be used, if this is corrected for in the calculations. The concentrations shall always be expressed to four decimal places.

4. **Apparatus**

Apparatus suitable for performing digestion, distillation and titration according to the Kjeldahl procedure.

5. **Procedure**

5.1. ***Digestion***

Weigh 1 g of the sample to the nearest 0,001 g and transfer the sample to the flask of the digestion apparatus. Add 15 g of potassium sulphate (3.1), an appropriate quantity of catalyst (3.2) (0,3 to 0,4 g of copper (II) oxide or 0,9 to 1,2 g of copper (II) sulphate pentahydrate), 25 ml of sulphuric acid (3.4) and if required, a few granules of pumice stone (3.12) and mix.

Heat the flask moderately at first, swirling from time to time if necessary until the mass has carbonised and the foam has disappeared; then heat more intensively until the liquid is boiling steadily. Heating is adequate if the boiling acid condenses on the wall of the flask. Prevent the sides from becoming overheated and organic particles from sticking to them.

When the solution becomes clear and light green continue to boil for another two hours, then leave to cool.

5.2. ***Distillation***

Add carefully enough water to ensure complete dissolution of the sulphates. Allow to cool and then add a few granules of zinc (3.3), if required. Proceed according to 5.2.1 or 5.2.2.

5.2.1. Distillation into sulphuric acid

Place in the collecting flask of the distillation apparatus an exactly measured quantity of 25 ml of sulphuric acid (3.5) or (3.7) depending on the presumed nitrogen content. Add a few drops of methyl red indicator (3.8).

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Connect the digestion flask to the condenser of the distillation apparatus and immerse the end of the condenser in the liquid contained in the collecting flask to a depth of at least 1 cm (see observation 8.3). Slowly pour 100 ml of sodium hydroxide solution (3.9) into the digestion flask without loss of ammonia (see observation 8.1). Heat the flask until the ammonia has distilled over.

5.2.2. Distillation into boric acid

Where titration of the ammonia content of the distillate is performed manually, the procedure mentioned below applies. Where the distillation unit is fully automated to include titration of the ammonia content of the distillate, follow the manufacturer's instructions for operation of the distillation unit.

Place a collecting flask containing 25 ml to 30 ml of the boric acid solution (3.18) under the outlet of the condenser in such a way that the delivery tube is below the surface of the excess boric acid solution. Adjust the distillation unit to dispense 50 ml of sodium hydroxide solution (3.9). Operate the distillation unit in accordance with the manufacturer's instructions and distil off the ammonia liberated by the addition of the sodium hydroxide solution. Collect distillate in the boric acid receiving solution. The amount of distillate (time of steam distillation) depends on the amount of nitrogen in the sample. Follow the instructions of the manufacturer.

Note: In a semi-automatic distillation unit, the addition of excess sodium hydroxide and the steam distillation are performed automatically.

5.3. ***Titration***

Proceed according to 5.3.1 or 5.3.2.

5.3.1. Sulphuric acid

Titrate the excess sulphuric acid in the collecting flask with sodium hydroxide solution (3.10 or 3.11) depending on the concentration of the sulphuric acid used, until the end-point is reached.

5.3.2. Boric acid

Titrate the contents of the collecting flask with the hydrochloric acid standard volumetric solution (3.19) or with the sulphuric acid standard volumetric solution (3.6) using a burette and read the amount of titrant used.

When colorimetric end-point detection is applied, the end-point is reached at the first trace of pink colour in the contents. Estimate the burette reading to the nearest 0,05 ml. An illuminated magnetic stirrer plate or a photometric detector may aid visualisation of the end-point.

This can be done automatically using a steam distiller with automatic titration.

Follow the manufacturers' instructions for operation of the specific distiller or distiller/titrator.

Note: When an automatic titration system is used, titration begins immediately after distillation starts and the 1 % boric acid solution (3.18) is used.

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Where a fully automatic distillation unit is employed, the automatic titration of the ammonia can also be carried out with end-point detection using a potentiometric pH system.

In this case an automatic titrator, with a pH-meter is used. The pH-meter shall be calibrated properly in the range of pH 4 to pH 7 following normal laboratory pH-calibration procedures.

The pH end-point of the titration is reached at pH 4,6, being the steepest point in the titration curve (inflection point).

5.4. ***Blank test***

To confirm that the reagents are free from nitrogen, carry out a blank test (digestion, distillation and titration) using 1 g of sucrose (3.14) in place of the sample.

6. **Calculation of results**

Calculations are performed according to 6.1 or 6.2.

6.1. ***Calculation for titration according to 5.3.1***

The content of crude protein, expressed as a percentage by weight, is calculated according to the following formula:

$$\frac{(V_0 - V_1) \times c \times 0{,}014 \times 100 \times 6{,}25}{m}$$

where:

V_o = is the volume (ml) of NaOH (3.10 or 3.11) used in the blank test,
V_1 = is the volume (ml) of NaOH (3.10 or 3.11) used in the sample titration,
c = is the concentration (mol/l) of sodium hydroxide (3.10 or 3.11),
m = is the weight (g) of sample.

6.2. ***Calculation for titration according to 5.3.2***

6.2.1. Titration with hydrochloric acid

The content of crude protein, expressed as a percentage by weight, is calculated according to the following formula:

$$\frac{(V_1 - V_0) \times c \times 1{,}4 \times 6{,}25}{m}$$

where:

m = is the weight (g) of the test portion,
c = is the concentration (mol/l) of the standard volumetric solution of the hydrochloric acid (3.19),
V_0 = is the volume (in ml) of hydrochloric acid used for the blank test,
V_1 = is the volume (in ml) of hydrochloric acid used for the test portion.

6.2.2. Titration with sulphuric acid

The content of crude protein, expressed as a percentage by weight, is calculated according to the following formula:

$$\frac{(V_1 - V_0) \times c \times 2{,}8 \times 6{,}25}{m}$$

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where:

m = is the weight (g) of the test portion,
c = is the concentration (mol/l) of the standard volumetric solution of sulphuric acid (3.6),
V_0 = is the volume (in ml) of sulphuric acid (3.6) used for the blank test,
V_1 = is the volume (in ml) of sulphuric acid (3.6) used for test portion.

7. **Verification of the method**

7.1. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

— 0,2 % in absolute value, for crude protein contents of less than 20 %,

— 1,0 % relative to the higher value, for crude protein contents from 20 % to 40 %,

— 0,4 % in absolute value, for crude protein contents of more than 40 %.

7.2. ***Accuracy***

Carry out the analysis (digestion, distillation and titration) on 1,5 to 2,0 g of acetanilide (3.13) in the presence of 1 g of sucrose (3.14); 1 g acetanilide consumes 14,80 ml of sulphuric acid (3.5). Recovery must be at least 99 %.

8. **Observations**

8.1. Apparatus may be of the manual, semi-automatic or automatic type. If the apparatus requires transference between the digestion and distillation steps, this transfer must be carried out without loss. If the flask of the distillation apparatus is not fitted with a dropping funnel, add the sodium hydroxide immediately before connecting the flask to the condenser, pouring the liquid slowly down the side.

8.2. If the digest solidifies, recommence the determination using a larger amount of sulphuric acid (3.4) than that specified above.

8.3. For products with a low nitrogen content, the volume of sulphuric acid (3.7) to be placed in the collecting flask may be reduced, if necessary, to 10 or 15 ml and made up to 25 ml with water.

8.4. For routine analysis, alternative methods of analysis can be applied for the determination of crude protein but the Kjeldahl method described in this Part C is the reference method. The equivalence of the results obtained with the alternative method (e.g. DUMAS) compared to the reference method must be demonstrated for each matrix individually. As the results obtained with an alternative method, even after having verified the equivalency, might deviate slightly from the results obtained with the reference method, it is necessary to mention in the analytical report the method of analysis used for the determination of crude protein.

D. DETERMINATION OF UREA

1. **Purpose and scope**

This method makes it possible to determine the level of urea in feed.

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2. **Principle**

The sample is suspended in water with a clarifying agent. The suspension is filtered. The urea content of the filtrate is determined after the addition of 4-dimethylaminobenzaldehyde (4-DMAB) by measuring the optical density at a wavelength of 420 nm.

3. **Reagents**

3.1. Solution of 4-dimethylaminobenzaldehyde: dissolve 1,6 g of 4-DMAB in 100 ml of 96 % ethanol and add 10 ml of hydrochloric acid (ρ_{20} 1,19 g/ml). This reagent keeps for a maximum period of two weeks.

3.2. Carrez solution I: dissolve in water 21,9 g of zinc acetate, $Zn(CH_3COO)_2$ $2H_2O$ and 3 g of glacial acetic acid. Make up to 100 ml with water.

3.3. Carrez solution II: dissolve in water 10,6 g of potassium ferrocyanide, K_4 Fe $(CN)_6$ $3H_2O$. Make up to 100 ml with water.

3.4. Active carbon which does not absorb urea (to be checked).

3.5. Urea, 0,1 % solution (w/v).

4. **Apparatus**

4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.

4.2. Test tubes: 160 × 16 mm with ground-glass stoppers.

4.3. Spectrophotometer.

5. **Procedure**

5.1. ***Analysis of sample***

Weigh out 2 g of the sample to the nearest mg and place with 1 g of active carbon (3.4) in a 500 ml volumetric flask. Add 400 ml of water and 5 ml of Carrez solution I (3.2), mix for approximately 30 seconds and add 5 ml of Carrez solution II (3.3). Mix for 30 minutes in the tumbler. Make up to volume with water, shake and filter.

Remove 5 ml of the transparent colourless filtrates, place in test tubes with ground-glass stoppers, add 5 ml of 4-DMAB solution (3.1) and mix. Place the tubes in a water bath at 20 °C (+/- 4 °C). After 15 minutes measure the optical density of the sample solution with the spectrophotometer at 420 nm. Compare with the blank test solution of the reagents.

5.2. ***Calibration curve***

Remove volumes of 1, 2, 4, 5 and 10 ml of the urea solution (3.5), place in 100 ml volumetric flasks and make up the volume with water. Remove 5 ml from each solution, add 5 ml of 4-DMAB solution (3.1) to each of them, homogenise and measure the optical density as shown above in comparison with a control solution containing 5 ml of 4-DMAB and 5 ml of water free from urea. Plot the calibration curve.

6. **Calculation of results**

Determine the amount of urea in the sample using the calibration curve.

Express the result as a percentage of the sample.

7. **Observations**

7.1. In the case of contents of urea exceeding 3 %, reduce the sample to 1 g or dilute the original solution so that there are not more than 50 mg of urea in 500 ml.

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7.2. In the case of low contents of urea, increase the sample as long as the filtrate remains transparent and colourless.

7.3. If the sample contains simple nitrogenous compounds such as amino acids, the optical density shall be measured at 435 nm.

E. DETERMINATION OF VOLATILE NITROGENOUS BASES

I. **BY MICRODIFFUSION**

1. **Purpose and scope**

This method makes it possible to determine the content of volatile nitrogenous bases, expressed as ammonia, in feed.

2. **Principle**

The sample is extracted with water and the solution clarified and filtered. The volatile nitrogenous bases are displaced by microdiffusion using a solution of potassium carbonate, collected in a solution of boric acid and titrated with sulphuric acid.

3. **Reagents**

3.1. Trichloroacetic acid, solution 20 % (w/v).

3.2. Indicator: dissolve 33 mg of bromocresol green and 65 mg of methyl red in 100 ml of 95 %-96 % (v/v) of ethanol.

3.3. Boric acid solution: in a 1 litre graduated flask dissolve 10 g of boric acid in 200 ml of 95 %-96 % (v/v) ethanol and 700 ml of water. Add 10 ml of indicator (3.2). Mix and, if necessary, adjust the colour of the solution to light red by adding a solution of sodium hydroxide. 1 ml of this solution will fix a maximum of 300 μg of NH_3.

3.4. Saturated potassium carbonate solution: dissolve 100 g of potassium carbonate in 100 ml of boiling water. Leave to cool, filter.

3.5. Sulphuric acid 0,01 mol/litre.

4. **Apparatus**

4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.

4.2. Glass or plastic Conway cells (see diagram).

4.3. Microburettes graduated in 1/100 ml.

5. **Procedure**

Weigh 10 g of sample to the nearest 1 mg and place with 100 ml of water in a 200 ml graduated flask. Mix or stir in the tumbler for 30 minutes. Add 50 ml of trichloroacetic acid solution (3.1), make up to volume with water, shake vigorously and filter through a pleated filter.

Using a pipette, introduce 1 ml of boric acid solution (3.3) into the central part of the Conway cell and 1 ml of the sample filtrate into the crown of the cell. Cover partially with the greased lid. Drop 1 ml of saturated potassium carbonate solution (3.4) quickly into the crown and close the lid so that the cell is airtight. Turn the cell carefully rotating it in a horizontal plane so that the two reagents are mixed. Leave to incubate either for at least four hours at room temperature or for one hour at 40 °C.

Using a microburette (4.3), titrate the volatile bases in the boric acid solution with sulphuric acid (3.5).

Carry out a blank test using the same procedure but without a sample to be analysed.

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6. **Calculation of results**

1 ml of H_2SO_4 0,01 mol/litre corresponds to 0,34 mg of ammonia.

Express the result as a percentage of the sample.

Repeatability

The difference between the results of two parallel determinations carried out on the same sample shall not exceed:

— 10 %, in relative value, for ammonia contents of less than 1,0 %,

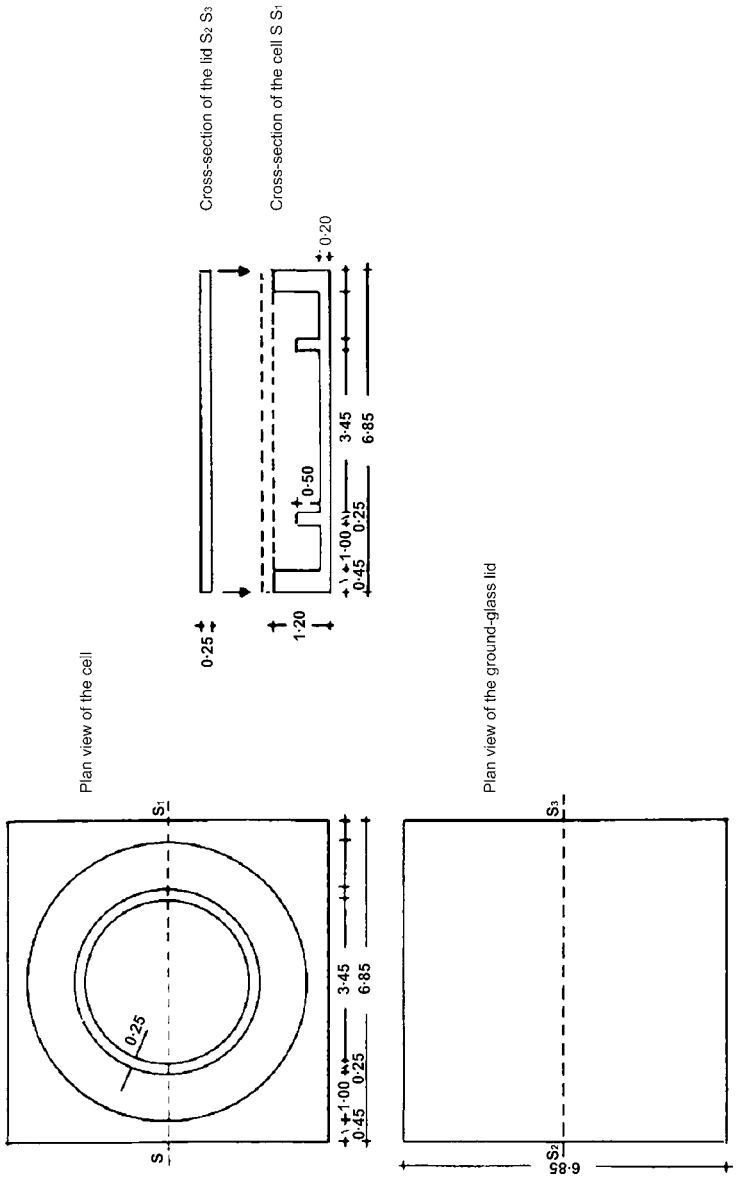
— 0,1 %, in absolute value, for ammonia contents of 1,0 % or more.

7. **Observation**

If the ammonia content of the sample exceeds 0,6 %, dilute the initial filtrate.

CONWAY CELL

Scale 1/1



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II. **BY DISTILLATION**

1. **Purpose and Scope**

This method makes it possible to determine the content of volatile nitrogenous bases, expressed as ammonia, in fish-meal containing practically no urea. It is applicable only to ammonia contents of less than 0,25 %.

2. **Principle**

The sample is extracted with water and the solution clarified and filtered. The volatile nitrogenous bases are displaced at boiling point by adding magnesium oxide and collected in a specific quantity of sulphuric acid, the excess of which is back-titrated with a solution of sodium hydroxide.

3. **Reagents**

3.1. Trichloroacetic acid, solution 20 % (w/v).

3.2. Magnesium oxide.

3.3. Anti-foaming emulsion (e.g. silicone).

3.4. Sulphuric acid 0,05 mol/litre.

3.5. Sodium hydroxide solution 0,1 mol/litre.

3.6. Methyl red solution 0,3 % in 95 %-96 % (v/v) ethanol.

4. **Apparatus**

4.1. Mixer (tumbler): approximately 35 to 40 r.p.m.

4.2. Distilling apparatus of the Kjeldahl type.

5. **Procedure**

Weigh 10 g of the sample to the nearest 1 mg and place with 100 ml of water in a 200 ml graduated flask. Mix or stir in the tumbler for 30 minutes. Add 50 ml of trichloroacetic acid solution (3.1), make up to volume with water, shake vigorously and filter through a pleated filter.

Take a quantity of clear filtrate appropriate for the presumed content of volatile nitrogenous bases (100 ml is usually suitable). Dilute to 200 ml and add 2 g of magnesium oxide (3.2) and a few drops of anti-foaming emulsion (3.3). The solution must be alkaline to litmus paper; otherwise add some magnesium oxide (3.2). Proceed according to 5.2 and 5.3 of the method of analysis for the determination of the crude protein content (Part C of this Annex).

Carry out a ***blank test*** using the same procedure but without a sample to be analysed.

6. **Calculation of results**

1 ml of H_2SO_4 0,05 mol/litre corresponds to 1,7 mg of ammonia.

Express the result as a percentage of the sample.

Repeatability

The difference between the results of two parallel determinations carried out on the same sample shall not exceed, in relative value, 10 % of ammonia.

F. DETERMINATION OF AMINO ACIDS (EXCEPT TRYPTOPHANE)

1. **Purpose and scope**

This method makes the determination possible of free (synthetic and natural) and total (peptide bound and free) amino acids in feed, using an amino acid analyser. It is applicable to the following amino acids:

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cyst(e)ine, methionine, lysine, threonine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, tyrosine and valine.

The method does not distinguish between the salts of amino acids and it cannot differentiate between D and L forms of amino acids. It is not valid for the determination of tryptophan or hydroxy analogues of amino acids.

2. **Principle**

2.1. ***Free amino acids***

The free amino acids are extracted with diluted hydrochloric acid. Co-extracted nitrogenous macromolecules are precipitated with sulfosalicylic acid and removed by filtration. The filtered solution is adjusted to pH 2,20. The amino acids are separated by ion exchange chromatography and determined by reaction with ninhydrin with photometric detection at 570 nm.

2.2. ***Total amino acids***

The procedure chosen depends on the amino acids under investigation. Cyst(e)ine and methionine must be oxidised to cysteic acid and methionine sulphone respectively prior to hydrolysis. Tyrosine must be determined in hydrolysates of unoxidised samples. All the other amino acids listed in paragraph 1 can be determined in either the oxidised or unoxidised sample.

Oxidation is performed at 0 °C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulphite. The oxidised or unoxidised sample is hydrolysed with hydrochloric acid (3.20) for 23 hours. The hydrolysate is adjusted to pH 2,20. The amino acids are separated by ion exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline).

3. **Reagents**

Double distilled water or water of equivalent quality must be used (conductivity < 10 μS).

3.1. Hydrogen peroxide, w (w/w) = 30 %.

3.2. Formic acid, w (w/w) = 98 %-100 %.

3.3. Phenol.

3.4. Sodium disulphite.

3.5. Sodium hydroxide.

3.6. 5-Sulfosalicylic acid dihydrate.

3.7. Hydrochloric acid, density approximately 1,18 g/ml.

3.8. tri-Sodium citrate dihydrate.

3.9. 2,2'-Thiodiethanol (thiodiglycol).

3.10. Sodium chloride.

3.11. Ninhydrin.

3.12. Light petroleum, boiling range 40-60 °C.

3.13. Norleucine, or other compound suitable for use as internal standard.

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3.14. Nitrogen gas (< 10 ppm oxygen).

3.15. 1-Octanol.

3.16. Amino acids.

3.16.1. Standard substances listed under paragraph 1. Pure compounds containing no water of crystallisation. Dry under vacuum over P_2O_5 or H_2SO_4 for 1 week prior to use.

3.16.2. Cysteic acid.

3.16.3. Methionine sulphone.

3.17. Sodium hydroxide solution, c = 7,5 mol/l:

Dissolve 300 g NaOH (3.5) in water and make up to 1 litre.

3.18. Sodium hydroxide solution, c = 1 mol/l:

Dissolve 40 g NaOH (3.5) in water and make up to 1 litre.

3.19. Formic acid — phenol solution:

Mix 889 g formic acid (3.2) with 111 g water and add 4,73 g phenol (3.3).

3.20. Hydrolysis mixture, c = 6 mol HCl/l containing 1 g phenol/l:

Add 1 g phenol (3.3) to 492 ml HCl (3.7) and make up to 1 litre with water.

3.21. Extraction mixture, c = 0,1 mol HCl/l containing 2 % thiodiglycol: Take 8,2 ml HCl (3.7), dilute with approximately 900 ml water, add 20 ml thiodiglycol (3.9) and make up to 1 litre with water, (do not mix 3.7 and 3.9 directly).

3.22. 5-Sulfosalicylic acid, ß = 6 %:

Dissolve 60 g 5-sulfosalicylic acid (3.6) in water and make up to 1 l with water.

3.23. Oxidation mixture (Performic acid — phenol):

Mix 0,5 ml hydrogen peroxide (3.1) with 4,5 ml formic acid-phenol solution (3.19) in a small beaker. Incubate at 20-30 °C for 1 hour in order to form performic acid, then cool on an ice-water bath (15 min.) before adding to the sample.

Caution: Avoid contact with skin and wear protective clothing.

3.24. Citrate buffer, c = 0,2 mol Na^+/l, pH 2,20:

Dissolve 19,61 g sodium citrate (3.8), 5 ml thiodiglycol (3.9), 1 g phenol (3.3) and 16,50 ml HCl (3.7) in approximately 800 ml water. Adjust pH to 2,20. Make up to 1 litre with water.

3.25. Elution buffers, prepared according to conditions for the analyser used (4.9).

3.26. Ninhydrin reagent, prepared according to conditions for the analyser used (4.9).

3.27. Standard solutions of amino acids. These solutions shall be stored below 5 °C.

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3.27.1. Stock standard solution of amino acids (3.16.1).

c = 2,5 μmol/ml of each in hydrochloric acid.

May be obtained commercially.

3.27.2. Stock standard solution of cysteic acid and methionine sulphone, c = 1,25 μmol/ml.

Dissolve 0,2115 g cysteic acid (3.16.2) and 0,2265 g methionine sulphone (3.16.3) in citrate buffer (3.24) in a 1 litre graduated flask and make up to mark with citrate buffer. Store below 5 °C for not more than 12 months. This solution is not used if the stock standard solution (3.27.1) contains cysteic acid and methionine sulphone.

3.27.3. Stock standard solution of the internal standard e.g. norleucine, c = 20 μmol/ml.

Dissolve 0,6560 g norleucine (3.13) in citrate buffer (3.24) in a graduated flask and make up to 250 ml with citrate buffer. Store below 5 °C for no more than 6 months.

3.27.4. Calibration solution of standard amino acids for use with hydrolysates, c = 5 nmol/50 μl of cysteic acid and methionine sulphone and c = 10 nmol/50 μl of the other amino acids. Dissolve 2,2 g sodium chloride (3.10) in 100 ml beaker with 30 ml citrate buffer (3.24). Add 4,00 ml stock standard solution of amino acids (3.27.1), 4,00 ml stock standard solution of cysteic acid and methionine sulphone (3.27.2) and 0,50 ml stock standard solution of internal standard (3.27.3) if used. Adjust pH to 2,20 with sodium hydroxide (3.18).

Transfer quantitatively to a 50 ml graduated flask and make up to the mark with citrate buffer (3.24) and mix.

Store below 5 °C for not more than 3 months.

See also observation 9.1.

3.27.5. Calibration solution of standard amino acids for use with hydrolysates prepared according to paragraph 5.3.3.1 and for use with extracts (5.2). The calibration solution is prepared according to 3.27.4 but omitting sodium chloride.

Store below 5 °C for not more than 3 months.

4. **Apparatus**

4.1. 100 or 250 ml round bottomed flask fitted with a reflux condenser.

4.2. 100 ml borosilicate glass bottle with screw cap with rubber/teflon liner (e.g. Duran, Schott) for use in the oven.

4.3. Oven with forced ventilation and a temperature regulator with an accuracy better than ± 2 °C.

4.4. pH-meter (three decimal places).

4.5. Membrane filter (0,22 μm).

4.6. Centrifuge.

4.7. Rotary vacuum evaporator.

4.8. Mechanical shaker or magnetic stirrer.

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4.9. Amino acid analyser or HPLC equipment with ion exchange column, device for ninhydrin, post column derivatisation and photometric detector.

The column is filled with sulfonated polystyrene resins capable of separating the amino acids from each other and from other ninhydrin-positive materials. The flow in the buffer and ninhydrin lines is provided by pumps having a flow stability of ± 0,5 % in the period covering both the standard calibration run and the analysis of the sample.

With some amino acid analysers hydrolysis procedures can be used in which the hydrolysate has a sodium concentration of c = 0,8 mol/l and contains all the residual formic acid from the oxidation step. Others do not give a satisfactory separation of certain amino acids if the hydrolysate contains excess formic acid and/or high sodium ion concentrations. In this case the volume of acid is reduced by evaporation to approx. 5 ml after the hydrolysis and prior to pH adjustment. The evaporation shall be performed under vacuum at 40 °C maximum.

5. **Procedure**

5.1. ***Preparation of the sample***

The sample is ground to pass through a 0,5 mm sieve. Samples high in moisture must be either air-dried at a temperature not exceeding 50 °C or freeze dried prior to grinding. Samples with a high fat content shall be extracted with light petroleum (3.12) prior to grinding.

5.2. ***Determination of free amino acids in feed and premixtures***

Weigh to the nearest 0,2 mg an appropriate amount (1-5 g) of the prepared sample (5.1), into a conical flask and add 100,0 ml of extraction mixture (3.21). Shake the mixture for 60 min. using a mechanical shaker or a magnetic stirrer (4.8). Allow the sediment to settle and pipette 10,0 ml of the supernatant solution into a 100 ml beaker.

Add 5,0 ml of sulfosalicylic acid solution (3.22), with stirring and continue to stir with the aid of magnetic stirrer for 5 min. Filter or centrifuge the supernatant in order to remove any precipitate. Place 10,0 ml of the resulting solution into a 100 ml beaker and adjust the pH to 2,20 using sodium hydroxide solution (3.18), transfer to a volumetric flask of appropriate volume using citrate buffer (3.24), and make up to the mark with the buffer solution (3.24).

If an internal standard is being used add 1,00 ml of internal standard (3.27.3) for each 100 ml final solution and make up to the mark with the buffer solution (3.24).

Proceed to the chromatography step according to paragraph 5.4.

If the extracts are not being examined the same day, they must be stored below 5 °C.

5.3. ***Determination of total amino acids***

5.3.1. Oxidation

Weigh to the nearest 0,2 mg from 0,1 to 1 g of the prepared sample (5.1) into:

— a 100 ml round-bottomed flask (4.1) for open hydrolysis (5.3.2.3) or,

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— a 250 ml round-bottomed flask (4.1) if a low sodium concentration is required (5.3.3.1) or,

— a 100 ml bottle fitted with a screw cap (4.2), for closed hydrolysis (5.3.2.4).

The weighed sample portion must have a nitrogen content of about 10 mg and a moisture content not exceeding 100 mg.

Place the flask/bottle in an ice-water bath and cool to 0 °C, add 5 ml of oxidation mixture (3.23) and mix using a glass spatula with a bent tip. Seal the flask/bottle containing the spatula with an air-tight film, place the ice-water bath containing the sealed container in a refrigerator at 0 °C and leave for 16 hours. After 16 hours remove from the refrigerator and decompose the excess oxidation reagent by the addition of 0,84 g of sodium disulphite (3.4).

Proceed to 5.3.2.1.

5.3.2. Hydrolysis

5.3.2.1. ***Hydrolysis of oxidised samples***

To the oxidised sample prepared according to 5.3.1 add 25 ml of hydrolysis mixture (3.20) taking care to wash down any sample residue adhering to the sides of the vessel and the spatula.

Depending on the hydrolysis procedure being used, proceed according to 5.3.2.3 or 5.3.2.4.

5.3.2.2. ***Hydrolysis of unoxidised samples***

Weigh into either a 100 ml or a 250 ml round-bottom flask (4.1) or a 100 ml bottle fitted with a screw cap (4.2), to the nearest 0,2 mg, from 0,1 to 1 g of the prepared sample (5.1). The weighed sample portion must have a nitrogen content of about 10 mg. Add carefully 25 ml of hydrolysis mixture (3.20) and mix with the sample. Proceed according to either 5.3.2.3 or 5.3.2.4.

5.3.2.3. ***Open hydrolysis***

Add 3 glass beads to the mixture in the flask (prepared in accordance with 5.3.2.1 or 5.3.2.2) and boil with continuous bubbling under reflux for 23 hours. On completion of hydrolysis, wash the condenser down with 5 ml of citrate buffer (3.24). Disconnect the flask and cool it in an ice bath.

Proceed according to 5.3.3.

5.3.2.4. ***Closed Hydrolysis***

Place the bottle containing the mixture prepared in accordance with 5.3.2.1 or 5.3.2.2 in an oven (4.3) at 110 °C. During the first hour in order to prevent a build up of pressure (due to the evolution of gaseous substances) and to avoid explosion, place the screw cap over the top of the vessel. Do not close the vessel with the cap. After one hour close the vessel with the cap and leave in the oven (4.3) for 23 hours. On completion of hydrolysis, remove the bottle from the oven, carefully open the cap of the bottle and place the bottle in an ice-water bath. Leave to cool.

Depending on the procedure for pH adjustment (5.3.3), quantitatively transfer the contents of the bottle to a 250 ml beaker or a 250 ml round-bottom flask, using citrate buffer (3.24).

Proceed according to 5.3.3.

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5.3.3. Adjustment of pH

Depending on the sodium tolerance of the amino acid analyser (4.9) proceed according to 5.3.3.1 or 5.3.3.2 for the pH adjustment.

5.3.3.1. ***For Chromatographic Systems (4.9) requiring a low sodium concentration***

It is advisable to use an internal stock standard solution (3.27.3) when amino acid analysers requiring a low sodium concentration are employed (when the acid volume has to be reduced).

In this case add 2,00 ml of the internal stock standard solution (3.27.3) to the hydrolysate before the evaporation.

Add 2 drops of 1-octanol (3.15) to the hydrolysate obtained in accordance with paragraph 5.3.2.3 or 5.3.2.4.

Using a rotary evaporator (4.7) reduce the volume to 5-10 ml under vacuum at 40 °C. If the volume is accidentally reduced to less than 5 ml the hydrolysate must be discarded and the analysis recommenced.

Adjust the pH to 2,20 with sodium hydroxide solution (3.18) and proceed to paragraph 5.3.4.

5.3.3.2. ***For all other Amino Acid Analysers (4.9)***

Take the hydrolysates obtained in accordance with 5.3.2.3 or 5.3.2.4 and partly neutralise them by carefully adding with stirring, 17 ml of sodium hydroxide solution (3.17), ensuring that the temperature is kept below 40 °C.

Adjust the pH to 2,20 at room temperature using sodium hydroxide solution (3.17) and finally sodium hydroxide solution (3.18). Proceed to 5.3.4.

5.3.4. Sample solution for chromatography

Quantitatively transfer the pH adjusted hydrolysate (5.3.3.1 or 5.3.3.2) with citrate buffer (3.24) to a 200 ml graduated flask, and make up to the mark with buffer (3.24).

If an internal standard has not already been used, add 2,00 ml of internal standard (3.27.3) and make up to the mark with citrate buffer (3.24). Mix thoroughly.

Proceed to the chromatography step (5.4).

If the sample solutions are not being examined the same day they must be stored below 5 °C.

5.4. ***Chromatography***

Before chromatography bring the extract (5.2) or hydrolysate (5.3.4) to room temperature. Shake the mixture and filter a suitable amount through a 0,22 µm membrane filter (4.5). The resulting clear solution is subjected to ion exchange chromatography, using an amino acid analyser (4.9).

The injection may be performed manually or automatically. It is important that the same quantity of solution ± 0,5 % is added to the column for the analysis of standards and samples except when an internal standard is used, and that the sodium:amino acid ratios in the standard and sample solutions are as similar as is practicable.

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In general the frequency of calibration runs depends on the stability of the ninhydrin reagent and the analytical system. The standard or sample is diluted with citrate buffer (3.24) to give a peak area of the standard of 30 %-200 % of the sample amino acid peak area.

The chromatography of amino acids will vary slightly according to the type of analyser employed and resin used. The chosen system must be capable of separating the amino acids from each other and from the ninhydrin-positive materials. In the range of operation the chromatographic system must give a linear response to changes in the amounts of amino acids added to the column.

During the chromatography step the valley:peak height ratios mentioned below apply, when an equimolar solution (of the amino acids being determined) is analysed. This equimolar solution must contain at least 30 % of the maximum load of each amino acid which can be accurately measured with the amino acid analyser system (4.9).

For separation of threonine-serine the valley:peak height ratio of the lower of the two overlapping amino acids on the chromatogram must not exceed 2:10. (if only cyst(e)ine, methionine, threonine and lysine are determined, insufficient separation from adjoining peaks will adversely influence the determination). For all other amino acids the separation must be better than 1:10.

The system must ensure that lysine is separated from ‘lysine artifacts’ and ornithine.

6. **Calculation of results**

The area of the sample and standard peaks is measured for each individual amino acid and the amount (X), in g amino acid per kg sample, is calculated as follows:

$$X = \frac{A \times c \times M \times V}{B \times m \times 1\,000}$$

If an internal standard is used multiply by: $\frac{D}{C}$

A = peak area, hydrolysate or extract
B = peak area, calibration standard solution
C = peak area, internal standard in hydrolysate or extract
D = peak area, internal standard, calibration standard solution
M = molar weight of the amino acid being determined
c = concentration of standard in µmol/ml
m = sample weight (g) (corrected to original weight if dried or defatted)
V = ml total hydrolysate (5.3.4) or ml calculated total dilution volume of extract (6.1)

Cystine and cysteine are both determined as cysteic acid in hydrolysates of oxidised sample, but calculated as cystine ($C_6H_{12}N_2O_4S_2$, M 240,30 g/mol) by using M 120,15 g/mol (= 0,5 x 240,30 g/mol).

Methionine is determined as methionine sulphone in hydrolysates of oxidised sample, but calculated as methionine by using M of methionine: 149,21 g/mol.

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Added free methionine is determined after extraction as methionine, for the calculation the same M is used.

6.1. The total dilution volume of extracts (F) for determination of free amino acids (5.2) is calculated as follows:

$$F = \frac{100\ ml \times (10\ ml + 5\ ml)}{10\ ml} \times \frac{V}{10}$$

V = Volume of final extract

7. **Evaluation of the method**

The method has been tested in an intercomparison made at international level in 1990 using four different feed (mixed pig feed, broiler compound, protein concentrate, premixture). The results, after elimination of outliers, of mean and standard deviation are given in the tables in this point:

Means in g/kg

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	6,94 n = 15	3,01 n = 17	3,27 n = 17	9,55 n = 13
Broiler Compound	9,31 n = 16	3,92 n = 18	5,08 n = 18	13,93 n = 16
Protein Concentrate	22,32 n = 16	5,06 n = 17	12,01 n = 17	47,74 n = 15
Premixture	58,42 N = 16	—	90,21 n = 16	98,03 n = 16

n = Number of participating laboratories.

7.1. ***Repeatability***

The repeatability expressed as 'within laboratory standard deviation' of the abovementioned intercomparison is given in the tables below:

Within Laboratory Standard Deviation (S_r) in g/kg

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	0,13 n = 15	0,10 n = 17	0,11 n = 17	0,26 n = 13
Broiler Compound	0,20 n = 16	0,11 n = 18	0,16 n = 18	0,28 n = 16
Protein Concentrate	0,48 n = 16	0,13 n = 17	0,27 n = 17	0,99 n = 15
Premixture	1,30 N = 16	—	2,19 n = 16	2,06 n = 16

n = Number of participating laboratories.

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Coefficient of Variation (%) for Within Laboratory Standard Deviation (S_r)

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	1,9 n = 15	3,3 n = 17	3,4 n = 17	2,8 n = 13
Broiler Compound	2,1 n = 16	2,8 n = 18	3,1 n = 18	2,1 n = 16
Protein Concentrate	2,7 n = 16	2,6 n = 17	2,2 n = 17	2,4 n = 15
Premixture	2,2 n = 16	—	2,4 n = 16	2,1 n = 16

n = Number of participating laboratories.

7.2 ***Reproducibility***

The results for between laboratory standard deviation by the abovementioned intercomparison are given in the table below:

Between Laboratory Standard Deviation (S_R) in g/kg

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	0,28 n = 15	0,30 n = 17	0,23 n = 17	0,30 n = 13
Broiler Compound	0,48 n = 16	0,34 n = 18	0,55 n = 18	0,75 n = 16
Protein Concentrate	0,85 n = 16	0,62 n = 17	1,57 n = 17	1,24 n = 15
Premixture	2,49 n = 16	—	6,20 n = 16	6,62 n = 16

n = Number of participating laboratories.

Coefficient of Variation (%) for Between Laboratory Standard Deviation (S_R)

Reference material	Amino Acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed Pig Feed	4,1 n = 15	9,9 n = 17	7,0 n = 17	3,2 n = 13
Broiler Compound	5,2 n = 16	8,8 n = 18	10,9 n = 18	5,4 n = 16
Protein Concentrate	3,8 n = 16	12,3 n = 17	13,0 n = 17	3,0 n = 15
Premixture	4,3 n = 16	—	6,9 n = 16	6,7 n = 16

n = Number of participating laboratories.

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8. **Use of reference materials**

The correct application of the method shall be verified by making replicate measurements of certified reference materials when available. Calibration with certified amino acid calibration solution is recommended.

9. **Observations**

9.1. Because of differences between amino acid analysers the final concentrations of the calibration solutions of standard amino acids (see 3.27.4 and 3.27.5) and of the hydrolysate (see 5.3.4) shall be taken as a guideline.

The range of linear response of the apparatus has to be checked for all amino acids.

The standard solution is diluted with citrate buffer to give peak areas in the middle of the range.

9.2. Where high performance liquid chromatographic equipment is used to analyse the hydrolysates, the experimental conditions must be optimised in accordance with the manufacturer's recommendations.

9.3. By applying the method to feed containing more than 1 % chloride (concentrate, mineral feeds, supplementary feeds) underestimation of methionine could occur and special treatment has to be done.

G. DETERMINATION OF TRYPTOPHAN

1. **Purpose and scope**

The method makes the determination possible of the total and free tryptophan in feed. It does not distinguish between D- and L- forms.

2. **Principle**

For the determination of the total tryptophan, the sample is hydrolysed under alkaline conditions with saturated barium hydroxide solution and heated to 110 °C for 20 hours. After hydrolysis internal standard is added.

For the determination of free tryptophan, the sample is extracted under mild acidic conditions in the presence of internal standard.

The tryptophan and the internal standard in the hydrolysate or in the extract are determined by HPLC with fluorescence detection.

3. **Reagents**

3.1. Double distilled water or water of equivalent quality must be used (conductivity < 10 μS/cm).

3.2. Standard substance: tryptophan (purity/content ≥ 99 %) dried under vacuum over phosphorous pentoxide.

3.3. Internal standard substance: α-methyl-tryptophan (purity/content ≥ 99 %), dried under vacuum over phosphorous pentoxide.

3.4. Barium hydroxide octa-hydrate (care shall be taken not to expose the $Ba(OH)_2$ .8 H_2O excessively to air in order to avoid formation of $BaCO_3$, which could disturb the determination) (see observation 9.3).

3.5. Sodium hydroxide.

3.6. Ortho-phosphoric acid, w (w/w) = 85 %.

3.7. Hydrochloric acid, ρ_{20} 1,19 g/ml.

3.8. Methanol, equivalent to HPLC grade.

3.9. Light petroleum, boiling range 40-60 °C.

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3.10. Sodium hydroxide solution, c = 1 mol/l:

Dissolve 40,0 g NaOH (3.5) in water and make up to 1 litre with water (3.1).

3.11. Hydrochloric acid, c = 6 mol/l:

Take 492 ml HCl (3.7) and make up to 1 litre with water.

3.12. Hydrochloric acid, c = 1 mol/l:

Take 82 ml HCl (3.7) and make up to 1 litre with water.

3.13. Hydrochloric acid, c = 0,1 mol/l:

Take 8,2 ml HCl (3.7) and make up to 1 litre with water.

3.14. Ortho-phosphoric acid, c = 0,5 mol/l:

Take 34 ml ortho-phosphoric acid (3.6) and make up to 1 litre with water (3.1).

3.15. Concentrated solution of tryptophan (3.2), c = 2,50 μmol/ml:

In a 500 ml volumetric flask dissolve 0,2553 g tryptophan (3.2) in hydrochloric acid (3.13) and make up to the mark with hydrochloric acid (3.13). Store at - 18 ^{o}C for a maximum of 4 weeks.

3.16. Concentrated internal standard solution, c = 2,50 μmol/ml:

In a 500 ml volumetric flask dissolve 0,2728 g α-methyl-tryptophan (3.3) in hydrochloric acid (3.13) and make up to the mark with hydrochloric acid (3.13). Store at - 18 ^{o}C for a maximum of 4 weeks.

3.17. Calibration standard solution of tryptophan and internal standard:

Take 2,00 ml concentrated solution of tryptophan (3.15), and 2,00 ml of concentrated internal standard (α-methyl-tryptophan) solution (3.16). Dilute with water (3.1) and methanol (3.8) to approximately the same volume and to approximately the same concentration of methanol (10 %-30 %) as the finished hydrolysate.

This solution must be prepared freshly before use.

Protect from direct sunlight during preparation.

3.18. Acetic acid

3.19. 1,1,1-trichloro-2-methyl-2-propanol.

3.20. Ethanolamine w (w/w) > 98 %.

3.21. Solution of 1 g 1,1,1-trichloro-2-methyl-2-propanol (3.19) in 100 ml methanol (3.8).

3.22. Mobile phase for HPLC: 3,00 g acetic acid (3.18) + 900 ml water (3.1) + 50,0 ml solution (3.21) of 1,1,1-trichloro-2-methyl-2-propanol (3.19) in methanol (3.8) (1g/100ml). Adjust pH to 5,00 using ethanolamine (3.20). Make up to 1 000 ml with water (3.1).

4. **Apparatus**

4.1. HPLC equipment with a spectrofluorometric detector.

4.2. Liquid chromatographic column, 125 mm x 4 mm, C_{18}, 3 μm packing, or equivalent.

4.3. pH-meter.

4.4. Polypropylene flask, capacity 125 ml, with wide neck and screw cap.

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4.5. Membrane filter, 0,45 μm.

4.6. Autoclave, 110 (± 2) °C, 1,4 (± 0,1) bar.

4.7. Mechanical shaker or magnetic stirrer.

4.8. Vortex mixer.

5. **Procedure**

5.1. ***Preparation of samples***

The sample is ground to pass through a 0,5 mm sieve. Samples high in moisture must be either air-dried at a temperature not exceeding 50 °C or freeze dried prior to grinding. Samples with high fat content shall be extracted with light petroleum (3.9) prior to grinding.

5.2. ***Determination of free tryptophan (extract)***

Weigh to the nearest 1 mg an appropriate amount (1-5 g) of the prepared sample (5.1), into a conical flask. Add 100,0 ml hydrochloric acid, (3.13) and 5,00 ml concentrated internal standard solution (3.16). Shake or mix for 60 min. using a mechanical shaker or a magnetic stirrer (4.7). Allow the sediment to settle and pipette 10,0 ml of the supernatant solution into a beaker. Add 5 ml ortho-phosphoric acid (3.14). Adjust the pH to 3 using sodium hydroxide (3.10). Add sufficient methanol (3.8) to give a concentration of between 10 % and 30 % of methanol in the final volume. Transfer to a volumetric flask of appropriate volume and dilute with water to a volume necessary for the chromatography (approx. the same volume as the calibration standard solution (3.17)).

Filter a few ml of the solution through a 0,45 μm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to paragraph 5.4.

Protect standard solution and extracts against direct sunlight. If it is not possible to analyse the extracts the same day, the extracts may be stored at 5 °C for a maximum of 3 days.

5.3. ***Determination of total tryptophan (hydrolysate)***

Weigh to the nearest 0,2 mg from 0,1 to 1 g of the prepared sample (5.1) into the polypropylene flask (4.4). The weighed sample portion shall have a nitrogen content of about 10 mg. Add 8,4 g barium hydroxide octa-hydrate (3.4) and 10 ml water. Mix on a vortex mixer (4.8) or magnetic stirrer (4.7). Leave the teflon coated magnet in the mixture. Wash down the walls of the vessel with 4 ml water. Put on the screw cap and close the flask loosely. Transfer to an autoclave (4.6) with boiling water and steam for 30-60 minutes. Close the autoclave and autoclave at 110 (± 2) °C for 20 hours.

Before opening the autoclave reduce the temperature to just under 100 °C. In order to avoid crystallisation of $Ba(OH)_2 \cdot 8\ H_2O$, add to the warm mixture 30 ml water which is at room temperature. Shake or stir gently. Add 2,00 ml concentrated internal standard (α-methyl-tryptophan) solution (3.16). Cool the vessels on water/ice bath for 15 minutes.

Then, add 5 ml ortho-phosphoric acid (3.14). Keep the vessel in the cooling bath and neutralise with HCl (3.11) whilst stirring and adjust the pH to 3,0 using HCl (3.12). Add sufficient methanol to give a concentration of between 10 % and 30 % of methanol in the final volume. Transfer to a volumetric flask of appropriate volume and dilute with water to the defined volume necessary for the chromatography (for example 100 ml). The addition of methanol shall not cause precipitation.

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Filter a few ml of the solution through a 0,45 µm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to paragraph 5.4.

Protect standard solution and hydrolysates against direct sunlight. If it is not possible to analyse the hydrolysates the same day, they may be stored at 5 °C for a maximum of 3 days.

5.4. ***HPLC determination***

The following conditions for isocratic elution are offered for guidance; other conditions may be used, provided they yield equivalent results (see also observations 9.1 and 9.2):

Liquid chromatographic column (4.2):	125 mm x 4 mm, C_{18}, 3 µm packing or equivalent
Column temperature:	Room temperature
Mobile phase (3.22):	3,00 g acetic acid (3.18) + 900 ml water (3.1) + 50,0 ml solution (3.21) of 1,1,1-trichloro-2- methyl-2-propanol (3.19) in methanol (3.8) (1 g/100 ml). Adjust pH to 5,00 using ethanolamine (3.20). Make up to 1 000 ml with water (3.1)
Flow rate:	1 ml/min.
Total run time:	approx. 34 min.
Detection wavelength:	excitation: 280 nm, emission: 356 nm.
Injection volume	20 µl

6. **Calculation of results**

The amount of tryptophane (X), in g per 100g sample, is calculated as follows:

$$X = \frac{A \times B \times V_1 \times \boldsymbol{c} \times V_2 \times M}{C \times D \times V_3 \times 10\,000 \times \boldsymbol{m}}$$

A = peak area of internal standard, calibration standard solution (3.17)
B = peak area of tryptophan, extract (5.2) or hydrolysate (5.3)
V_1 = volume in ml (2 ml) of concentrated tryptophan solution (3.15) added to the calibration solution (3.17)
c = concentration in µmol/ml (= 2,50) of concentrated tryptophan solution (3.15) added to calibration solution (3.17)
V_2 = volume in ml of concentrated internal standard solution (3.16) added at the extraction (5.2) (= 5,00 ml) or to the hydrolysate (5.3) (= 2,00 ml)
C = peak area of internal standard, extract (5.2) or hydrolysate (5.3)
D = peak area of tryptophan, calibration standard solution (3.17)
V_3 = volume in ml (= 2,00 ml) of concentrated internal standard solution (3.16) added to calibration standard solution (3.17)
m = sample weight in g (corrected to original weight if dried and/or defatted)
M = molar weight of tryptophan (= 204,23 g/mol)

7. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample must not exceed 10 % relative to the highest result.

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8. **Results of a collaborative study**

An EC collaborative study (4th intercomparison) was arranged in which three samples were analysed by up to 12 laboratories to certify the method for hydrolysis. Replicate (5) analyses were performed on each sample. The results are given in the following table:

	Sample 1Pig feed	Sample 2 Pig feed supplemented with L-tryptophan	Sample 3 Feed concentrate for pigs
L	12	12	12
n	50	55	50
Mean [g/kg]	2,42	3,40	4,22
s_r [g/kg]	0,05	0,05	0,08
r [g/kg]	0,14	0,14	0,22
CV_r [%]	1,9	1,6	1,9
S_R [g/kg]	0,15	0,20	0,09
R [g/kg]	0,42	0,56	0,25
CV_R [%]	6,3	6,0	2,2

L = number of laboratories submitting results
n = number of single results retained eliminating outliers (identified by Cochran, Dixon outlier test)
s_r = standard deviation of repeatability
S_R = standard deviation of reproducibility
r = repeatability
R = reproducibility
CV_r = coefficient of variation of repeatability, %
CV_R = coefficient of variation of reproducibility, %

Another EC collaborative study (3rd intercomparison) was arranged in which two samples were analysed by up to 13 laboratories to certify the method for extraction of free tryptophan. Replicate (5) analyses were performed on each sample. The results are given in the following table:

	Sample 4 Wheat and soya mixture	Sample 5 Wheat and soya mixture (= sample 4) with added tryptophan (0,457g/kg1)
L	12	12
n	55	60
Mean [g/kg]	0,391	0,931
s_r [g/kg]	0,005	0,012
r [g/kg]	0,014	0,034
CV_r [%]	1,34	1,34
S_R [g/kg]	0,018	0,048
R [g/kg]	0,050	0,134
CV_R [%]	4,71	5,11

L = number of laboratories submitting results
n = number of single results retained after eliminating outliers (identified by Cochran, Dixon outlier test)
s_r = standard deviation of repeatability
S_R = standard deviation of reproducibility
r = repeatability
R = reproducibility
CV_r = coefficient of variation of repeatability, %
CV_R = coefficient of variation of reproducibility, %

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Another EC intercomparison study was arranged in which four samples were analysed by up to 7 laboratories with the aim of a tryptophan certification for hydrolysis. The results are given below. Replicate (5) analyses were performed on each sample.

	Sample 1 Mixed pig feed (CRM 117)	Sample 2 Low fat fish meal (CRM 118)	Sample 3 Soybean meal (CRM 119)	Sample 4 Skimmed milk powder (CRM 120)
L	7	7	7	7
n	25	30	30	30
Mean [g/kg]	2,064	8,801	6,882	5,236
s_r [g/kg]	0,021	0,101	0,089	0,040
r [g/kg]	0,059	0,283	0,249	0,112
CV_r [%]	1,04	1,15	1,30	0,76
S_R [g/kg]	0,031	0,413	0,283	0,221
R [g/kg]	0,087	1,156	0,792	0,619
CV_R [%]	1,48	4,69	4,11	4,22

L = number of laboratories submitting results
n = number of single results retained after eliminating outliers (identified by Cochran, Dixon outlier test)
s_r = standard deviation of repeatability
S_R = standard deviation of reproducibility
r = repeatability
R = reproducibility
CV_r = coefficient of variation of repeatability, %
CV_R = coefficient of variation of reproducibility, %

9. **Observations**

9.1. Following special chromatographic conditions may give better separation between tryptophan and α-methyl-tryptophan.

Isocratic elution followed by gradient column cleaning:

Liquid chromatographic column:	125 mm x 4 mm, C_{18}, 5 µm packing or equivalent		
Column temperature:	32 °C		
Mobile phase:	A: 0,01 mol/l KH_2PO_4/méthanol, 95+5 (V+V). B: methanol		
Gradient program:	0 min.	100 % A	0 % B
	15 min.	100 % A	0 % B
	17 min.	60 % A	40 % B
	19 min.	60 % A	40 % B
	21 min.	100 % A	0 % B
	33 min.	100 % A	0 % B
Flow rate:	1,2 ml/min.		
Total run time:	approx. 33 min.		

9.2. The chromatography will vary according to the type of HPLC and column packing material used. The chosen system must be capable of giving baseline separation between the tryptophan and the internal standard. Moreover it is important that degradation products are well separated from the tryptophan and the internal standard. Hydrolysates without internal standard shall be run in order to check the base line under the internal standard for impurities. It is important that the run

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time is sufficiently long for the elution of all the degradation products, otherwise late eluting peaks may interfere with subsequent chromatographic runs.

In the range of operation, the chromatographic system shall give linear response. The linear response shall be measured with a constant (the normal) concentration of the internal standard and varying concentrations of tryptophan. It is of importance that the size of both the tryptophan and internal standard peaks are within the linear range of the HPLC/fluorescence system. If either the tryptophan and/or the internal standard peak(s) is (are) too small or too high the analysis shall be repeated with another sample size and/or a changed final volume.

9.3. ***Barium hydroxide***

With age barium hydroxide becomes more difficult to dissolve. This results in an unclear solution for the HPLC determination, which may produce low results for tryptophan.

H. DETERMINATION OF CRUDE OILS AND FATS

1. **Purpose and scope**

This method is for the determination of crude oils and fats in feed. It does not cover the analysis of oil seeds and oleaginous fruit.

The use of the two procedures described below depends on the nature and composition of the feed and the reason for carrying out the analysis.

1.1. ***Procedure A — Directly extractable crude oils and fats***

This method is applicable to feed materials of plant origin, except those included within the scope of Procedure B.

1.2. ***Procedure B — Total crude oils and fats***

This method is applicable to feed materials of animal origin and to all compound feeds. It is to be used for all materials from which the oils and fats cannot be completely extracted without prior hydrolysis (e.g. glutens, yeast, potato proteins and products subjected to processes such as extrusion, flaking and heating).

1.3. ***Interpretation of results***

In all cases where a higher result is obtained by using Procedure B than by Procedure A, the result obtained by Procedure B shall be accepted as the true value.

2. **Principle**

2.1. ***Procedure A***

The sample is extracted with light petroleum. The solvent is distilled off and the residue dried and weighed.

2.2. ***Procedure B***

The sample is treated under heating with hydrochloric acid. The mixture is cooled and filtered. The residue is washed and dried and submitted to the determination according to Procedure A.

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3. **Reagents**

3.1. Light petroleum, boiling range: 40 to 60 °C. The bromine value must be less than 1 and the residue on evaporation less than 2 mg/100 ml.

3.2. Sodium sulfate, anhydrous.

3.3. Hydrochloric acid, c = 3 mol/l

3.4. Filtration aid, e.g. Kieselguhr, Hyflo-supercel.

4. **Apparatus**

4.1. Extraction apparatus. If fitted with a siphon (Soxhlet apparatus), the reflux rate shall be such as to produce about 10 cycles per hour; if of the non-siphoning type, the reflux rate shall be about 10 ml per minute.

4.2. Extraction thimbles, free of matter soluble in light petroleum and having a porosity consistent with the requirements of point 4.1.

4.3. Drying oven, either a vacuum oven set at 75 ± 3 °C or an air-oven set at 100 ± 3 °C.

5. **Procedure**

5.1. ***Procedure A (see point 8.1)***

Weigh 5 g of the sample to the nearest 1 mg, transfer it to an extraction thimble (4.2) and cover with a fat-free wad of cotton wool.

Place the thimble in an extractor (4.1) and extract for six hours with light petroleum (3.1). Collect the light petroleum extract in a dry, weighed flask containing fragments of pumice stone ([1]).

Distil off the solvent. Dry the residue maintaining the flask for one and a half hours in the drying oven (4.3). Leave to cool in a desiccator and weigh. Dry again for 30 minutes to ensure that the weight of the oils and fats remains constant (loss in weight between two successive weighings must be less than or equal to 1 mg).

5.2. ***Procedure B***

Weigh 2,5 g of the sample to the nearest 1 mg (see point 8.2), place in a 400 ml beaker or a 300 ml conical flask and add 100 ml of hydrochloric acid (3.3) and fragments of pumice stone. Cover the beaker with a watch glass or fit the conical flask with a reflux condenser. Bring the mixture to a gentle boil over a low flame or a hot-plate and keep it there for one hour. Do not allow the product to stick to the sides of the container.

Cool and add a quantity of filtration aid (3.4) sufficient to prevent any loss of oil and fat during filtration. Filter through a moistened, fat-free, double filter paper. Wash the residue in cold water until a neutral filtrate is obtained. Check that the filtrate does not contain any oil or fats. Their presence indicates that the sample must be extracted with light petroleum, using Procedure A, before hydrolysis.

Place the double filter paper containing the residue on a watch glass and dry for one and a half hours in the air oven (4.3) at 100 ± 3 °C.

([1]) Where the oil or fat has to undergo subsequent quality tests, replace the fragments of pumice stone by glass beads.

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Place the double filter paper containing the dry residue in an extraction thimble (4.2) and cover with a fat-free wad of cotton wool. Place the thimble in an extractor (4.1) and proceed as indicated in the second and third paragraphs of point 5.1.

6. **Expression of result**

Express the weight of the residue as a percentage of the sample.

7. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample by the same analyst shall not exceed:

— 0,2 %, in absolute value, for contents of crude oils and fats lower than 5 %,

— 4,0 % relative to the highest result for contents of 5 % to 10 %,

— 0,4 %, in absolute value, for contents above 10 %.

8. **Observations**

8.1. For products with a high content of oils and fats, which are difficult to crush or unsuitable for drawing a homogeneous reduced test sample, proceed as follows.

Weigh 20 g of the sample to the nearest 1 mg and mix with 10 g or more of anhydrous sodium sulfate (3.2). Extract with light petroleum (3.1) as indicated in point 5.1. Make up the extract obtained to 500 ml with light petroleum (3.1) and mix. Take 50 ml of the solution and place in a small, dry, weighed flask containing fragments of pumice stone. Distil off the solvent, dry and proceed as indicated in the last paragraph of point 5.1.

Eliminate the solvent from the extraction residue left in the thimble, crush the residue to a fineness of 1 mm, return it to the extraction thimble (do not add sodium sulfate) and proceed as indicated in the second and third paragraphs of point 5.1.

Calculate the content of oils and fats as a percentage of the sample by using the following formula:

$$(10m_1 + m_2) \times 5$$

where:

m_1 = weight in grams of the residue after the first extraction (aliquot part of the extract),
m_2 = weight in grams of the residue after the second extraction.

8.2. For products low in oils and fats the test sample may be increased to 5 g.

8.3. Pet foods containing a high content of water may need to be mixed with anhydrous sodium sulfate prior to hydrolysis and extraction as per Procedure B.

8.4. In paragraph 5.2 it may be more effective to use hot water in place of cold water to wash the residue after filtration.

8.5. The drying time of 1,5 h may need to be extended for some feed. Excessive drying shall be avoided as this can lead to low results. A microwave oven can also be used.

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8.6. Pre-extraction by Procedure A prior to hydrolysis and re-extraction by Procedure B is recommended if the crude oil/fat content is greater than 15 %. To some extent this depends on the nature of the feed and the nature of the oil/fat in the feed.

I. DETERMINATION OF CRUDE FIBRE

1. **Purpose and scope**

This method makes it possible to determine fat-free organic substances in feed which are insoluble in acid and alkaline media and are conventionally described as crude fibre.

2. **Principle**

The sample, defatted where necessary, is treated successively with boiling solutions of sulphuric acid and potassium hydroxide of specified concentrations. The residue is separated by filtration on a sintered-glass filter washed, dried, weighed and ashed within a range of 475 to 500 °C. The loss of weight resulting from ashing corresponds to the crude fibre present in the test sample.

3. **Reagents**

3.1. Sulphuric acid, c = 0,13 mol/l.

3.2. Anti-foaming agent (e.g. n-octanol).

3.3. Filter aid (Celite 545 or equivalent), heated at 500 °C for four hours (8.6).

3.4. Acetone.

3.5. Light petroleum boiling-range 40 to 60 °C.

3.6. Hydrochloric acid, c = 0,5 mol/l.

3.7. Potassium hydroxide solution, c = 0,23 mol/l.

4. **Apparatus**

4.1. Heating unit for digestion with sulphuric acid and potassium hydroxide solution, equipped with a support for the filter crucible (4.2) and provided with an outlet tube with a tap to the liquid outlet and vacuum, possibly with compressed air. Before use each day preheat the unit with boiling water for five minutes.

4.2. Glass filter crucible with fused sintered glass filter plate pore size 40-90 µm. Before first use, heat to 500 °C for a few minutes and cool (8.6).

4.3. Cylinder of at least 270 ml with a reflux condenser, suitable for boiling.

4.4. Drying oven with thermostat.

4.5. Muffle furnace with thermostat.

4.6. Extraction unit consisting of a support plate for the filter crucible (4.2) and with a discharge pipe with a tap to the vacuum and liquid outlet.

4.7. Connecting rings to assemble the heating unit (4.1), crucible (4.2) and cylinder (4.3) and to connect the cold extraction unit (4.6) and crucible.

5. **Procedure**

Weigh out 1 g of the prepared sample to the nearest 1 mg and place it in the crucible (4.2), (see observations 8.1, 8.2 and 8.3) and add 1 g of filter aid (3.3).

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Assemble the heating unit (4.1) and the filter crucible (4.2), then attach the cylinder (4.3) to the crucible. Pour 150 ml of boiling sulphuric acid (3.1) into the assembled cylinder and crucible and if necessary add a few drops of anti-foaming agent (3.2).

Bring the liquid to the boil within 5 ± 2 minutes and boil vigorously for exactly 30 minutes.

Open the tap to the discharge pipe (4.1) and, under vacuum, filter the sulphuric acid through the filter crucible and wash the residue with three consecutive 30 ml portions of boiling water, ensuring that the residue is filtered dry after each washing.

Close the outlet tap and pour 150 ml boiling potassium hydroxide solution (3.7) to the assembled cylinder and crucible and add a few drops of anti-foaming agent (3.2). Bring the liquid to boiling point within 5 ± 2 minutes and boil vigorously for exactly 30 minutes. Filter and repeat the washing procedure used for the sulphuric acid step.

After the final washing and drying, disconnect the crucible and its contents and reconnect it to the cold extraction unit (4.6). Apply the vacuum and wash the residue in the crucible with three consecutive 25 ml portions of acetone (3.4) ensuring that the residue is filtered dry after each washing.

Dry the crucible to constant weight in the oven at 130 °C. After each drying cool in the desiccator and weigh rapidly. Place the crucible in a muffle furnace and ash to constant weight (loss in weight between two successive weightings must be less than or equal to 2 mg) at 475 °C to 500 °C for at least 30 minutes.

After each heating cool first in the furnace and then in the desiccator before weighing.

Carry out a blank test without the sample. Loss of weight resulting from ashing must not exceed 4 mg.

6. **Calculation of results**

The crude fibre content as a percentage of the sample is given by the expression:

$$X = \frac{(m_0 - m_1) \times 100}{m}$$

where:

m = weight of sample in g,
m_0 = loss of weight after ashing during the determination, in g,
m_1 = loss of weight after ashing during the blank test, in g.

7. **Repeatability**

The difference between two parallel determinations carried out on the same sample must not exceed:

— 0,6 % in absolute value for crude fibre contents lower than 10 %,

— 6 % relative to the higher result, for crude fibre contents equal to or greater than 10 %.

8. **Observations**

8.1. Feed containing more than 10 % crude fat must be defatted prior to analysis with light petroleum (3.5). Connect the filter crucible (4.2) and its contents to the cold extraction unit (4.6) and apply vacuum

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and wash the residue with three consecutive 30 ml portions of light petroleum, ensuring that the residue is dry. Connect the crucible and its contents to the heating unit (4.1) and continue as described under 5.

8.2. Feed containing fats which cannot be extracted directly with light petroleum (3.5) must be defatted as shown in 8.1 and defatted once more after boiling with acid. After boiling with acid and the subsequent washing connect the crucible and its contents to the cold extraction unit (4.6) and wash three times with 30 ml acetone followed by three further washings with 30 ml portions of light petroleum. Filter under vacuum until dry and continue the analysis as described under 5, beginning with potassium hydroxide treatment.

8.3. If the feed contains over 5 % of carbonates, expressed as calcium carbonate, connect the crucible (4.2) with the weighed sample to the heating unit (4.1). Wash the sample three times with 30 ml hydrochloric acid (3.6). After each addition let the sample stand for about one minute before filtering. Wash once with 30 ml water and then continue as described under 5.

8.4. If an apparatus in the form of a stand is used (several crucibles attached to the same heating unit) no two individual determinations on the same sample for analysis may be carried out in the same series.

8.5. If after boiling it is difficult to filter the acidic and basic solutions, use compressed air through the discharge pipe of the heating unit and then continue filtering.

8.6. The temperature for ashing shall not be higher than 500 °C in order to extend the lifetime of the glass filter crucibles. Care must be taken to avoid excessive thermal shock during heating and cooling cycles.

J. DETERMINATION OF SUGAR

1. **Purpose and scope**

This method makes it possible to determine the amount of reducing sugars and total sugars after inversion, expressed as glucose or where appropriate as sucrose, converting by the factor 0,95. It is applicable to compound feed. Special methods are provided for other feed. Where necessary, lactose shall be measured separately and taken into account when calculating the results.

2. **Principle**

The sugars are extracted in dilute ethanol; the solution is clarified with Carrez solutions I and II. After eliminating the ethanol, the quantities before and after inversion are determined by the Luff-Schoorl method.

3. **Reagents**

3.1. Ethanol solution 40 % (v/v) density: 0,948 g/ml at 20 °C, neutralised to phenolphthalein.

3.2. Carrez solution I: dissolve in water 21,9 g of zinc acetate Zn $(CH_3COO)_2$ $2H_2O$ and 3 g of glacial acetic acid. Make up to 100 ml with water.

3.3. Carrez solution II: dissolve in water 10,6 g of potassium ferrocyanide $K_4Fe(CN)_6$ $3H_2O$. Make up to 100 ml with water.

3.4. Methyl orange, solution 0,1 % (w/v).

3.5. Hydrochloric acid 4 mol/litre.

3.6. Hydrochloric acid 0,1 mol/litre.

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3.7. Sodium hydroxide solution 0,1 mol/litre.

3.8. Luff-Schoorl reagent:

Stirring carefully, pour the citric acid solution (3.8.2) into the sodium carbonate solution (3.8.3). Add the copper sulphate solution (3.8.1) and make up to 1 litre with water. Leave to settle overnight and filter.

Check the concentration of the reagent thus obtained (Cu 0,05 mol/litre; $Na_2 CO_3$ 1 mol/litre), see (5.4) last paragraph. The solution's pH shall be approximately 9,4.

3.8.1. Copper sulphate solution: dissolve 25 g of copper sulphate, $Cu SO_4 5H_2O$, free from iron, in 100 ml of water.

3.8.2. Citric acid solution: dissolve 50 g of citric acid, $C_6H_8O_7 \cdot H_2O$ in 50 ml of water.

3.8.3. Sodium carbonate solution: dissolve 143,8 g of anhydrous sodium carbonate in approximately 300 ml of warm water. Leave to cool.

3.9. Sodium thiosulphate solution 0,1 mol/litre.

3.10. Starch solution: add a mixture of 5 g of soluble starch in 30 ml of water to 1 litre of boiling water. Boil for three minutes, leave to cool and if necessary add 10 mg of mercuric iodide as a preservative.

3.11. Sulphuric acid 3 mol/litre.

3.12. Potassium iodide, solution 30 % (w/v).

3.13. Granulated pumice stone boiled in hydrochloric acid, washed in water and dried.

3.14. 3-methylbutan-l-ol.

4. **Apparatus**

Mixer (tumbler): approximately 35 to 40 r.p.m.

5. **Procedure**

5.1. ***Extraction of sample***

Weigh 2,5 g of the sample to the nearest mg and place in a 250 ml volumetric flask. Add 200 ml of ethanol (3.1) and mix in the tumbler for one hour. Add 5 ml of Carrez solution I (3.2) and stir for approximately 30 seconds. Add 5 ml of Carrez solution II (3.3) and again stir for one minute. Make up to volume with ethanol (3.1), homogenise and filter. Remove 200 ml of the filtrate and evaporate to approximately half volume in order to eliminate most of the ethanol. Transfer the evaporation residue quantitatively to a 200 ml volumetric flask using warm water, cool, bring up to volume with water, homogenise and filter if necessary. This solution will be used to determine the amount of reducing sugars and, after inversion, of total sugars.

5.2. ***Determination of reducing sugars***

Using a pipette, remove not more than 25 ml of the solution containing less than 60 mg of reducing sugars expressed as glucose. If necessary, make up to 25 ml with distilled water and determine the content of reducing sugars by the Luff-Schoorl method. The result is expressed as the percentage content of glucose in the sample.

5.3. ***Determination of total sugars after inversion***

Using a pipette take 50 ml of the solution and transfer to a 100 ml volumetric flask. Add a few drops of methyl orange solution (3.4) then, carefully and stirring continuously, add hydrochloric acid (3.5) until the liquid turns a definite red. Add 15 ml of hydrochloric acid (3.6), immerse the flask in a fast boiling water bath and keep there for 30 minutes. Cool rapidly to approximately 20 °C and add 15 ml of sodium hydroxide solution (3.7). Make up to 100 ml with water and homogenise. Remove not more than 25 ml containing less than 60 mg of reducing sugars expressed as glucose. If necessary, make up to 25 ml with distilled water and determine the content of reducing sugars by the

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Luff-Schoorl method. The result is expressed as the percentage of glucose or, where appropriate, sucrose, by multiplying by the factor 0,95.

5.4. ***Titration by the Luff-Schoorl method***

Using a pipette, take 25 ml of Luff-Schoorl reagent (3.8) and transfer to a 300 ml Erlenmeyer flask; add exactly 25 ml of the clarified sugar solution. Add 2 granules of pumice stone (3.13), heat, stirring by hand, over a free flame of medium height and bring the liquid to the boil in approximately two minutes. Place the Erlenmeyer immediately on an asbestos-coated wire gauze with a hole approximately 6 cm in diameter under which a flame has been lit. The flame shall be regulated in such a way that only the base of the Erlenmeyer is heated. Fit a reflux condenser to the Erlenmeyer flask. Boil for exactly 10 minutes. Cool immediately in cold water and after approximately five minutes titrate as follows:

Add 10 ml of potassium iodide solution (3.12) and immediately afterwards (carefully, because of the risk of abundant foaming), add 25 ml of sulphuric acid (3.11). Titrate with sodium thiosulphate solution (3.9) until a dull yellow colour appears, add the starch indicator (3.10) and complete titration.

Carry out the same titration on an accurately measured mixture of 25 ml of Luff-Schoorl reagent (3.8) and 25 ml of water, after adding 10 ml of potassium iodide solution (3.12) and 25 ml of sulphuric acid (3.11) without boiling.

6. **Calculation of results**

Using the table establish the amount of glucose in mg which corresponds to the difference between the values of the two titrations, expressed in mg of sodium thiosulphate 0,1 mol/litre. Express the result as a percentage of the sample.

7. **Special procedures**

7.1. In the case of feed which are rich in molasses and other feed which are not particularly homogeneous, weigh out 20 g and place with 500 ml of water in a 1 litre volumetric flask. Mix for one hour in the tumbler. Clarify using Carrez 1 (3.2) and II (3.3) reagents as described under 5.1, this time however using four times the quantities of each reagent. Bring up to volume with 80 % ethanol (v/v).

Homogenise and filter. Eliminate the ethanol as described under 5.1. If there is no dextrinised starch, bring up to volume with distilled water.

7.2. In the case of molasses and feed materials which are rich in sugar and almost starch-free (carobs, dried beetroot cossettes etc.), weigh out 5 g, place in a 250 ml volumetric flask, add 200 ml of distilled water and mix in the tumbler for one hour, or more if necessary. Clarify using Carrez I (3.2) and II (3.3) reagents as described under 5.1. Bring up to volume with cold water, homogenise and filter. In order to determine the amount of total sugars, continue as described under 5.3.

8. **Observations**

8.1. In order to prevent foaming it is advisable to add (irrespective of the volume) approximately 1 ml of 3-methylbutan-l-ol (3.14) before boiling with Luff-Schoorl reagent.

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8.2. The difference between the content of total sugars after inversion, expressed as glucose, and the content of reducing sugars, expressed as glucose, multiplied by 0,95, gives the percentage content of sucrose.

8.3. In order to determine the content of reducing sugars, excluding lactose, two methods may be adopted:

8.3.1. For an approximate calculation, multiply by 0,675 the lactose content established by a different method of analysis and subtract the result obtained from the content of reducing sugars.

8.3.2. For an accurate calculation of reducing sugars, excluding lactose, the same sample must be used for the two final determinations. One of the analyses is carried out on part of the solution obtained under 5.1, the other on part of the solution obtained during the determination of lactose by the method laid down for that purpose (after fermenting the other types of sugar and clarifying).

In both cases the amount of sugar present is determined by the Luff-Schoorl method and calculated in mg of glucose. One of the values is subtracted from the other and the difference is expressed as a percentage of the sample.

Example:

The two volumes taken correspond, for each determination, to a sample of 250 mg.

In the first case 17 ml of sodium thiosulphate solution 0,1 mol/litre corresponding to 44,2 mg of glucose is consumed; in the second, 11 ml, corresponding to 27,6 mg of glucose.

The difference is 16,6 mg of glucose.

The content of reducing sugars (excluding lactose), calculated as glucose, is therefore:

$$\frac{4 \times 16{,}6}{10} = 6{,}64\ \%$$

Table of values for 25 ml of Luff-Schoorl reagent

ml of $Na_2\ S_2\ O_3$ 0,1 mol/litre, two minutes' heating, 10 minutes' boiling

$Na_2\ S_2\ O_3$ 0,1 mol/ litre	Glucose, fructose invert sugars $C_6\ H_{12}\ O_6$		Lactose $C_{12}\ H_{22}\ O_{11}$		Maltose $C_{12}\ H_{22}\ O_{11}$		$Na_2\ S_2\ O_3$ 0,1 mol/ litre
ml	mg	difference	mg	difference	mg	difference	ml
1	2,4	2,4	3,6	3,7	3,9	3,9	1
2	4,8	2,4	7,3	3,7	7,8	3,9	2
3	7,2	2,5	11,0	3,7	11,7	3,9	3
4	9,7	2,5	14,7	3,7	15,6	4,0	4
5	12,2	2,5	18,4	3,7	19,6	3,9	5
6	14,7	2,5	22,1	3,7	23,5	4,0	6
7	17,2	2,6	25,8	3,7	27,5	4,0	7
8	19,8	2,6	29,5	3,7	31,5	4,0	8
9	22,4	2,6	33,2	3,8	35,5	4,0	9
10	25,0	2,6	37,0	3,8	39,5	4,0	10
11	27,6	2,7	40,8	3,8	43,5	4,0	11
12	30,3	2,7	44,6	3,8	47,5	4,1	12
13	33,0	2,7	48,4	3,8	51,6	4,1	13
14	35,7	2,8	52,2	3,8	55,7	4,1	14
15	38,5	2,8	56,0	3,9	59,8	4,1	15
16	41,3	2,9	59,9	3,9	63,9	4,1	16
17	44,2	2,9	63,8	3,9	68,0	4,2	17
18	47,1	2,9	67,7	4,0	72,2	4,3	18
19	50,0	3,0	71,7	4,0	76,5	4,4	19
20	53,0	3,0	75,7	4,1	80,9	4,5	20
21	56,0	3,1	79,8	4,1	85,4	4,6	21
22	59,1	3,1	83,9	4,1	90,0	4,6	22
23	62,2		88,0		94,6		23

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K. DETERMINATION OF LACTOSE

1. **Purpose and scope**

This method makes it possible to determine the level of lactose in feed containing more than 0,5 % of lactose.

2. **Principle**

The sugars are dissolved in water. The solution is subjected to fermentation by the yeast ***Saccharomyces cerevisiae*** which leaves the lactose intact. After clarification and filtration the lactose content of the filtrate is determined by the Luff-Schoorl method.

3. **Reagents**

3.1. Suspension of ***Saccharomyces cerevisiae:*** suspend 25 g of fresh yeast in 100 ml of water. The suspension will keep for a maximum period of one week in a refrigerator.

3.2. Carrez solution I: dissolve in water 21,9 g of zinc acetate, Zn $(CH_3 COO)_2$ $2H_2O$ and 3 g of glacial acetic acid. Make up to 100 ml with water.

3.3. Carrez solution II: dissolve in water 10,6 g of potassium ferrocyanide $K_4Fe (CN)_6$ $3H_2O$. Make up to 100 ml with water.

3.4. Luff-Schoorl reagent:

Stirring carefully, pour the citric acid solution (3.4.2) into the sodium carbonate solution (3.4.3). Add the copper sulphate solution (3.4.1) and make up to 1 litre with water. Leave to settle overnight and filter. Check the concentration of the reagent thus obtained (Cu 0,05 mol/litre; $Na_2 CO_3$ 1 mol/litre). The solution's pH shall be approximately 9,4.

3.4.1. Copper sulphate solution: dissolve 25 g of copper sulphate Cu SO_4 $5H_2O$, free from iron, in 100 ml of water.

3.4.2. Citric acid solution: dissolve 50 g of citric acid $C_6H_8O_7 \cdot H_2O$ in 50 ml of water.

3.4.3. Sodium carbonate solution: dissolve 143,8 g of anhydrous sodium carbonate in approximately 300 ml of warm water. Leave to cool.

3.5. Granulated pumice stone boiled in hydrochloric acid, washed in water and dried.

3.6. Potassium iodide, solution 30 % (w/v).

3.7. Sulphuric acid 3 mol/litre.

3.8. Solution of sodium thiosulphate 0,1 mol/litre.

3.9. Starch solution: add a mixture of 5 g of soluble starch in 30 ml of water to 1 litre of boiling water. Boil for three minutes, leave to cool, and if necessary add 10 mg of mercuric iodide as a preservative.

4. **Apparatus**

Water bath with thermostat set at 38-40 °C.

5. **Procedure**

Weigh 1 g of the sample to the nearest mg and place this portion of the sample in a 100 ml volumetric flask. Add 25 to 30 ml of water. Place the flask in a boiling water bath for 30 minutes and then cool to approximately 35 °C. Add 5 ml of yeast suspension (3.1) and homogenise. Leave the flask to stand for two hours in a water bath, at a temperature of 38-40° C. Cool to approximately 20 °C.

Add 2,5 ml of Carrez solution I (3.2) and stir for 30 seconds, then add 2,5 ml of Carrez solution II (3.3) and again stir for 30 seconds. Make up to 100 ml with water, mix and filter. Using a pipette, remove an amount

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of filtrate which does not exceed 25 ml and which preferably contains from 40 to 80 mg of lactose and transfer it to a 300 ml Erlenmeyer flask. If necessary, make up to 25 ml with water.

Carry out a blank test in the same way with 5 ml of yeast suspension (3.1). Determine the lactose content according to Luff-Schoorl, as follows: add exactly 25 ml of Luff-Schoorl reagent (3.4) and two granules of pumice stone (3.5). Stir by hand-while heating over a free flame of medium height and bring the liquid to the boil in approximately two minutes. Place the Erlenmeyer immediately on an asbestos-coated wire gauze with a hole approximately 6 cm in diameter under which a flame has been lit. The flame shall be regulated in such a way that only the base of the Erlenmeyer is heated. Fit a reflux condenser to the Erlenmeyer flask. Boil for exactly 10 minutes. Cool immediately in cold water and after approximately five minutes titrate as follows:

Add 10 ml of potassium iodide solution (3.6) and immediately afterwards (carefully, because of the risk of abundant foaming) add 25 ml of sulphuric acid (3.7). Titrate with sodium thiosulphate solution (3.8) until a dull yellow colour appears, add the starch indicator (3.9) and complete titration.

Carry out the same titration on an accurately measured mixture of 25 ml of Luff-Schoorl reagent (3.4) and 25 ml of water, after adding 10 ml of potassium iodide solution (3.6) and 25 ml of sulphuric acid (3.7) without boiling.

6. **Calculation of results**

Using the attached table, establish the amount of lactose in mg which corresponds to the difference between the results of the two titrations, expressed in ml of sodium thiosulphate 0,1 mol/litre.

Express the result of anhydrous lactose as a percentage of the sample.

7. **Observation**

For products containing more than 40 % of fermentable sugar, use more than 5 ml of yeast suspension (3.1).

Table of values for 25 ml of Luff-Schoorl reagent

ml of $Na_2 S_2 O_3$ 0,1 mol/litre, two minutes' heating, 10 minutes' boiling

$Na_2 S_2 O_3$ 0,1 mol/ litre	Glucose, fructose invert sugars $C_6 H_{12} O_6$		Lactose $C_{12} H_{22} O_{11}$		Maltose $C_{12} H_{22} O_{11}$		$Na_2 S_2 O_3$ 0,1 mol/ litre
ml	mg	difference	mg	difference	mg	difference	ml
1	2,4	2,4	3,6	3,7	3,9	3,9	1
2	4,8	2,4	7,3	3,7	7,8	3,9	2
3	7,2	2,5	11,0	3,7	11,7	3,9	3
4	9,7	2,5	14,7	3,7	15,6	4,0	4
5	12,2	2,5	18,4	3,7	19,6	3,9	5
6	14,7	2,5	22,1	3,7	23,5	4,0	6
7	17,2	2,6	25,8	3,7	27,5	4,0	7
8	19,8	2,6	29,5	3,7	31,5	4,0	8
9	22,4	2,6	33,2	3,8	35,5	4,0	9
10	25,0	2,6	37,0	3,8	39,5	4,0	10
11	27,6	2,7	40,8	3,8	43,5	4,0	11
12	30,3	2,7	44,6	3,8	47,5	4,1	12
13	33,0	2,7	48,4	3,8	51,6	4,1	13
14	35,7	2,8	52,2	3,8	55,7	4,1	14
15	38,5	2,8	56,0	3,9	59,8	4,1	15
16	41,3	2,9	59,9	3,9	63,9	4,1	16
17	44,2	2,9	63,8	3,9	68,0	4,2	17
18	47,1	2,9	67,7	4,0	72,2	4,3	18
19	50,0	3,0	71,7	4,0	76,5	4,4	19
20	53,0	3,0	75,7	4,1	80,9	4,5	20
21	56,0	3,1	79,8	4,1	85,4	4,6	21
22	59,1	3,1	83,9	4,1	90,0	4,6	22
23	62,2		88,0		94,6		23

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L. DETERMINATION OF STARCH

POLARIMETRIC METHOD

1. **Purpose and scope**

This method makes it possible to determine the levels of starch and of high molecular weight starch degradation products in feed for the purpose of checking compliance with the declared energy value (provisions in Annex VII) and Council Directive 96/25/EC ([1]).

2. **Principle**

The method comprises two determinations. In the first, the sample is treated with dilute hydrochloric acid. After clarification and filtration the optical rotation of the solution is measured by polarimetry.

In the second, the sample is extracted with 40 % ethanol. After acidifying the filtrate with hydrochloric acid, clarifying and filtering, the optical rotation is measured as in the first determination.

The difference between the two measurements, multiplied by a known factor, gives the starch content of the sample.

3. **Reagents**

3.1. Hydrochloric acid, solution 25 % (w/w) density: 1,126 g/ml.

3.2. Hydrochloric acid. solution 1,13 % (w/v)

The concentration must be checked by titration using a sodium hydroxide solution 0,1 mol/litre in the presence of 0,1 % (w/v) methyl red in 94 % (v/v) ethanol. For the neutralisation of 10 ml, 30,94 ml of NaOH 0,1 mol/litre is needed.

3.3. Carrez solution I: dissolve 21,9 g of zinc acetate $Zn(CH_3COO)_2\ 2H_2O$ and 3 g of glacial acetic acid in water. Make up to 100 ml with water.

3.4. Carrez solution II: dissolve 10,6 g of potassium ferrocyanide $K_4\ Fe(CN)_6\ 3H_2O$ in water. Make up to 100 ml with water.

3.5. Ethanol, solution 40 % (v/v), density: 0,948 g/ml at 20 °C.

4. **Apparatus**

4.1. 250 ml Erlenmeyer flask with standard ground-glass joint and with reflux condenser.

4.2. Polarimeter or saccharimeter.

5. **Procedure**

5.1. ***Preparation of the sample***

Crush the sample until it is fine enough for all of it to pass through a 0,5 mm round-meshed sieve.

5.2. ***Determination of the total optical rotation (P or S) (see observation 7.1)***

Weigh 2,5 g of the crushed sample to the nearest mg and place in a 100 ml graduated flask. Add 25 ml of hydrochloric acid (3.2), shake to obtain even distribution of the test sample and add a further 25 ml of hydrochloric acid (3.2). Immerse the flask in a boiling water bath shaking vigorously and steadily for the first three minutes to prevent the formation of agglomerates. The quantity of water in the water bath

([1]) OJ L 125, 23.5.1996, p. 35.

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must be sufficient for the bath to remain at boiling point when the flask is introduced into it. The flask must not be taken out of the bath whilst being shaken. After exactly 15 minutes, remove from the bath, add 30 ml of cold water and cool immediately to 20 °C.

Add 5 ml of Carrez solution I (3.3) and shake for approximately 30 seconds. Then add 5 ml of Carrez solution II (3.4) and shake again for approximately 30 seconds. Make up to volume with water, mix and filter. If the filtrate is not perfectly clear (which is rare), repeat the determination using a larger quantity of Carrez solutions I and II, for example 10 ml.

Measure the optical rotation of the solution in a 200 mm tube with the polarimeter or saccharimeter.

5.3. ***Determination of the optical rotation (P' or S') of substances soluble in 40 % ethanol***

Weigh 5 g of the sample to the nearest mg, place in a 100 ml graduated flask and add about 80 ml of ethanol (3.5) (see observation 7.2). Leave the flask to stand for 1 hour at room temperature; during this time, shake vigorously on six occasions so that the test sample is thoroughly mixed with the ethanol. Make up to volume with ethanol (3.5), mix and filter.

Pipette 50 ml of the filtrate (corresponds to 2,5 g of the sample) into a 250 ml Erlenmeyer flask, add 2,1 ml of hydrochloric acid (3.1) and shake vigorously. Fit a reflux condenser to the Erlenmeyer flask and immerse the latter in a boiling water bath. After exactly 15 minutes, remove the Erlenmeyer flask from the bath, transfer the contents to a 100 ml graduated flask, rinsing with a little cold water, and cool to 20 °C.

Clarify using Carrez solutions I (3.3) and II (3.4), make up to volume with water, mix, filter and measure the optical rotation as indicated in the 2nd and 3rd paragraphs of 5.2.

6. **Calculation of results**

The starch content (%) is calculated as follows:

6.1. ***Measurement by polarimeter***

$$\text{Starch content } (\%) = \frac{2\,000(P - P')}{[\alpha]_D^{20^\circ}}$$

P = Total optical rotation in angle degrees

P' = Optical rotation in angle degrees of the substances soluble in 40 % (V/V) ethanol

$[\alpha]_D^{20^\circ}$ = Specific optical rotation of pure starch. The numerical values conventionally accepted for this factor are the following:

+185,9°: rice starch
+185,7°: potato starch
+184,6°: maize starch
+182,7°: wheat starch
+181,5°: barley starch
+181,3°: oat starch
+184,0°: other types of starch and starch mixtures in compound feed

6.2. ***Measurement by saccharimeter***

$$\text{Starch content } (\%) = \frac{2\,000}{[\alpha]_D^{20^\circ}} \times \frac{(2\,N \times 0{,}665) \times (S - S')}{100} - \frac{26{,}6\,N \times (S - S')}{[\alpha]_D^{20^\circ}}$$

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S = Total optical rotation in saccharimeter degrees

S' = Optical rotation in saccharimeter degrees of the substances soluble in 40 % (v/v) ethanol

N = weight (g) of saccharose in 100 ml of water yielding an optical rotation of 100 saccharimeter degrees when measured using a 200 mm tube

16,29 g for the French saccharimeters

26,00 g for the German saccharimeters

20,00 g for mixed saccharimeters.

$[\alpha]_D^{20°}$ = Specific optical rotation of pure starch (see 6.1)

6.3. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed 0,4 in absolute value for a starch content lower than 40 % and 1 % relative for starch contents equal to or greater than 40 %.

7. **Observations**

7.1. If the sample contains more than 6 % of carbonates, calculated in terms of calcium carbonate, they must be destroyed by treatment with an exactly appropriate quantity of dilute sulphuric acid before determination of the total optical rotation.

7.2. In the case of products with a high lactose content, such as powdered milk serum or skimmed milk powder, proceed as follows after adding 80 ml of ethanol (3.5). Fit a reflux condenser to the flask and immerse the latter in a water bath at 50 °C for 30 minutes. Leave to cool and continue the analysis as indicated in 5.3.

7.3. The following feed materials, where they are present in significant amounts in feed, are known to give rise to interferences when determining the starch content by the polarimetric method and thereby incorrect results could be yielded:

— (sugar) beet products such as (sugar)beet pulp, (sugar) beet molasses, (sugar) beet pulp — molassed, (sugar) beet vinasse, (beet) sugar,

— citrus pulp,

— linseed; linseed expeller; linseed extracted,

— rape seed; rape seed expeller; rape seed extracted; rape seed hulls,

— sunflower seed; sunflower seed extracted; sunflower seed, partially decorticated, extracted,

— copra expeller; copra extracted,

— potato pulp,

— dehydrated yeast,

— products rich in inulin (e.g. Chips and meal of Jerusalem artichokes),

— greaves.

M. DETERMINATION OF CRUDE ASH

1. **Purpose and Scope**

This method makes it possible to determine the crude ash content of feed.

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2. **Principle**

The sample is ashed at 550 °C; the residue is weighed.

3. **Reagents**

Ammonium nitrate, solution 20 % (w/v).

4. **Apparatus**

4.1. Hot-plate.

4.2. Electric muffle-furnace with thermostat.

4.3. Crucibles for ashing made of silica, porcelain or platinum either rectangular (approx. 60 × 40 × 25 mm) or circular (diameter: 60 to 75 mm, height: 20 to 40 mm).

5. **Procedure**

Weigh out to the nearest mg approximately 5 g of the sample (2,5 in the case of products which have a tendency to swell) and place in a crucible for ashing which has first been heated at 550 °C, cooled down and tared. Place the crucible on the hot-plate and heat gradually until the substance carbonises. Ash according to 5.1 or 5.2.

5.1. Put the crucible into the calibrated muffle furnace set at 550 °C. Keep at this temperature until white, light grey or reddish ash is obtained which appears to be free from carbonaceous particles. Place the crucible in a desiccator, leave to cool and weigh immediately.

5.2. Put the crucible into the calibrated muffle-furnace set at 550 °C. Ash for 3 hours. Place the crucible in a desiccator, leave to cool and weigh immediately. Ash again for 30 minutes to ensure that the weight of the ash remains constant (loss in weight between two successive weightings must be less than or equal to 1 mg).

6. **Calculation of results**

Calculate the weight of the residue by deducting the tare.

Express the result as a percentage of the sample.

7. **Observations**

7.1. The ash of ***substances which are difficult to ash*** must be subjected to an initial ashing of at least three hours, cooled and then a few drops of 20 % solution of ammonium nitrate or water added to it (carefully, to avoid dispersal of the ash or the formation of lumps). Continue calcining after drying in the oven. Repeat the operation as necessary until ashing is complete.

7.2. In the case of ***substances resistant to the treatment*** described under 7.1, proceed as follows: after ashing for three hours, place the ash in warm water and filter through a small, ash-free filter. Ash the filter and its contents in the original crucible. Place the filtrate in the cooled crucible, evaporate until dry, ash and weigh.

7.3. In the case of ***oils and fats***, weigh accurately a sample of 25 g in a suitably sized crucible. Carbonise by setting light to the substance with a strip of ash-free filter paper. After combustion, moisten with as little water as possible. Dry and ash as described under 5.

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N. DETERMINATION OF ASH WHICH IS INSOLUBLE IN HYDROCHLORIC ACID

1. Purpose and Scope

This method makes it possible to determine the level in feed of mineral substances which are insoluble in hydrochloric acid. Two methods can be used, depending on the nature of the sample.

1.1. ***Method A:*** applicable to organic feed materials and to most compound feed.

1.2. ***Method B:*** applicable to mineral compounds and mixtures and to compound feed, whose content in substances insoluble in hydrochloric acid, as determined by Method A, is greater than 1 %.

2. Principle

2.1. ***Method A:*** the sample is ashed, the ash boiled in hydrochloric acid and the insoluble residue filtered and weighed.

2.2. ***Method B:*** the sample is treated with hydrochloric acid. The solution is filtered, the residue ashed and the ash thus obtained treated in accordance with Method A.

3. Reagents

3.1. Hydrochloric acid 3 mol/litre.

3.2. Trichloroacetic acid, solution 20 % solution (w/v).

3.3. Trichloroacetic acid, solution 1 % (w/v).

4. Apparatus

4.1. Hot plate.

4.2. Electric muffle-furnace with thermostat.

4.3. Crucibles for ashing made of silica, porcelain or platinum, either rectangular (approx. 60 × 40 × 25 mm) or circular (diameter: 60 to 75 mm, height: 20 to 40 mm).

5. Procedure

5.1. ***Method A***

Ash the sample using the method described for the determination of crude ash. Ash obtained from that analysis may also be used.

Place the ash in a 250 to 400 ml beaker using 75 ml of hydrochloric acid (3.1). Bring slowly to the boil and boil gently for 15 minutes. Filter the warm solution through an ash-free filter paper and wash the residue with warm water until the acid reaction is no longer visible. Dry the filter containing the residue and ash in a tared crucible at a temperature of not less than 550 ^{o}C and not more than 700 ^{o}C. Cool in a desiccator and weigh.

5.2. ***Method B***

Weigh 5 g of the sample to the nearest mg and place in a 250 to 400 ml beaker. Add 25 ml of water and 25 ml of hydrochloric acid (3.1) successively, mix and wait for effervescence to cease. Add a further 50 ml of hydrochloric acid (3.1). Wait for any release of gas to cease then place the beaker in a boiling water bath and keep it there for 30 minutes or longer, if necessary, in order to hydrolyse thoroughly any starch which may be present. Filter while warm through an ash-free filter

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and wash the filter in 50 ml of warm water (see observation 7). Place the filter containing the residue in a crucible for ashing, dry and ash at a temperature of not less than 550 °C and not more than 700 °C. Place the ash in a 250 to 400 ml beaker using 75 ml of hydrochloric acid (3.1); continue as described in the second subparagraph of 5.1.

6. **Calculation of results**

Calculate the weight of the residue by deducting the tare. Express the result as a percentage of the sample.

7. **Observation**

If filtration proves difficult recommence the analysis, replacing the 50 ml of hydrochloric acid (3.1) by 50 ml of 20 % trichloroacetic acid (3.2) and washing the filter in a warm solution of 1 % trichloroacetic acid (3.3).

O. DETERMINATION OF CARBONATES

1. **Purpose and Scope**

This method makes it possible to determine the amount of carbonates, conventionally expressed as calcium carbonate, in most feed.

However in certain cases (for example, with iron carbonate) a special method must be used.

2. **Principle**

The carbonates are decomposed in hydrochloric acid; the carbon dioxide released is collected in a graduated tube, and its volume compared with that released under the same conditions by a known quantity of calcium carbonate.

3. **Reagents**

3.1. Hydrochloric acid, density 1,10 g/ml.

3.2. Calcium carbonate.

3.3. Sulphuric acid, approximately 0,05 mol/litre, coloured with methyl red.

4. **Apparatus**

Scheibler-Dietrich apparatus (see diagram) or equivalent apparatus.

5. **Procedure**

According to the sample's carbonate content, weigh a portion of the sample as shown below:

— 0,5 g for products containing from 50 % to 100 % of carbonates, expressed as calcium carbonate,

— 1 g for products containing from 40 % to 50 % of carbonates, expressed as calcium carbonate,

— 2 to 3 g for other products.

Place the portion of the sample in the special flask (4) of the apparatus, fitted with a small tube of unbreakable material containing 10 ml of hydrochloric acid (3.1), and connect the flask to the apparatus. Turn the three-way cock (5) so that the tube (1) connects with the outside. Using the mobile tube (2), which is filled with coloured sulphuric acid (3.3) and connected to the graduated tube (1), bring the level of the liquid up to the zero mark. Turn the cock (5) in order to connect up tubes (1) and (3) and check that the level is at zero.

Run the hydrochloric acid (3.1) slowly over the portion of the sample, tilting the flask (4). Make the pressure equal by lowering the tube (2). Shake the flask (4) until the release of carbon dioxide has stopped completely.

Restore pressure by bringing the liquid back to the same level in tubes (1) and (2). After a ***few minutes***, when the volume of gas has become constant, take the reading.

Carry out a control test in the same conditions on 0,5 g of calcium carbonate (3.2).

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6. **Calculation of results**

The content of carbonates, expressed as calcium carbonate, is calculated by using the formula:

$$X = \frac{V \times 100}{V_1 \times 2m}$$

where:

X = % (w/w) of carbonates in the sample, expressed as calcium carbonate

V = ml of CO_2 released by the portion of the sample.

V_1 = ml of CO_2 released by 0,5 g of $CaCO_3$.

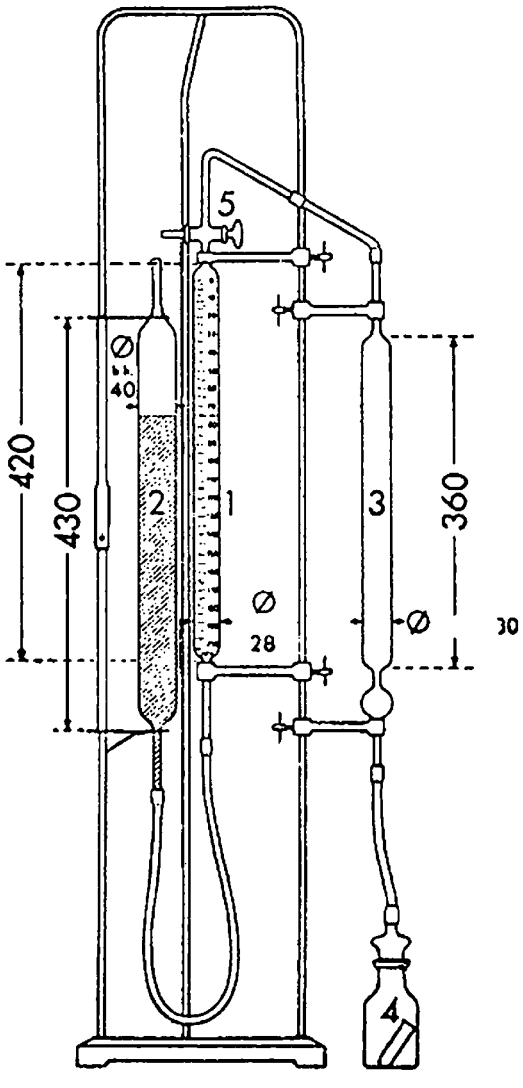
m = weight, in grammes, of the portion of the sample.

7. **Observations**

7.1. When the portion of the sample weighs more than 2 g, first place 15 ml of distilled water in the flask (4) and mix before beginning the test. Use the same volume of water for the control test.

7.2. If the apparatus used has a different volume from that of the Scheibler-Dietrich apparatus, the portions taken from the sample and from the control substance and the calculation of the results must be adapted accordingly.

SCHEIBER-DIETRICH APPARATUS FOR THE DETERMINATION OF CO_2



(measured in mm)

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P. DETERMINATION OF TOTAL PHOSPHORUS

PHOTOMETRIC METHOD

1. **Purpose and Scope**

This method makes it possible to determine the content of total phosphorus in feed. It is particularly appropriate for the analysis of products low in phosphorus. In certain cases (product rich in phosphorus), a gravimetric method may be used.

2. **Principle**

The sample is mineralised, either by dry combustion (in the case of organic feed) or by acid digestion (in the case of mineral compounds and liquid feed), and placed in an acid solution. The solution is treated with molybdovanadate reagent. The optical density of the yellow solution thus formed is measured in a spectrophotometer at 430 nm.

3. **Reagents**

3.1. Calcium carbonate.

3.2. Hydrochloric acid, ρ_{20} = 1,10 g/ml (approx 6 mol/litre).

3.3. Nitric acid, ρ_{20} = 1,045 g/ml.

3.4. Nitric acid, ρ_{20} = 1,38 to 1,42 g/ml.

3.5. Sulphuric acid, ρ_{20} = 1,84 g/ml.

3.6. Molybdovanadate reagent: mix 200 ml of ammonium heptamolybdate solution (3.6.1), 200 ml of ammonium monovanadate solution (3.6.2) and 134 ml of nitric acid (3.4) in a 1 litre graduated flask. Make up to volume with water.

3.6.1. Ammonium heptamolybdate solution: dissolve in hot water 100 g of ammonium heptamolybdate $(NH_4)\ 6Mo_7O_{24}{\cdot}4H_2O$. Add 10 ml of ammonia (density 0,91 g/ml) and make up to 1 litre with water.

3.6.2. Ammonium monovanadate solution: dissolve 2,35 g of ammonium monovanadate NH_4VO_3 in 400 ml of hot water. Stirring constantly, slowly add 20 ml of dilute nitric acid (7 ml of HNO_3 (3.4) + 13 ml of H_2O and make up to 1 litre with water.

3.7. Standard solution of 1 mg phosphorus per ml: dissolve 4,387 g of potassium dihydrogen phosphate KH_2PO_4 in water. Make up to 1 litre with water.

4. **Apparatus**

4.1. Silica, porcelain or platinum ashing crucibles.

4.2. Electric muffle-furnace with thermostat set at 550 °C.

4.3. 250 ml Kjeldahl flask.

4.4. Graduated flasks and precision pipettes.

4.5. Spectrophotometer.

4.6. Test tubes about 16 mm in diameter, with stoppers graded to a diameter of 14,5 mm; capacity: 25 to 30 ml.

5. **Procedure**

5.1. ***Preparation of the solution***

According to the nature of the sample, prepare a solution as indicated in 5.1.1 or 5.1.2.

5.1.1. Usual procedure

Weigh 1 g or more of the sample to the nearest 1 mg. Place the test sample in a Kjeldahl flask, add 20 ml of sulphuric acid (3.5), shake to impregnate the substance completely with acid and to prevent it from

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sticking to the sides of the flask, heat and keep at boiling point for 10 minutes. Leave to cool slightly, add 2 ml of nitric acid (3.4), heat gently, leave to cool slightly, add a little more nitric acid (3.4) and bring back to boiling point. Repeat this procedure until a colourless solution is obtained. Cool, add a little water, decant the liquid into a 500 ml graduated flask, rinsing the Kjeldahl flask with hot water. Leave to cool, make up to volume with water, homogenise and filter.

5.1.2. Samples containing organic substances and free from calcium and magnesium dihydrogen phosphates

Weigh about 2,5 g of the sample to the nearest 1 mg in an ashing crucible. Mix the test sample until completely merged with 1 g of calcium carbonate (3.1). Ash in the oven at 550 °C until white or grey ash is obtained (a little charcoal does not matter). Transfer the ash into a 250 ml beaker. Add 20 ml of water and hydrochloric acid (3.2) until effervescence ceases. Add a further 10 ml of hydrochloric acid (3.2). Place the beaker on a sand bath and evaporate until dry to make the silica insoluble. Redissolve the residue in 10 ml of nitric acid (3.3) and boil on the sand bath or hot plate for 5 minutes without evaporating until dry. Decant the liquid into a 500 ml graduated flask, rinsing the beaker several times with hot water. Leave to cool, make up to volume with water, homogenise and filter.

5.2. ***Development of coloration and measurement of optical density***

Dilute an aliquot part of the filtrate obtained by 5.1.1 or 5.1.2 to obtain a phosphorus concentration of not more than 40 µg/ml. Place 10 ml of this solution in a test tube (4.6) and add 10 ml of molybdovanadate reagent (3.6). Homogenise and leave to stand for at least 10 minutes at 20 °C. Measure the optical density in a spectrophotometer at 430 nm against a solution obtained by adding 10 ml of the molybdovanadate reagent (3.6) to 10 ml of water.

5.3. ***Calibration curve***

From the standard solution (3.7) prepare solutions containing respectively 5, 10, 20, 30 and 40 µg of phosphorus per ml. Take 10 ml of each of these solutions and add thereto 10 ml of molybdovanadate reagent (3.6). Homogenise and leave to stand for at least 10 minutes at 20 °C. Measure the optical density as indicated in 5.2. Trace the calibration curve by plotting the optical densities against the corresponding quantities of phosphorus. For concentrations between 0 and 40 µg/ml, the curve will be linear.

6. **Calculation of results**

Determine the amount of phosphorus in the test sample by using the calibration curve.

Express the result as a percentage of the sample.

Repeatability

The difference between the results of two parallel determinations carried out on the same sample shall not exceed:

— 3 %, relative to the higher result, for phosphorus contents of less than 5 %,

— 0,15 % in absolute value, for phosphorus contents of 5 % or more.

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Q. DETERMINATION OF CHLORINE FROM CHLORIDES

1. **Purpose and Scope**

This method makes it possible to determine the amount of chlorine in chlorides which are soluble in water, conventionally expressed as sodium chloride. It is applicable to all feed.

2. **Principle**

The chlorides are dissolved in water. If the product contains organic matter it is clarified. The solution is slightly acidified with nitric acid and the chlorides precipitated in the form of silver chloride by means of a solution of silver nitrate. The excess silver nitrate is titrated with a solution of ammonium thiocyanate, by Volhard's method.

3. **Reagents**

3.1. Solution of ammonium thiocyanate 0,1 mol/litre.

3.2. Solution of silver nitrate 0,1 mol/litre.

3.3. Saturated solution of ammonium ferric sulphate $(NH_4)Fe(SO_4)_2$.

3.4. Nitric acid, density: 1,38 g/ml.

3.5. Diethyl ether.

3.6. Acetone.

3.7. Carrez I solution: dissolve in water 21,9 g of zinc acetate, $Zn(CH_3COO)_2 \cdot 2H_2O$ and 3 g of glacial acetic acid. Make up to 100 ml with water.

3.8. Carrez II solution: dissolve in water 10,6 g of potassium ferrocyanide $K_4Fe(CN)_6 \cdot 3H_2O$. Make up to 100 ml with water.

3.9. Active carbon, free from chlorides and not absorbing them.

4. **Apparatus**

Mixer (tumbler): approximately 35 to 40 r.p.m.

5. **Procedure**

5.1. ***Preparation of the solution***

According to the nature of the sample, prepare a solution as shown under 5.1.1, 5.1.2 or 5.1.3.

At the same time carry out a ***blank test*** omitting the sample to be analysed.

5.1.1. Samples free from organic matter

Weigh to the nearest mg a sample of not more than 10 g and containing not more than 3 g of chlorine in the form of chlorides. Place with 400 ml of water in a 500 ml volumetric flask at approximately 20 °C. Mix for 30 minutes in the tumbler, bring up to volume, homogenise and filter.

5.1.2. Samples containing organic matter, excluding the products listed under 5.1.3.

Weigh approximately 5 g of the sample to the nearest mg and place with 1 g of active carbon in a 500 ml volumetric flask. Add 400 ml of water at approximately 20 °C and 5 ml of Carrez solution I (3.7), stir for 30 seconds then add 5 ml of Carrez solution II (3.8). Mix for 30 minutes in the tumbler, bring up to volume, homogenise and filter.

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5.1.3. Cooked feed, flax cakes and flour, products rich in flax flour and other products rich in mucilage or in colloidal substances (for example, dextrinated starch)

Prepare the solution as described under 5.1.2 but do not filter. Decant (if necessary centrifuge), remove 100 ml of the supernatant liquid and transfer to a 200 ml measuring flask. Mix with acetone (3.6) and bring up to volume with this solvent, homogenise and filter.

5.2. ***Titration***

Using a pipette, transfer to an Erlenmeyer flask from 25 ml to 100 ml of the filtrate (according to the assumed chlorine content) obtained as described under 5.1.1, 5.1.2 or 5.1.3. The aliquot portion must not contain more than 150 mg of chlorine (Cl). Dilute if necessary to not less than 50 ml with water, add 5 ml of nitric acid (3.4), 20 ml of saturated solution of ammonium ferric sulphate (3.3) and two drops of ammonium thiocyanate solution (3.1) transferred by means of a burette filled up to the zero mark. Using a burette, transfer the silver nitrate solution (3.2) in such a way that an excess of 5 ml is obtained. Add 5 ml of diethyl ether (3.5) and shake hard to coagulate the precipitate. Titrate the excess silver nitrate with the ammonium thiocyanate solution (3.1) until the reddish-brown tint has lasted for one minute.

6. **Calculation of results**

The amount of chlorine (X), expressed as % sodium chloride is calculated by using the following formula:

$$X = \frac{5{,}845 \times (V_1 - V_2)}{m}$$

where:

V_1 = ml of silver nitrate solution 0,1 mol/l added
V_2 = ml of ammonium thiocyanate solution 0,1 mol/l used for titration
m = weight of sample.
If the blank test indicates that silver nitrate solution 0,1 mol/l has been consumed deduct this value from the volume ($V_1 - V_2$).

7. **Observations**

7.1. Titration may also be carried out by potentiometry.

7.2. In the case of products which are very rich in oils and fats, first de-fat with diethyl ether or light petroleum.

7.3. In the case of fish-meal, titration may be carried out by Mohr's method.

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ANNEX IV

METHODS OF ANALYSIS TO CONTROL THE LEVEL OF AUTHORISED ADDITIVES IN FEED

A. DETERMINATION OF VITAMIN A

1. **Purpose and Scope**

This method makes it possible to determine the level of vitamin A (retinol) in feed and premixtures. Vitamin A includes all-***trans***-retinyl alcohol and its ***cis***-isomers which are determined by this method. The content of vitamin A is expressed in International Units (IU) per kg. One IU corresponds to the activity of 0,300 μg all-***trans***-vitamin A alcohol or 0,344 μg all-***trans***-vitamin A acetate or 0,550 μg all-***trans***-vitamin A palmitate.

The limit of quantification is 2 000 IU vitamin A/kg.

2. **Principle**

The sample is hydrolysed with ethanolic potassium hydroxide solution and the vitamin A is extracted into light petroleum. The solvent is removed by evaporation and the residue is dissolved in methanol and, if necessary, diluted to the required concentration. The content of vitamin A is determined by reversed phase high performance liquid chromatography (RP-HPLC) using a UV or a fluorescence detector. The chromatographic parameters are chosen so that there is no separation between the all-***trans***-vitamin A alcohol and its ***cis*** isomers.

3. **Reagents**

3.1. Ethanol, σ = 96 %

3.2. Light petroleum, boiling range 40 °C-60 °C

3.3. Methanol

3.4. Potassium hydroxide solution, c = 50 g/100 ml

3.5. Sodium ascorbate solution, c = 10 g/100 ml (see 7.7 observations)

3.6. Sodium sulphide, $Na_2S \cdot x\ H_2O$ (x = 7-9)

3.6.1. Sodium sulphide solution, c = 0,5 mol/l in glycerol, ß = 120 g/l (for x = 9) (see 7.8 observations)

3.7. Phenolphthalein solution, c = 2 g/100 ml in ethanol (3.1)

3.8. 2-Propanol

3.9. Mobile phase for HPLC: mixture of methanol (3.3) and water, e.g. 980 + 20 (v + v). The exact ratio will be determined by the characteristics of the column employed.

3.10. Nitrogen, oxygen free

3.11. All-***trans***-vitamin A acetate, extra pure, of certified activity, e.g. 2,80 x 10^6 IU/g

3.11.1. Stock solution of all-***trans***-vitamin A acetate: Weigh to the nearest 0,1 mg, 50 mg of vitamin A acetate (3.11) into a 100 ml graduated flask. Dissolve in 2-propanol (3.8) and make up to the mark with the same solvent. The nominal concentration of this solution is 1 400 IU vitamin A per ml. The exact content has to be determined according to 5.6.3.1.

3.12. All-***trans***-vitamin A palmitate, extra pure, of certified activity, e.g. 1,80 x 10^6 IU/g

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3.12.1. Stock solution of all-***trans***-vitamin A palmitate: Weigh to the nearest 0,1 mg, 80 mg of vitamin A palmitate (3.12) into a 100 ml graduated flask. Dissolve in 2-propanol (3.8) and make up to the mark with the same solvent. The nominal concentration of this solution is 1 400 IU vitamin A per ml. The exact content has to be determined according to 5.6.3.2.

3.13. 2,6-Di-***tert***-butyl-4-methylphenol (BHT) (see 7.5 observations)

4. **Apparatus**

4.1. Vacuum rotary evaporator

4.2. Amber glassware

4.2.1. Flat bottom or conical flasks, 500 ml, with ground-glass socket

4.2.2. Graduated flasks with ground-glass stoppers, narrow-necked, 10, 25, 100 and 500 ml

4.2.3. Separating funnels, conical, 1 000 ml, with ground-glass stoppers

4.2.4. Pear shaped flasks, 250 ml, with ground-glass sockets

4.3. Allihn condenser, jacket length 300 mm, with ground-glass joint, with adapter for gas feed pipe

4.4. Pleated filter paper for phase separation, diameter 185 mm (e.g. Schleicher & Schuell 597 HY 1/2)

4.5. HPLC equipment with injection system

4.5.1. Liquid chromatographic column, 250 mm x 4 mm, C_{18}, 5 or 10 µm packing, or equivalent (performance criterion: only a single peak for all retinol isomers under the HPLC-conditions)

4.5.2. UV or fluorescence detector, with variable wavelength adjustment

4.6. Spectrophotometer with 10 mm quartz cells

4.7. Water-bath with magnetic stirrer

4.8. Extraction apparatus (see figure 1) consisting of:

4.8.1. Glass cylinder of 1 l capacity fitted with a ground glass neck and stopper

4.8.2. Ground glass insert equipped with a side-arm and an adjustable tube passing through the centre. The adjustable tube shall have a U-shaped lower end and a jet at the opposite end so that the upper liquid layer in the cylinder may be transferred into a separating funnel.

5. **Procedure**

Note: Vitamin A is sensitive to (UV-) light and to oxidation. All operations shall be carried out in the absence of light (using amber glassware, or glassware protected with aluminium foil) and oxygen (flush with nitrogen). During extraction air above the liquid shall be replaced by nitrogen (avoid excess pressure by loosening the stopper from time to time).

5.1. ***Preparation of the sample***

Grind the sample so that it passes a 1 mm mesh sieve, taking care to avoid generation of heat. Grinding must be carried out **immediately** before weighing and saponification otherwise there may be losses of vitamin A.

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5.2. ***Saponification***

Depending on the vitamin A content weigh, to the nearest 1 mg, 2 g to 25 g of the sample into a 500 ml flat bottom or conical flask (4.2.1). Add successively with swirling 130 ml ethanol (3.1), approximately 100 mg BHT (3.13), 2 ml sodium ascorbate solution (3.5) and 2 ml sodium sulphide solution (3.6). Fit a condenser (4.3) to the flask and immerse the flask in a water-bath with magnetic stirrer (4.7). Heat to boiling and allow to reflux for 5 minutes. Then add 25 ml potassium hydroxide solution (3.4) through the condenser (4.3) and allow to reflux for a further 25 min., with stirring under a slow stream of nitrogen. Then rinse the condenser with approximately 20 ml water and cool the content of the flask to room temperature.

5.3. ***Extraction***

Transfer by decantation the saponification solution quantitatively by rinsing with a total volume of 250 ml water to a 1 000 ml separating funnel (4.2.3) or to the extraction apparatus (4.8). Rinse the saponification flask successively with 25 ml ethanol (3.1) and 100 ml light petroleum (3.2) and transfer the rinsings to the separating funnel or to the extraction apparatus. The proportion of water and ethanol in the combined solutions must be about 2:1. Shake vigorously for 2 min. and allow to settle for 2 minutes.

5.3.1. Extraction using a separating funnel (4.2.3)

When the layers have separated (see observation 7.3) transfer the light petroleum layer to another separating funnel (4.2.3). Repeat this extraction twice, with 100 ml light petroleum (3.2) and twice, with 50 ml light petroleum (3.2).

Wash the combined extracts in the separating funnel twice by gently swirling (to avoid formation of emulsions) with 100 ml portions of water and then by repeated shaking with further 100 ml portions of water until the water remains colourless on addition of phenolphthalein solution (3.7) (washing four times is usually sufficient). Filter the washed extract through a dry pleated filter for phase separation (4.4) to remove any suspended water into a 500 ml graduated flask (4.2.2). Rinse the separating funnel and the filter with 50 ml light petroleum (3.2), make up to the mark with light petroleum (3.2) and mix well.

5.3.2. Extraction using an extraction apparatus (4.8)

When the layers have separated (see observation 7.3) replace the stopper of the glass cylinder (4.8.1) by the ground glass insert (4.8.2) and position the U-shaped lower end of the adjustable tube so that it is just above the level of the interface. By application of pressure from a nitrogen line to the side-arm, transfer the upper light petroleum-layer to a 1 000 ml separating funnel (4.2.3). Add 100 ml light petroleum (3.2) to the glass cylinder, stopper and shake well. Allow the layers to separate and transfer the upper layer to the separating funnel as before. Repeat the extraction procedure with further 100 ml of light petroleum (3.2), then twice with 50 ml portions of light petroleum (3.2) and add the light petroleum layers to the separating funnel.

Wash the combined light petroleum extracts as described in 5.3.1 and proceed as described there.

5.4. ***Preparation of the sample solution for HPLC***

Pipette an aliquot portion of the light petroleum solution (from 5.3.1 or 5.3.2) into a 250 ml pear shaped flask (4.2.4). Evaporate the solvent nearly to dryness on the rotary evaporator (4.1) with reduced pressure at a bath temperature not exceeding 40 °C. Restore atmospheric pressure by admitting nitrogen (3.10) and remove the flask from the rotary

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evaporator. Remove the remaining solvent with a stream of nitrogen (3.10) and dissolve the residue immediately in a known volume (10-100 ml) of methanol (3.3) (the concentration of vitamin A must be in the range of 5 IU/ml to 30 IU/ml).

5.5. ***Determination by HPLC***

Vitamin A is separated on a C_{18} reversed phase column (4.5.1) and the concentration is measured by means of a UV detector (325 nm) or a fluorescence detector (excitation: 325 nm, emission: 475 nm) (4.5.2).

Inject an aliquot portion (e.g. 20 µl) of the methanolic solution obtained in 5.4 and elute with the mobile phase (3.9). Calculate the mean peak height (area) of several injections of the same sample solution and the mean peak heights (areas) of several injections of the calibration solutions (5.6.2).

HPLC conditions

The following conditions are offered for guidance; other conditions may be used provided that they give equivalent results.

Liquid chromatographic column (4.5.1):	250 mm × 4 mm, C_{18}, 5 or 10 µm packing, or equivalent
Mobile phase (3.9):	Mixture of methanol (3.3) and water e.g. 980 + 20 (v + v).
Flow rate:	1-2 ml/min.
Detector (4.5.2):	UV detector (325 nm) or fluorescence detector (excitation: 325 nm/emission: 475 nm)

5.6. ***Calibration***

5.6.1. Preparation of the working standard solutions

Pipette 20 ml of the vitamin A acetate stock solution (3.11.1) or 20 ml of the vitamin A palmitate stock solution (3.12.1) into a 500 ml flat bottom or conical flask (4.2.1) and hydrolyse as described under 5.2, but without addition of BHT. Subsequently extract with light petroleum (3.2) according to 5.3 and make up to 500 ml with light petroleum (3.2). Evaporate 100 ml of this extract on the rotary evaporator (see 5.4) nearly to dryness, remove the remaining solvent with a stream of nitrogen (3.10) and redissolve the residue in 10,0 ml of methanol (3.3). The nominal concentration of this solution is 560 IU vitamin A per ml. The exact content has to be determined according to 5.6.3.3. The working standard solution has to be freshly prepared before use.

Pipette 2,0 ml of this working standard solution into a 20 ml graduated flask, make up to the mark with methanol (3.3) and mix. The nominal concentration of this **diluted** working standard solution is 56 IU vitamin A per ml.

5.6.2. Preparation of the calibration solutions and calibration graph

Transfer 1,0, 2,0, 5,0 and 10,0 ml of the **diluted** working standard solution into a series of 20 ml graduated flasks, make up to the mark with methanol (3.3) and mix. The nominal concentrations of these solutions are 2,8, 5,6, 14,0 and 28,0 IU vitamin A per ml.

Inject 20 µl of each calibration solution several times and determine the mean peak heights (areas). Using the mean peak heights (areas) plot a calibration graph considering the results of the UV control (5.6.3.3).

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5.6.3. UV standardisation of the standard solutions

5.6.3.1. ***Vitamin A acetate stock solution***

Pipette 2,0 ml of the vitamin A acetate stock solution (3.11.1) into a 50 ml graduated flask (4.2.2) and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 56 IU vitamin A per ml. Pipette 3,0 ml of this diluted vitamin A acetate solution into a 25 ml graduated flask and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 6,72 IU vitamin A per ml. Measure the UV spectrum of this solution against 2-propanol (3.8) in the spectrophotometer (4.6) between 300 nm and 400 nm. The extinction maximum must be between 325 nm and 327 nm.

Calculation of the vitamin A content:

$$\text{IU vitamin A/ml} = E_{326} \times 19{,}0$$

($E^{1\,\%}_{1\,cm}$ for vitamin A acetate = 1 530 at 326 nm in 2-propanol)

5.6.3.2. ***Vitamin A palmitate stock solution***

Pipette 2,0 ml of the vitamin A palmitate stock solution (3.12.1) into a 50 ml graduated flask (4.2.2) and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 56 IU vitamin A per ml. Pipette 3,0 ml of this diluted vitamin A palmitate solution into a 25 ml graduated flask and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 6,72 IU vitamin A per ml. Measure the UV spectrum of this solution against 2-propanol (3.8) in the spectrophotometer (4.6) between 300 nm and 400 nm. The extinction maximum must be between 325 nm and 327 nm.

Calculation of the vitamin A content:

$$\text{IU vitamin A/ml} = E_{326} \times 19{,}0$$

($E^{1\,\%}_{1\,cm}$ for vitamin A palmitate = 957 at 326 nm in 2-propanol)

5.6.3.3. ***Vitamin A working standard solution***

Pipette 3,0 ml of the **undiluted** vitamin A working standard solution, prepared according to 5.6.1 into a 50 ml graduated flask (4.2.2) and make up to the mark with 2-propanol (3.8). Pipette 5,0 ml of this solution into a 25 ml graduated flask and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 6,72 IU vitamin A per ml. Measure the UV spectrum of this solution against 2-propanol (3.8) in the spectrophotometer (4.6) between 300 nm and 400 nm. The extinction maximum must be between 325 nm and 327 nm.

Calculation of the vitamin A content:

$$\text{IU vitamin A/ml} = E_{325} \times 18{,}3$$

($E^{1\,\%}_{1\,cm}$ for vitamin A alcohol = 1 821 at 325 nm in 2-propanol)

6. **Calculation of the results**

From the mean height (area) of the vitamin A peaks of the sample solution determine the concentration of the sample solution in IU/ml by reference to the calibration graph (5.6.2).

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The vitamin A content w in IU/kg of the sample is given by the following formula:

$$w = \frac{500 \times c \times V_2 \times 1\,000}{V_1 \times m} \text{ [IU/kg]}$$

in which:

c = vitamin A concentration of the sample solution (5.4) in IU/ml
V_1 = volume of sample solution (5.4) in ml
V_2 = volume of aliquot taken in 5.4 in ml
m = weight of the test portion in g

7. **Observations**

7.1. For samples with low vitamin A concentration it may be useful to combine the light petroleum-extracts of two saponification-charges (amount weighed: 25 g) to one sample solution for HPLC-determination.

7.2. The weight of the sample taken for the analysis shall not contain more than 2 g fat.

7.3. If phase separation does not occur add approximately 10 ml ethanol (3.1) to break the emulsion.

7.4. With cod-liver oil and other pure fats the saponification time shall be extended to 45-60 minutes.

7.5. Hydroquinone can be used instead of BHT.

7.6. Using a normal phase-column the separation of retinol isomers is possible. But in that case, the heights (areas) of all cis and trans isomers peaks have to be summed for calculations.

7.7. Approximately 150 mg ascorbic acid can be used instead of sodium ascorbate solution.

7.8. Approximately 50 mg EDTA can be used instead of sodium sulphide solution.

7.9. In cases of analysis of vitamin A in milk replacers, specific attention has to be paid

— at saponification (5.2): due to the amount of fat present in the sample, increasing of potassium hydroxide solution amount (3.4) may be necessary,

— at extraction (5.3): due to the presence of emulsions, adaptation of the water/ethanol 2:1 ratio may be necessary.

To check if the applied method of analysis generates reliable results on this specific matrix (milk replacer), a recovery test shall be applied on an additional test portion. If the recovery rate is lower than 80 %, the analytical result has to be corrected for recovery.

8. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample must not exceed 15 % relative to the higher result.

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9. **Results of a collaborative study** ([1])

	Premix	Premix feed	Mineral concentrate	Protein feed	Piglet
L	13	12	13	12	13
n	48	45	47	46	49
mean [IU/kg]	$17{,}02 \times 10^6$	$1{,}21 \times 10^6$	537 100	151 800	18 070
S_r [IU/kg]	$0{,}51 \times 10^6$	$0{,}039 \times 10^6$	22 080	12 280	682
r [IU/kg]	$1{,}43 \times 10^6$	$0{,}109 \times 10^6$	61 824	34 384	1 910
CV_r[%]	3,0	3,5	4,1	8,1	3,8
S_R [IU/kg]	$1{,}36 \times 10^6$	$0{,}069 \times 10^6$	46 300	23 060	3 614
R [IU/kg]	$3{,}81 \times 10^6$	$0{,}193 \times 10^6$	129 640	64 568	10 119
CV_R [%]	8,0	6,2	8,6	15	20

L = number of laboratories
n = number of single values
s_r = standard deviation of repeatability
S_R = standard deviation of reproducibility
r = repeatability
R = reproducibility
CV_r = coefficient of variation of repeatability
CV_R = coefficient of variation of reproducibility.

([1]) Conducted by the Feed Working Group of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA).

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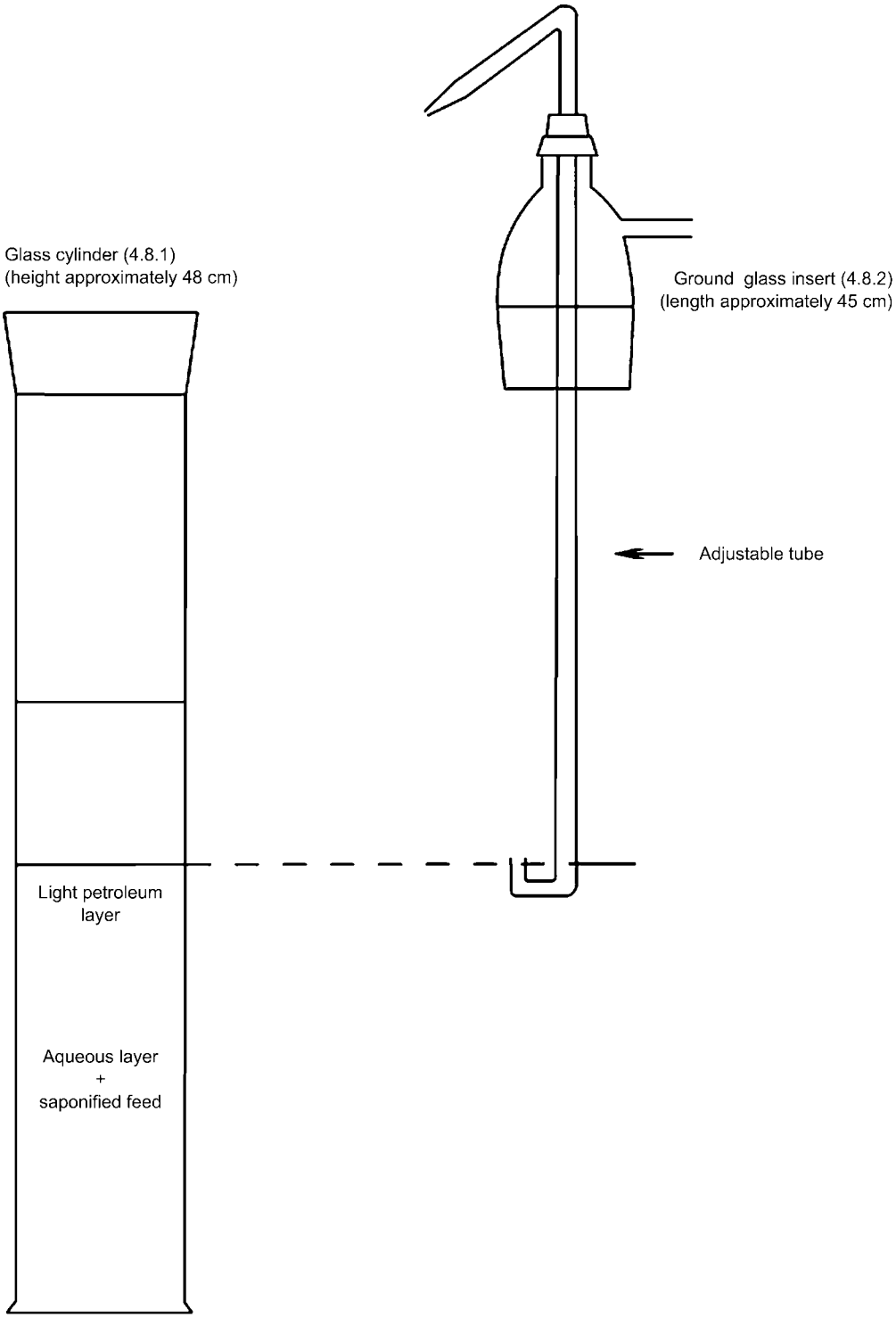
Figure 1: Extraction apparatus (4.8)



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B. DETERMINATION OF VITAMIN E

1. **Purpose and Scope**

This method makes it possible to determine the level of vitamin E in feed and premixtures. The content of vitamin E is expressed as mg DL-α-tocopherol acetate per kg. 1 mg DL-α-tocopherol acetate corresponds to 0,91 mg DL-α-tocopherol (vitamin E).

The limit of quantification is 2 mg vitamin E/kg. This limit of quantification is only achievable with fluorescence detector. With an UV detector the limit of quantification is 10 mg/kg.

2. **Principle**

The sample is hydrolysed with ethanolic potassium hydroxide solution and the vitamin E is extracted into light petroleum. The solvent is removed by evaporation and the residue is dissolved in methanol and, if necessary, diluted to the required concentration. The content of vitamin E is determined by reversed phase high performance liquid chromatography (RP-HPLC) using a fluorescence or a UV detector.

3. **Reagents**

3.1. Ethanol, σ = 96 %.

3.2. Light petroleum, boiling range 40 °C-60 °C.

3.3. Methanol.

3.4. Potassium hydroxide solution, c = 50 g/100 ml.

3.5. Sodium ascorbate solution, c = 10 g/100 ml (see 7.7 observations).

3.6. Sodium sulphide, $Na_2S \cdot x\ H_2O$ (x = 7-9).

3.6.1. Sodium sulphide solution, c = 0,5 mol/l in glycerol, β = 120 g/l. (for x = 9) (see 7.8 observations)

3.7. Phenolphthalein solution, c = 2 g/100 ml in ethanol (3.1).

3.8. Mobile phase for HPLC: mixture of methanol (3.3) and water, e.g. 980 + 20 (v + v). The exact ratio will be determined by the characteristics of the column employed.

3.9. Nitrogen, oxygen free.

3.10. DL-α-tocopherol acetate, extra pure, of certified activity.

3.10.1. Stock solution of DL-α-tocopherol acetate: Weigh to the nearest 0,1 mg, 100 mg of DL-α-tocopherol acetate (3.10) into a 100 ml graduated flask. Dissolve in ethanol (3.1) and make up to the mark with the same solvent. 1 ml of this solution contains 1 mg DL-α-tocopherol acetate. (UV control see 5.6.1.3; stabilisation see 7.4 observations).

3.11. DL-α-tocopherol, extra pure, of certified activity.

3.11.1. Stock solution of DL-α-tocopherol: Weigh to the nearest 0,1 mg, 100 mg of DL-α-tocopherol (3.10) into a 100 ml graduated flask. Dissolve in ethanol (3.1) and make up to the mark with the same solvent. 1 ml of this solution contains 1 mg DL-α-tocopherol. (UV control see 5.6.2.3; stabilisation see 7.4 observations).

3.12. 2,6-Di-***tert***-butyl-4-methylphenol (BHT) (see 7.5 observations).

4. **Apparatus**

4.1. Rotary film evaporator.

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4.2. Amber glassware.

4.2.1. Flat bottom or conical flasks, 500 ml, with ground-glass socket.

4.2.2. Graduated flasks with ground-glass stoppers, narrow-necked, 10, 25, 100 and 500 ml.

4.2.3. Separating funnels, conical, 1 000 ml, with ground-glass stoppers.

4.2.4. Pear shaped flasks, 250 ml, with ground-glass sockets.

4.3. Allihn condenser, jacket length 300 mm, with ground-glass joint, with adapter for gas feed pipe.

4.4. Pleated filter paper for phase separation, diameter 185 mm (e.g. Schleicher & Schuell 597 HY 1/2).

4.5. HPLC equipment with injection system.

4.5.1. Liquid chromatographic column, 250 mm × 4 mm, C_{18}, 5 or 10 µm packing, or equivalent.

4.5.2. Fluorescence or UV detector, with variable wavelength adjustment.

4.6. Spectrophotometer with 10 mm quartz cells.

4.7. Water-bath with magnetic stirrer.

4.8. Extraction apparatus (see figure 1) consisting of:

4.8.1. Glass cylinder of 1 l capacity fitted with a ground glass neck and stopper.

4.8.2. Ground glass insert equipped with a side-arm and an adjustable tube passing through the centre. The adjustable tube shall have a U-shaped lower end and a jet at the opposite end so that the upper liquid layer in the cylinder may be transferred into a separating funnel.

5. **Procedure**

Note: Vitamin E is sensitive to (UV-) light and to oxidation. All operations shall be carried out in the absence of light (using amber glassware, or glassware protected with aluminium foil) and oxygen (flush with nitrogen). During extraction air above the liquid shall be replaced by nitrogen (avoid excess pressure by loosening the stopper from time to time).

5.1. ***Preparation of the sample***

Grind the sample so that it passes a 1 mm mesh sieve, taking care to avoid generation of heat. Grinding must be carried out **immediately** before weighing and saponification otherwise there may be losses of vitamin E.

5.2. ***Saponification***

Depending on the vitamin E content weigh, to the nearest 0,01 g, 2 g to 25 g of the sample into a 500 ml flat bottom or conical flask (4.2.1). Add successively with swirling 130 ml ethanol (3.1), approximately 100 mg BHT (3.12), 2 ml sodium ascorbate solution (3.5) and 2 ml sodium sulphide solution (3.6). Fit the condenser (4.3) to the flask and immerse the flask in a water-bath with magnetic stirrer (4.7). Heat to boiling and allow to reflux for 5 minutes. Then add 25 ml potassium hydroxide solution (3.4) through the condenser (4.3) and allow to reflux for a further 25 min. with stirring under a slow stream of nitrogen. Then rinse the condenser with approximately 20 ml water and cool the content of the flask to room temperature.

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5.3. ***Extraction***

Transfer by decantation the saponification solution quantitatively by rinsing with a total volume of 250 ml water to a 1 000 ml separating funnel (4.2.3) or to the extraction apparatus (4.8). Rinse the saponification flask successively with 25 ml ethanol (3.1) and 100 ml light petroleum (3.2) and transfer the rinsings to the separating funnel or to the extraction apparatus. The proportion of water and ethanol in the combined solutions must be about 2:1. Shake vigorously for 2 min. and allow to settle for 2 minutes.

5.3.1. Extraction using a separating funnel (4.2.3)

When the layers have separated (see observation 7.3) transfer the light petroleum layer to another separating funnel (4.2.3). Repeat this extraction twice, with 100 ml light petroleum (3.2) and twice, with 50 ml light petroleum (3.2).

Wash the combined extracts in the separating funnel twice by gently swirling (to avoid formation of emulsions) with 100 ml portions of water and then by repeated shaking with further 100 ml portions of water until the water remains colourless on addition of phenolphthalein solution (3.7) (washing four times is usually sufficient). Filter the washed extract through a dry pleated filter for phase separation (4.4) to remove any suspended water into a 500 ml graduated flask (4.2.2). Rinse the separating funnel and the filter with 50 ml light petroleum (3.2), make up to the mark with light petroleum (3.2) and mix well.

5.3.2. Extraction using an extraction apparatus (4.8)

When the layers have separated (see observation 7.3) replace the stopper of the glass cylinder (4.8.1) by the ground glass insert (4.8.2) and position the U-shaped lower end of the adjustable tube so that it is just above the level of the interface. By application of pressure from a nitrogen line to the side-arm, transfer the upper light petroleum-layer to a 1 000 ml separating funnel (4.2.3). Add 100 ml light petroleum (3.2) to the glass cylinder, stopper and shake well. Allow the layers to separate and transfer the upper layer to the separating funnel as before. Repeat the extraction procedure with further 100 ml of light petroleum (3.2), then twice with 50 ml portions of light petroleum (3.2) and add the light petroleum layers to the separating funnel.

Wash the combined light petroleum extracts as described in 5.3.1 and proceed as described there.

5.4. ***Preparation of the sample solution for HPLC***

Pipette an aliquot portion of the light petroleum solution (from 5.3.1 or 5.3.2) into a 250 ml pear shaped flask (4.2.4). Evaporate the solvent nearly to dryness on the rotary evaporator (4.1) with reduced pressure at a bath temperature not exceeding 40 °C. Restore atmospheric pressure by admitting nitrogen (3.9) and remove the flask from the rotary evaporator. Remove the remaining solvent with a stream of nitrogen (3.9) and dissolve the residue immediately in a known volume (10-100 ml) of methanol (3.3) (the concentration of DL-α-tocopherol must be in the range 5 μg/ml to 30 μg/ml).

5.5. ***Determination by HPLC***

Vitamin E is separated on a C_{18} reversed phase column (4.5.1) and the concentration is measured using a fluorescence detector (excitation: 295 nm, emission: 330 nm) or a UV detector (292 nm) (4.5.2).

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Inject an aliquot portion (e.g. 20 µl) of the methanolic solution obtained in 5.4 and elute with the mobile phase (3.8). Calculate the mean peak heights (areas) of several injections of the same sample solution and the mean peak heights (areas) of several injections of the calibration solutions (5.6.2).

HPLC conditions

The following conditions are offered for guidance; other conditions may be used provided that they give equivalent results.

Liquid chromatographic column (4.5.1):	250 mm × 4 mm, C_{18}, 5 or 10 µm packing, or equivalent
Mobile phase (3.8):	Mixture of methanol (3.3) and water e.g. 980 + 20 (v + v).
Flow rate:	1-2 ml/min.
Detector (4.5.2)	Fluorescence detector (excitation: 295 nm/emission: 330 nm) or UV detector (292 nm)

5.6. ***Calibration (DL-α-tocopherol acetate or DL-α-tocopherol)***

5.6.1. DL-α-tocopherol acetate standard

5.6.1.1. ***Preparation of the working standard solution***

Transfer by pipette 25 ml of the DL-α-tocopherol acetate stock solution (3.10.1) into a 500 ml flat bottom or conical flask (4.2.1) and hydrolyse as described under 5.2. Subsequently extract with light petroleum (3.2) according to 5.3 and make up to 500 ml with light petroleum. Evaporate 25 ml of this extract on the rotary evaporator (see 5.4) nearly to dryness, remove the remaining solvent with a stream of nitrogen (3.9) and redissolve the residue in 25,0 ml of methanol (3.3). The nominal concentration of this solution is 45,5 µg DL-α-tocopherol per ml, equivalent to 50 µg DL-α-tocopherol acetate per ml. The working standard solution has to be freshly prepared before use.

5.6.1.2. ***Preparation of the calibration solutions and calibration graph***

Transfer 1,0, 2,0, 4,0 and 10,0 ml of the working standard solution into a series of 20 ml graduated flasks, make up to the mark with methanol (3.3) and mix. The nominal concentrations of these solutions are 2,5, 5,0, 10,0 and 25,0 µg/ml DL-α-tocopherol acetate, i.e. 2,28, 4,55, 9,10 µg/ml and 22,8 µg/ml DL-α-tocopherol.

Inject 20 µl of each calibration solution several times and determine the mean peak heights (areas). Using the mean peak heights (areas) plot a calibration graph.

5.6.1.3. ***UV standardisation of the DL-α-tocopherol acetate stock solution (3.10.1)***

Dilute 5,0 ml of the DL-α-tocopherol acetate stock solution (3.10.1) to 25,0 ml with ethanol and measure the UV spectrum of this solution against ethanol (3.1) in the spectrophotometer (4.6) between 250 nm and 320 nm.

The absorption maximum shall be at 284 nm:

$E^{1\,\%}_{1\,cm}$ = 43,6 at 284 nm in ethanol

At this dilution an extinction value of 0,84 to 0,88 must be obtained.

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5.6.2. DL-α-tocopherol standard

5.6.2.1. ***Preparation of the working standard solution***

Transfer by pipette 2 ml of the DL-α-tocopherol stock solution (3.11.1) into a 50 ml graduated flask, dissolve in methanol (3.3) and make up to the mark with methanol. The nominal concentration of this solution is 40 µg DL-α-tocopherol per ml, equivalent to 44,0 µg DL-α-tocopherol acetate per ml. The working standard solution has to be freshly prepared before use.

5.6.2.2. ***Preparation of the calibration solutions and calibration graph***

Transfer 1,0, 2,0, 4,0 and 10,0 ml of the working standard solution into a series of 20 ml graduated flasks, make up to the mark with methanol (3.3) and mix. The nominal concentrations of these solutions are 2,0, 4,0, 8,0 and 20,0 µg/ml DL-α-tocopherol, i.e. 2,20, 4,40, 8,79 µg/ml and 22,0 µg/ml DL-α-tocopherol acetate.

Inject 20 µl of each calibration solution several times and determine the mean peak heights (areas). Using the mean peak heights (areas) plot a calibration graph.

5.6.2.3. ***UV standardisation of the DL-α-tocopherol stock solution (3.11.1)***

Dilute 2,0 ml of the DL-α-tocopherol stock solution (3.11.1) to 25,0 ml with ethanol and measure the UV spectrum of this solution against ethanol (3.1) in the spectrophotometer (4.6) between 250 nm and 320 nm. The absorption maximum shall be at 292 nm:

$E_{1cm}^{1\%}$ = 75,8 at 292 nm in ethanol

At this dilution an extinction value of 0,6 must be obtained.

6. **Calculation of the results**

From the mean height (area) of the vitamin E peaks of the sample solution determine the concentration of the sample solution in µg/ml (calculated as α-tocopherol acetate) by reference to the calibration graph (5.6.1.2 or 5.6.2.2).

The vitamin E content w in mg/kg of the sample is given by the following formula:

$$w = \frac{500 \times c \times V_2}{V_1 \times m} \text{ [mg/kg]}$$

in which:

c = vitamin E concentration (as α-tocopherol acetate) of the sample solution (5.4) in µg/ml
V_1 = volume of sample solution (5.4), in ml
V_2 = volume of aliquot taken in (5.4), in ml
m = weight of the test portion in g

7. **Observations**

7.1. For samples with low vitamin E concentration it may be useful to combine the light petroleum-extracts of two saponification-charges (amount weighed: 25 g) to one sample solution for HPLC-determination.

7.2. The weight of the sample taken for the analysis shall not contain more than 2 g fat.

7.3. If phase separation does not occur add approximately 10 ml ethanol (3.1) to break the emulsion.

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7.4. After the spectrophotometric measurement of the DL-α-tocopherol acetate or DL-α-tocopherol solution according to 5.6.1.3 or 5.6.2.3 respectively add approximately 10 mg BHT (3.12) to the solution (3.10.1 or 3.10.2) and keep the solution in a refrigerator (storage life max. 4 weeks).

7.5. Hydroquinone can be used instead of BHT.

7.6. Using a normal phase-column the separation of α-, β-, γ- and δ-tocopherol is possible.

7.7. Approximately 150 mg ascorbic acid can be used instead of sodium ascorbate solution.

7.8. Approximately 50 mg EDTA can be used instead of sodium sulphide solution.

7.9. Vitamin E acetate hydrolyses very fast under alkaline conditions and is therefore very sensitive to oxidation, especially in the presence of trace elements like iron or copper. In case of the determination of vitamin E in premixtures at levels higher than 5 000 mg/kg, a degradation of vitamin E could be the consequence. Therefore a HPLC method including an enzymatic digestion of the vitamin E formulation without an alkaline saponification step is to be recommended for confirmation.

8. **Repeatability**·

The difference between the results of two parallel determinations carried out on the same sample must not exceed 15 % relative to the higher result.

9. **Results of a collaborative study** ([1])

	Premix	Premix feed	Mineral concentrate	Protein feed	Piglet
L	12	12	12	12	12
n	48	48	48	48	48
mean [mg/kg]	17 380	1 187	926	315	61,3
S_r [mg/kg]	384	45,3	25,2	13,0	2,3
r [mg/kg]	1 075	126,8	70,6	36,4	6,4
CV_r [%]	2,2	3,8	2,7	4,1	3,8
S_R [mg/kg]	830	65,0	55,5	18,9	7,8
R [mg/kg]	2 324	182,0	155,4	52,9	21,8
CV_R [%]	4,8	5,5	6,0	6,0	12,7

L = number of laboratories
n = number of single values
s_r = standard deviation of repeatability
s_R = standard deviation of reproducibility
r = repeatability
R = reproducibility
CV_r = coefficient of variation of repeatability
CV_R = coefficient of variation of reproducibility

([1]) Conducted by the Feed Working Group of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA).

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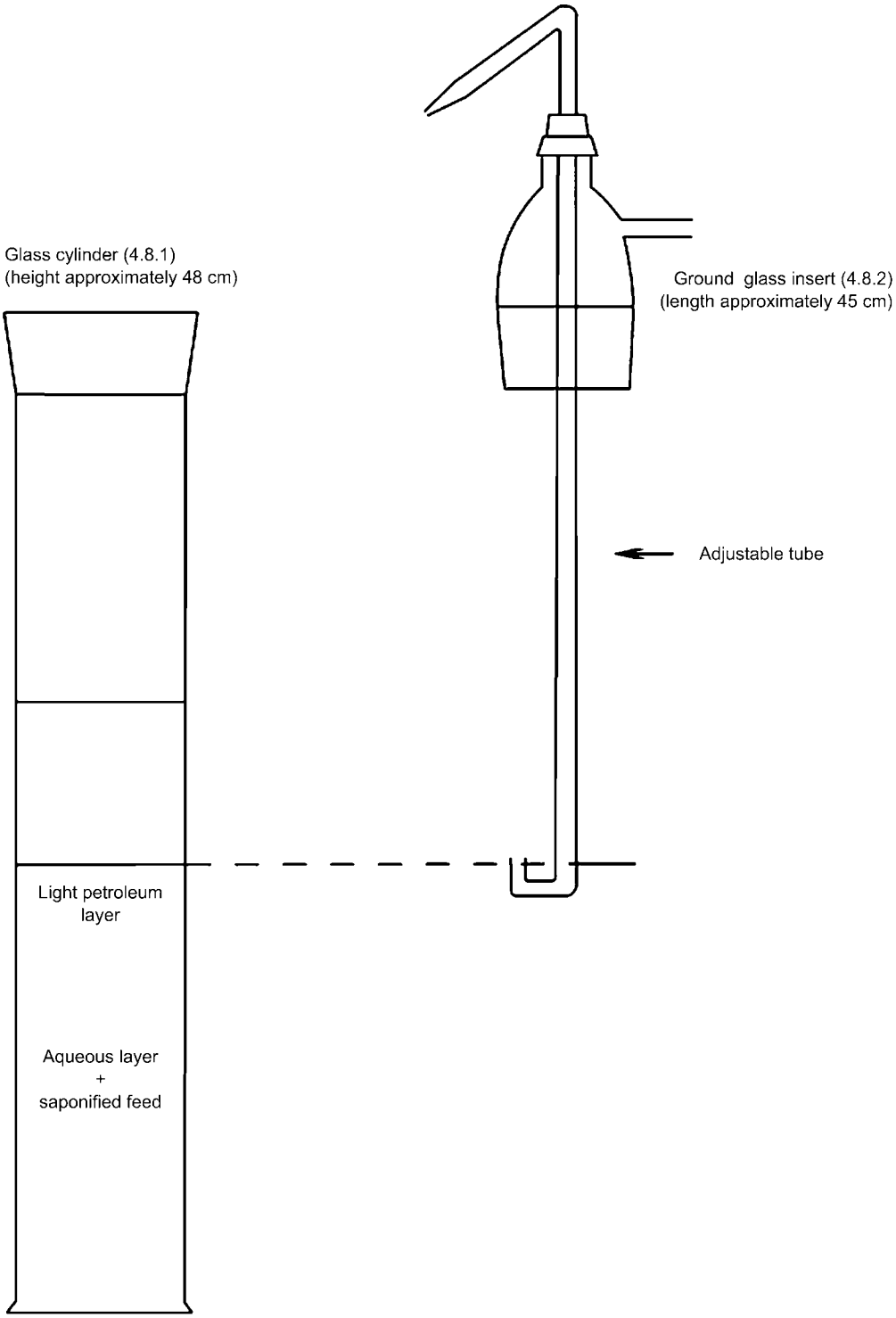
Figure 1: Extraction apparatus (4.8)



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C. DETERMINATION OF THE TRACE ELEMENTS IRON, COPPER, MANGANESE AND ZINC

1. **Purpose and scope**

The method makes it possible to determine the trace elements iron, copper, manganese and zinc in feed. The limits of quantification are:

— iron (Fe): 20 mg/kg

— copper (Cu): 10 mg/kg

— manganese (Mn): 20 mg/kg

— zinc (Zn): 20 mg/kg.

2. **Principle**

The sample is brought into solution in hydrochloric acid after destruction of organic matter, if any. The elements iron, copper, manganese and zinc are determined, after appropriate dilution, by atomic absorption spectrometry.

3. **Reagents**

Introductory comments

For preparation of the reagents and analytical solutions use water free from the cations to be determined, obtained either by double distilling water in a borosilicate glass or quartz still or by double treatment on ion exchange resin.

The reagents must be of at least analytical grade. Freedom from the element to be determined must be checked in a blank experiment. If necessary, the reagents must be further purified.

In place of the standard solutions described below, commercial standard solutions may be used provided that they are guaranteed and have been checked before use.

3.1. Hydrochloric acid (d:1,19 g/ml).

3.2. Hydrochloric acid (6 mol/litre).

3.3. Hydrochloric acid (0,5 mol/litre).

3.4. Hydrofluoric acid 38 % to 40 % (v/v) having an iron (Fe) content of less than 1 mg/litre and a residue after evaporation of less than 10 mg (as sulphate)/litre.

3.5. Sulphuric acid (d: 1,84 g/ml).

3.6. Hydrogen peroxide (approximately 100 volumes of oxygen (30 % by weight)).

3.7. Standard iron solution (1 000 µg Fe/ml) prepared as follows or equivalent commercially available solution: dissolve 1 g of iron wire in 200 ml of 6 mol/litre hydrochloric acid (3.2), add 16 ml of hydrogen peroxide (3.6) and make up to one litre with water.

3.7.1. Working standard iron solution (100 µg Fe/ml) prepared by diluting one part of the standard solution (3.7) with 9 parts of water.

3.8. Standard copper solution (1 000 µg Cu/ml) prepared as follows or equivalent commercially available solution:

— dissolve 1 g of copper in powder form in 25 ml of 6 mol/litre hydrochloric acid (3.2), add 5 ml of hydrogen peroxide (3.6) and make up to one litre with water.

3.8.1. Working standard copper solution (10 µg Cu/ml) prepared by diluting 1 part of the standard solution (3.8) with 9 parts of water and then diluting 1 part of the resulting solution with 9 parts of water.

▼B

3.9. Standard manganese solution (1 000 µg Mn/ml) prepared as follows or equivalent commercially available solution:

— dissolve 1 g of manganese in powder form in 25 ml of 6 mol/litre hydrochloric acid (3.2) and make up to one litre with water.

3.9.1. Working standard manganese solution (10 µg Mn/ml) prepared by diluting 1 part of the standard solution (3.9) with 9 parts of water and then diluting 1 part of the resulting solution with 9 parts of water.

3.10. Standard zinc solution (1 000 µg Zn/ml) prepared as follows or equivalent commercially available solution:

— dissolve 1 g of zinc in strip or leaf form in 25 ml of 6 mol/litre hydrochloric acid (3.2) and make up to one litre with water.

3.10.1. Working standard zinc solution (10 µg Zn/ml) prepared by diluting 1 part of the standard solution (3.10) with 9 parts of water and then diluting 1 part of the resulting solution with 9 parts of water.

3.11. Lanthanum chloride solution: dissolve 12 g of lanthanum oxide in 150 ml of water, add 100 ml of 6 mol/litre hydrochloric acid (3.2) and make up to one litre with water.

4. **Apparatus**

4.1. Muffle furnace with temperature regulation and preferably recorder.

4.2. Glassware must be of resistant borosilicate type and it is recommended to use apparatus which is reserved exclusively for trace element determinations.

4.3. Atomic absorption spectrophotometer meeting the requirements of the method with regard to sensitivity and precision in the required range.

5. **Procedure** ([1])

5.1. ***Samples containing organic matter***

5.1.1. Ashing and preparation of the solution for analysis ([2])

5.1.1.1. Place 5 to 10 g of sample weighed to the nearest 0,2 mg in a quartz or platinum crucible (see Note (b)), dry in an oven at 105 °C and introduce the crucible into the cold muffle furnace (4.1). Close the furnace (see Note (c)) and gradually raise the temperature to 450 to 475 °C over about 90 minutes. Maintain this temperature for 4 to 16 hours (e.g. overnight) to remove carbonaceous material and then open the furnace and allow to cool (see Note (d)).

([1]) Other methods of digestion may be used provided they have been demonstrated to have similar results (such as microwave pressure digestion).

([2]) Green fodder (fresh or dried) is liable to contain large amounts of vegetable silica, which may retain trace elements and must be removed. For samples of these feed, therefore, the following modified procedure must be followed. Carry out operation 5.1.1.1. as far as the filtration. Wash the filter paper containing the insoluble residue twice with boiling water and place it in a quartz or platinum crucible. Ignite in the muffle furnace (4.1) at a temperature below 550 °C until all carbonaceous material has completely disappeared. Allow to cool, add a few drops of water followed by 10 to 15 ml of hydrofluoric acid (3.4) and evaporate to dryness at about 150 °C. If any silica remains in the residue, redissolve it in a few millilitres of hydrofluoric acid (3.4) and evaporate to dryness. Add five drops of sulphuric acid (3.5) and heat until no more white fumes are given off. After the addition of 5 ml of 6 mol/litre hydrochloric acid (3.2) and about 30 ml of water, heat, filter the solution into the 250 ml volumetric flask and make up to the mark with water (HCl concentration about 0,5 mol/l). Proceed then with the determination from point 5.1.2.

▼B

Moisten the ashes with water and transfer these in a beaker of 250 ml. Wash the crucible out with a total of about 5 ml of hydrochloric acid (3.1) and add the latter slowly and carefully to the beaker (there may be a vigorous reaction due to CO_2 formation). Add hydrochloric acid (3.1) dropwise with agitation until all effervescence has stopped. Evaporate to dryness, occasionally stirring with a glass rod.

Next add 15 ml of 6 mol/litre hydrochloric acid (3.2) to the residue followed by about 120 ml of water. Stir with the glass rod, which shall be left in the beaker, and cover the beaker with a watch-glass. Bring gently to the boil and maintain at boiling point until no more ash can be seen to dissolve. Filter on ash-free filter paper and collect the filtrate in a 250 ml volumetric flask. Wash the beaker and filter with 5 ml of hot 6 mol/litre hydrochloric acid (3.2) and twice with boiling water. Fill the volumetric flask up to the mark with water (HCl concentration about 0,5 mol/litre).

5.1.1.2. If the residue in the filter appears black (carbon), put it back in the furnace and ash again at 450 to 475 °C. This ashing, which only requires a few hours (about three to five hours), is complete when the ash appears white or nearly white. Dissolve the residue with about 2 ml of hydrochloric acid (3.1), evaporate to dryness and add 5 ml of 6 mol/litre hydrochloric acid (3.2). Heat, filter the solution into the volumetric flask and make up to the mark with water (HCl concentration about 0,5 mol/litre.

Notes:

(a) In determining trace elements it is important to be alert to the risks of contamination, particularly by zinc, copper and iron. For this reason, the equipment used in preparing the samples must be free of these metals.

To reduce the general risk of contamination, work in a dust-free atmosphere with scrupulously clean equipment and carefully washed glassware. The determination of zinc is particularly sensitive to many types of contamination, e.g. from glassware, reagents, dust, etc.

(b) The weight of sample to be ashed is calculated from the approximate trace element content of the feed in relation to the sensitivity of the spectrophotometer used. For certain feed low in trace elements it may be necessary to start with a 10 to 20 g sample and make up the final solution to only 100 ml.

(c) Ashing must be carried out in a closed furnace without injection of air or oxygen.

(d) The temperature indicated by the pyrometer must not exceed 475 °C.

5.1.2. Spectrophotometric determination

5.1.2.1. ***Preparation of calibration solutions***

For each of the elements to be determined, prepare from the working standard solutions given in points 3.7.1, 3.8.1, 3.9.1 and 3.10.1 a range of calibration solutions, each calibration solution having an HCl concentration of about 0,5 mol/litre (and (in the cases of iron, manganese and zinc) a lanthanum chloride concentration equivalent to 0,1 % La (w/v).

The trace element concentrations selected must lie within the range of sensitivity of the spectrophotometer used. The tables below show, by way of example, the compositions of typical ranges of calibration solutions; depending, however, on the type and sensitivity of spectrophotometer used it may be necessary to select other concentrations.

▼B

Iron

µg Fe/ml	0	0,5	1	2	3	4	5
ml working standard solution (3.7.1) (1 ml = 100 µg Fe)	0	0,5	1	2	3	4	5
ml HCl (3.2)	7	7	7	7	7	7	7
+ 10 ml of lanthanum chloride solution (3.11) and make up to 100 ml with water							

Copper

µg Cu/ml	0	0,1	0,2	0,4	0,6	0,8	1,0
ml working standard solution (3.8.1) (1 ml = 10 µg Cu)	0	1	2	4	6	8	10
ml HCl (3.2)	8	8	8	8	8	8	8

Manganese

µg Mn/ml	0	0,1	0,2	0,4	0,6	0,8	1,0
ml working standard solution (3.9.1) (1 ml = 10 µg Mn)	0	1	2	4	6	8	10
ml HCl (3.2)	7	7	7	7	7	7	7
+ 10 ml of lanthanum chloride solution (3.11) and make up to 100 ml with water							

Zinc

µg Zn/ml	0	0,05	0,1	0,2	0,4	0,6	0,8
ml working standard solution (3.10.1) (1 ml = 10 µg Zn)	0	0,5	1	2	4	6	8
ml HCl (3.2)	7	7	7	7	7	7	7
+ 10 ml of lanthanum chloride solution (3.11) and make up to 100 ml with water							

5.1.2.2. ***Preparation of solution for analysis***

For the determination of copper, the solution prepared from point 5.1.1 can normally be used directly. If necessary to bring its concentration within the range of the calibration solutions, an aliquot portion may be pipetted into a 100 ml volumetric flask and made up to the mark with 0,5 mol/litre hydrochloric acid (3.3).

For the determination of iron, manganese and zinc, pipette an aliquot portion of the solution prepared from point 5.1.1 into a 100 ml volumetric flask, add 10 ml of lanthanum chloride solution (3.11) and make up to the mark with 0,5 mol/litre hydrochloric acid (3.3) (see also point 8 ‘Observation’).

5.1.2.3. ***Blank experiment***

The blank experiment must include all the prescribed steps of the procedure except that the sample material is omitted. The calibration solution ‘0’ must not be used as the blank.

5.1.2.4. ***Measurement of the atomic absorption***

Measure the atomic absorption of the calibration solutions and of the solution to be analysed using an oxidising air-acetylene flame at the following wavelengths:

Fe: 248,3 nm

Cu: 324,8 nm

▼B

Mn: 279,5 nm

Zn: 213,8 nm

Carry out each measurement four times.

5.2. ***Mineral feed***

If the sample contains no organic matter, prior ashing is unnecessary. Proceed as described in point 5.1.1.1 starting from the second paragraph. Evaporation with hydrofluoric acid may be omitted.

6. **Calculation of results**

Using a calibration curve, calculate the trace element concentration in the solution to be analysed and express the result in milligrams of trace element per kilogram of sample (ppm).

7. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample by the same analyst shall not exceed:

— 5 mg/kg, in absolute value, for contents of the trace element concerned up to 50 mg/kg,

— 10 % of the higher result for contents of the trace element concerned from 50 and up to 100 mg/kg,

— 10 mg/kg, in absolute value, for contents of the trace element concerned from 100 and up to 200 mg/kg,

— 5 % of the higher result for contents of the trace element concerned above 200 mg/kg.

8. **Observation**

The presence of large quantities of phosphates may interfere with the determination of iron, manganese and zinc. Such interference must be corrected by addition of lanthanum chloride solution (3.11). If, however, in the sample the weight ratio Ca + Mg/P is > 2, addition of lanthanum chloride solution (3.11) to the solution for analysis and to the calibration solutions may be omitted.

D. DETERMINATION OF HALOFUGINONE

DL-trans-7-bromo-6-chloro-3- [3-(3-hydroxy-2-piperidyl)acetonyl]-quinazolin-4-(3H)-one hydrobromide

1. **Purpose and scope**

The method makes it possible to determine the level of halofuginone in feed. The limit of quantification is 1 mg/kg.

2. **Principle**

After treatment with hot water, halofuginone is extracted as the free base into ethyl acetate and subsequently partitioned as the hydrochloride into an aqueous acid solution. The extract is purified by ion-exchange chromatography. The content of halofuginone is determined by reversed-phase high performance liquid chromatography (HPLC) using an UV detector.

3. **Reagents**

3.1. Acetonitrile, equivalent to HPLC grade.

3.2. Amberlite XAD-2 resin.

3.3. Ammonium acetate.

3.4. Ethyl acetate.

3.5. Acetic acid, glacial.

▼B

3.6. Halofuginone standard substance (DL-trans-7-brome-6-chloro-3-[3-hydroxy-2-piperidyl)acetonyl] quinazoline-4-(3H)-one hydrobromide, E 764).

3.6.1. Halofuginone stock standard solution, 100 µg/ml

Weight to the nearest 0,1 mg, 50 mg of halofuginone (3.6) in a 500 ml graduated flask, dissolve in ammonium acetate buffer solution (3.18), make up to the mark with the buffer solution and mix. This solution is stable for three weeks at 5 °C if stored in the dark.

3.6.2. Calibration solutions

Into a series of 100 ml graduated flasks transfer 1,0, 2,0, 3,0, 4,0 and 6,0 ml of the stock standard solution (3.6.1). Make up to the mark with mobile phase (3.21) and mix. These solutions have concentrations of 1,0, 2,0, 3,0, 4,0 and 6,0 µg/ml of halofuginone respectively. These solutions must be freshly prepared before use.

3.7. Hydrochloric acid (ρ_{20} approximately 1,16 g/ml).

3.8. Methanol.

3.9. Silver nitrate.

3.10. Sodium ascorbate.

3.11. Sodium carbonate.

3.12. Sodium chloride.

3.13. EDTA (ethylenediaminetetraacetic acid, disodium salt).

3.14. Water, equivalent to HPLC grade.

3.15. Sodium carbonate solution, c = 10 g/100 ml.

3.16. Sodium chloride-saturated sodium carbonate solution, c = 5 g/100 ml.

Dissolve 50 g of sodium carbonate (3.11) in water, dilute to 1 litre and add sodium chloride (3.12) until the solution is saturated.

3.17. Hydrochloric acid, approximately 0,1 mol/l.

Dilute 10 ml of HCI (3.7) with water to 1 litre.

3.18. Ammonium acetate buffer solution, approximately 0,25 mol/l.

Dissolve 19,3 g of ammonium acetate (3.3) and 30 ml of acetic acid (3.5) in water (3.14) and dilute to 1 1itre.

3.19. Amberlite XAD-2 resin preparation.

Wash an appropriate quantity of Amberlite (3.2) with water until all chloride ions have been removed, as indicated by a silver nitrate (3.20) test performed on the discarded aqueous phase. Then wash the resin with 50 ml of methanol (3.8), discard the methanol and store the resin under fresh methanol.

3.20. Silver nitrate solution, approximately 0,1 mol/l.

Dissolve 0,17 g of silver nitrate (3.9) in 10 ml of water.

3.21. HPLC Mobile phase.

Mix 500 ml of acetonitrile (3.1) with 300 ml of ammonium acetate buffer solution (3.18) and 1 200 ml of water (3.14). Adjust the pH to 4,3 using acetic acid (3.5). Filter through a 0,22 µm filter (4.8) and degas the solution (e.g. by ultrasonification for 10 minutes). This solution is stable for one month, if stored in the dark in a closed container.

▼B

4. **Apparatus**

4.1. Ultrasonic bath

4.2. Rotary film evaporator

4.3. Centrifuge

4.4. HPLC equipment with variable wavelength ultraviolet detector or diode-array detector

4.4.1. Liquid chromatographic column, 300 mm x 4 mm, C_{18}, 10 µm packaging, or an equivalent column

4.5. Glass column (300 mm x 10 mm) fitted with a sintered-glass filter and a stopcock

4.6. Glass-fibre filters, diameter 150 mm

4.7. Membrane filters, 0,45 µm

4.8. Membrane filters, 0,22 µm

5. **Procedure**

Note: Halofuginone as the free base is unstable in alkaline and ethyl acetate solutions. It shall not remain in ethyl acetate for more than 30 minutes.

5.1. ***General***

5.1.1. A blank feed shall be analysed to check that neither halofuginone nor interfering substances are present.

5.1.2. A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of halofuginone, similar to that present in the sample. To fortify at a level of 3 mg/kg, add 300 µl of the stock standard solution (3.6.1) to 10 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Note: for the purpose of this method, the blank feed shall be similar in type to that of the sample and on analysis halofuginone shall not be detected.

5.2. ***Extraction***

Weigh to the nearest 0,1 g, 10 g of the prepared sample, into a 200 ml centrifuge tube, add 0,5 g of sodium ascorbate (3.10), 0,5 g of EDTA (3.13) and 20 ml of water and mix. Place the tube for 5 minutes in a water bath (80 °C). After cooling down to room temperature, add 20 ml of sodium carbonate solution (3.15) and mix. Add immediately 100 ml of ethyl acetate (3.4) and shake vigorously by hand for 15 seconds. Then place the tube for three minutes in the ultrasonic bath (4.1) and loosen the stopper. Centrifuge for two minutes and decant the ethyl acetate phase through a glass fibre filter (4.6), into a 500 ml separating funnel. Repeat the extraction of the sample with a second portion of 100 ml of ethyl acetate. Wash the combined extracts for one minute with 50 ml of sodium chloride saturated sodium carbonate solution (3.16) and discard the aqueous layer.

Extract the organic layer for 1 min. with 50 ml of hydrochloric acid (3.17). Run the lower acid layer into a 250 ml separating funnel. Re-extract the organic layer for 1,5 minutes with a further 50 ml of hydrochlorid acid and combine with the first extract. Wash the combined acid extracts by swirling for approximately 10 seconds with 10 ml of ethyl acetate (3.4).

▼B

Quantitatively transfer the aqueous layer into a 250 ml round-bottomed flask and discard the organic phase. Evaporate all the remaining ethyl acetate from the acid solution using a rotary film evaporator (4.2). The temperature of the water bath must not exceed 40 °C. Under a vacuum of approximately 25 mbar all of the residual ethyl acetate will be removed within 5 minutes at 38 °C.

5.3. ***Clean up***

5.3.1. Preparation of the Amberlite column

An XAD-2 column is prepared for each sample extract. Transfer 10 g of prepared Amberlite (3.19) into a glass column (4.5) with methanol (3.8). Add a small plug of glass-wool to the top of the resin bed. Drain the methanol from the column and wash the resin with 100 ml of water, stopping the flow as the liquid reaches the top of the resin bed. Allow the column to equilibrate for 10 minutes before use. Never allow the column to run dry.

5.3.2. Sample clean up

Transfer the extract (5.2) quantitatively to the top of the prepared Amberlite column (5.3.1) and elute, discarding the eluate. The rate of elution must not exceed 20 ml/min. Rinse the round-bottomed flask with 20 ml of hydrochlorid acid (3.17) and use this to wash the resin column. Blow through any remaining acid solution with a stream of air. Discard the washings. Add 100 ml of methanol (3.8) to the column and allow 5 to 10 ml to elute, collecting the eluate in a 250 ml round-bottomed flask. Leave the remaining methanol for 10 minutes to equilibrate with the resin and continue the elution at a rate not exceeding 20 ml/min. collecting the eluate in the same round-bottomed flask. Evaporate the methanol on the rotary film evaporator (4.2), the temperature of the water bath must not exceed 40 °C. Transfer the residue quantitatively into a 10 ml calibrated flask using the mobile phase (3.21). Make up to the mark with mobile phase and mix. An aliquot is filtered through a membrane filter (4.7). Reserve this solution for the HPLC determination (5.4).

5.4. ***HPLC determination***

5.4.1. Parameters

The following conditions are offered for guidance, other conditions may be used provided they yield equivalent results.

Liquid chromatographic column (4.4.1)

HPLC Mobile phase (3.21)

Flow rate: 1,5 to 2 ml/min.

Detection wavelength: 243 nm

Injection volume: 40 to 100 µl.

Check the stability of the chromatographic system, injecting the calibration solution (3.6.2) containing 3,0 µg/ml several times, until constant peak heights (or areas) and retention times are achieved.

5.4.2. Calibration graph

Inject each calibration solution (3.6.2) several times and measure the peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights or areas of the calibration solutions as the ordinates and the corresponding concentrations in µg/ml as the abscissae.

▼B

5.4.3. Sample solution

Inject the sample extract (5.3.2) several times, using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the halofuginone peaks.

6. **Calculation of results**

Determine the concentration of the sample solution in µg/ml, from the mean height (area) of the halofuginone peaks of the sample solution by reference to the calibration graph (5.4.2).

The content of halofuginone w (mg/kg) of the sample is given by the following formula:

$$w = \frac{c \times 10}{m}$$

in which:

c = halofuginone concentration of the sample solution in µg/ml,
m = weight of the test portion in grams.

7. **Validation of the results**

7.1. ***Identity***

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract and the calibration solution (3.6.2) containing 6,0 µg/ml are compared.

7.1.1. Co-chromatography

A sample extract is fortified by addition of an appropriate amount of a calibration solution (3.6.2). The amount of added halofuginone must be similar to the estimated amount of halofuginone found in the sample extract.

Only the height of the halofuginone peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within ± 10 % of the original width.

7.1.2. Diode-array detection

The results are evaluated according to the following criteria:

(a) the wavelength of maximum absorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within ± 2 nm;

(b) between 225 and 300 nm, the sample and standard spectra recorded at the peak apex on the chromatogram, must not be different for those parts of the spectrum within the range 10 % to 100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte;

(c) between 225 and 300 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 % to 100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the apex.

▼B

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. ***Repeatability***

The difference between results of two parallel determinations carried out on the same sample must not exceed 0,5 mg/kg for halofuginone contents up to 3 mg/kg.

7.3. ***Recovery***

For the fortified blank sample the recovery shall be at least 80 %.

8. **Results of a collaborative study**

A collaborative study ([1]) was arranged in which three samples were analysed by eight laboratories.

Results

	Sample A (blank) On receipt	Sample B (Meal)		Sample C (Pellets)	
		On receipt	After two months	On receipt	After two months
Mean [mg/kg]	ND	2,80	2,42	2,89	2,45
S_R [mg/kg]	—	0,45	0,43	0,40	0,42
CV_R [%]	—	16	18	14	17
Rec. [%]		86	74	88	75

ND = not detected
S_R = standard deviation of reproducibility
CV_R = coefficient of variation of reproducibility (%)
Rec. = recovery (%)

E. DETERMINATION OF ROBENIDINE

1,3-bis [(4-chlorobenzylidene)amino]guanidine — hydrochloride

1. **Purpose and scope**

This method makes it possible to determine the levels of robenidine in feed. The limit of quantification is 5 mg/kg.

2. **Principle**

The sample is extracted with acidified methanol. The extract is dried and an aliquot portion subjected to a clean-up on an aluminium oxide column. Robenidine is eluted from the column with methanol, concentrated, and made up to a suitable volume with mobile phase. The content of robenidine is determined by reversed-phase high-performance liquid chromatography (HPLC) using an UV detector.

3. **Reagents**

3.1. Methanol.

3.2. Acidified methanol.

Transfer 4,0 ml hydrochloric acid (ρ20 = 1,18 g/ml) into a 500 ml graduated flask, make up to the mark with methanol (3.1) and mix. This solution shall be freshly prepared before use.

([1]) The Analyst 108, 1983, pp. 1252 to 1256.

▼B

3.3. Acetonitrile, equivalent to HPLC grade.

3.4. Molecular sieve.

Type 3A, 8 to 12 mesh beads (1,6-2,5 mm beads, crystalline alumino-silicate, diameter of pores 0,3 mm).

3.5. Aluminium oxide acidic activity grade I for column chromatography.

Transfer 100 g aluminium oxide into a suitable container and add 2,0 ml of water. Stopper and shake for approximately 20 minutes. Store in a well stoppered container.

3.6. Potassium dihydrogen phosphate solution, c = 0,025 mol/l.

Dissolve 3,40 g of potassium dihydrogen phosphate in water (HPLC grade) in a 1 000 ml graduated flask, make up to the mark and mix.

3.7. Di-sodium hydrogen phosphate solution, c = 0,025 mol/l.

Dissolve 3,55 g of anhydrous (or 4,45 g of dihydrate or 8,95 g of dodecahydrate) di-sodium hydrogen phosphate in water (equivalent to HPLC grade) in a 1 litre graduated flask, make up to the mark and mix.

3.8. HPLC mobile phase.

Mix together the following reagents:

650 ml acetonitrile (3.3),

250 ml water (equivalent to HPLC-grade),

50 ml potassium di-hydrogen phosphate solution (3.6),

50 ml di-sodium hydrogen phosphate solution (3.7).

Filter through a 0,22 µm filter (4.6) and degas the solution, (e.g. by ultrasonification for 10 minutes).

3.9. Standard substance.

Pure robenidine: 1,3-bis [(4-chlorobenzylidene)amino]guanidine — hydrochloride.

3.9.1. Robenidine stock standard solution: 300 µg/ml

Weigh to the nearest 0,1 mg, 30 mg of robenidine standard substance (3.9). Dissolve in acidified methanol (3.2) in a 100 ml graduated flask, make up to the mark with the same solvent and mix. Wrap the flask with aluminium foil and store in a dark place.

3.9.2. Robenidine intermediate standard solution: 12 µg/ml

Transfer 10,0 ml of the stock standard solution (3.9.1) into a 250 ml graduated flask, make up to the mark with the mobile phase (3.8) and mix. Wrap the flask with aluminium foil and store in a dark place.

3.9.3. Calibration solutions

Into a series of 50 ml calibrated flasks, transfer 5,0, 10,0, 15,0, 20,0 and 25,0 ml of the intermediate standard solution (3.9.2). Make up to the mark with mobile phase (3.8) and mix. These solutions correspond to 1,2, 2,4, 3,6, 4,8 and 6,0 µg/ml of robenidine respectively. These solutions must be freshly prepared before use.

3.10. Water equivalent to HPLC grade.

▼B

4. **Apparatus**

4.1. Glass column.

Constructed of amber glass fitted with a stopcock and a reservoir of approximately 150 ml capacity, internal diameter 10 to 15 mm, length 250 mm.

4.2. Mechanical shaker or magnetic stirrer.

4.3. Rotary film evaporator.

4.4. HPLC equipment with variable wavelength ultraviolet detector or diode array detector operating in the range of 250 to 400 nm.

4.4.1. Liquid chromatographic column: 300 mm x 4 mm, C_{18} 10 µm packing or equivalent.

4.5. Glass fibre filter paper (Whatman GF/A or equivalent).

4.6. Membrane filters, 0,22 µm.

4.7. Membrane filters, 0,45 µm.

5. **Procedure**

Note: Robenidine is light-sensitive. Amber glassware shall be used in all operations.

5.1. ***General***

5.1.1. A blank feed shall be analysed to check that neither robenidine nor interfering substances are present.

5.1.2. A recovery test shall be carried out by analysing the blank feed (5.1.1) which has been fortified by addition of a quantity of robenidine, similar to that present in the sample. To fortify at a level of 60 mg/kg, transfer 3,0 ml of the stock standard solution (3.9.1) to a 250 ml conical flask. Evaporate the solution to ca. 0,5 ml in a stream of nitrogen. Add 15 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Note: For the purpose of this method, the blank feed shall be similar in type to that of the sample and on analysis robenidine shall not be detected.

5.2. ***Extraction***

Weigh to the nearest 0,01 g, approximately 15 g of the prepared sample. Transfer to a 250 ml conical flask and add 100,0 ml of acidified methanol (3.2), stopper and shake for one hour on the shaker (4.2). Filter the solution through a glass fibre filter paper (4.5) and collect the whole filtrate in a 150 ml conical flask. Add 7,5 g molecular sieve (3.4), stopper and shake for five minutes. Filter immediately through a glass-fibre filter paper. Retain this solution for the purification step (5.3).

5.3. ***Purification***

5.3.1. Preparation of the aluminium-oxide column

Insert a small glass-wool plug into the lower end of a glass column (4.1) and tamp it down using a glass rod. Weigh out 11,0 g of the prepared aluminium oxide (3.5) and transfer to the column. Care shall be taken to minimise the exposure to the atmosphere during this stage. Gently tap the loaded column at its lower end to settle the aluminium oxide.

▼B

5.3.2. Sample purification

Transfer onto the column by pipette 5,0 ml of the sample extract prepared in (5.2) Rest the pipette tip close to the column wall and allow the solution to be absorbed onto the aluminium oxide. Elute the robenidine from the column using 100 ml methanol (3.1), at a flow rate of 2 to 3 ml/minute and collect the eluate in a 250 ml round bottomed flask. Evaporate the methanol solution to dryness under reduced pressure at 40 °C by means of a rotary film evaporator (4.3). Re-dissolve the residue in 3 to 4 ml of mobile phase (3.8) and transfer quantitatively to a 10 ml graduated flask. Rinse the flask with several 1 to 2 ml portions of mobile phase and transfer these rinsings to the graduated flask. Make up to the mark with the same solvent and mix. An aliquot is filtered through a 0,45 µm membrane filter (4.7). Reserve this solution for HPLC determination (5.4).

5.4. ***HPLC determination***

5.4.1. Parameters

The following conditions are offered for guidance, other conditions may be used provided they yield equivalent results:

Liquid chromatographic column (4.4.1),

HPLC mobile phase (3.8),

Flow rate: 1,5 to 2 ml/minute,

Detector wavelength: 317 nm,

Injection volume: 20 to 50 µl.

Check the stability of the chromatographic system, injecting the calibration solution (3.9.3) containing 3,6 µg/ml several times, until constant peak heights and retention times are achieved.

5.4.2. Calibration graph

Inject each calibration solution (3.9.3) several times and measure the peak heights (areas) for each concentration. Plot a calibration curve using the mean peak heights or areas of the calibration solutions as the ordinates and corresponding concentrations in µg per ml as abscissae.

5.4.3. Sample solution

Inject the sample extract (5.3.2) several times, using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the robenidine peaks.

6. **Calculation of results**

From the mean height (area) of the robenidine peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.4.2).

The content of robenidine w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times 200}{m}$$

in which:

c = robenidine concentration of the sample solution in µg/ml,
m = weight of the test portion in grams.

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7. **Validation of the results**

7.1. ***Identity***

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract and the calibration solution (3.9.3) containing 6 µg/ml are compared.

7.1.1. Co-chromatography

A sample extract is fortified by addition of an appropriate amount of calibration solution (3.9.3). The amount of added robenidine must be similar to the estimated amount of robenidine found in the sample extract.

Only the height of the robenidine peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within approximately 10 % of the original width.

7.1.2. Diode-array detection

The results are evaluated according to the following criteria:

(a) the wavelength of maximum absorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within approximately 2 nm;

(b) between 250 and 400 nm, the sample and standard spectra recorded at the peak apex on the chromatogram, must not be different for those parts of the spectrum within the range 10 % to 100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte;

(c) between 250 and 400 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 % to 100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed 10 % of the higher result for robenidine content higher than 15 mg/kg.

7.3. ***Recovery***

For a fortified blank sample the recovery shall be at least 85 %.

8. **Results of a collaborative study**

An EC collaborative study was arranged in which four samples of poultry and rabbit feed, in meal or pelleted form were analysed by 12 laboratories. Duplicate analyses were performed on each sample. The results are given in the table below:

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	Poultry		Rabbit	
	Meal	Pellet	Meal	Pellet
Mean [mg/kg]	27,00	27,99	43,6	40,1
s_r [mg/kg]	1,46	1,26	1,44	1,66
CV_r [%]	5,4	4,5	3,3	4,1
S_R [mg/kg]	4,36	3,36	4,61	3,91
CV_R [%]	16,1	12,0	10,6	9,7
Recovery [%]	90,0	93,3	87,2	80,2

s_r = standard deviation of repeatability,
CV_r = coefficient of variation of repeatability, %
S_R = standard deviation of reproducibility,
CV_R = coefficient of variation of reproducibility. %

F. DETERMINATION OF DICLAZURIL

(+)-4-chlorphenyl [2,6-dichloro-4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl)phenyl] acetonitrile

1. **Purpose and scope**

The method makes it possible to determine the level of diclazuril in feed and premixtures. The limit of detection is 0,1 mg/kg, the limit of quantification is 0,5 mg/kg.

2. **Principle**

After addition of an internal standard, the sample is extracted with acidified methanol. For feed, an aliquot of the extract is purified on a C_{18} solid phase extraction cartridge. Diclazuril is eluted from the cartridge with a mixture of acidified methanol and water. After evaporation, the residue is dissolved in DMF/water. For premixtures, the extract is evaporated and the residue is dissolved in DMF/water. The content of diclazuril is determined by ternary gradient reversed-phase high-performance liquid chromatography (HPLC) using a UV detector.

3. **Reagents**

3.1. Water, equivalent to HPLC-grade

3.2. Ammonium acetate

3.3. Tetrabutylammonium hydrogen sulphate (TBHS)

3.4. Acetonitrile, equivalent to HPLC grade

3.5. Methanol, equivalent to HPLC grade

3.6. N, N-dimethylformamide (DMF)

3.7. Hydrochloric acid, ρ_{20} = 1,19 g/ml

3.8. Standard substance: diclazuril II-24: (+)-4-chlorphenyl [2,6-dichloro-4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl) phenyl] acetonitrile with guaranteed purity, E771

3.8.1. Diclazuril stock standard solution, 500 µg/ml

Weigh to the nearest 0,1 mg, 25 mg of diclazuril standard substance (3.8) in a 50 ml graduated flask. Dissolve in DMF (3.6), make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

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3.8.2. Diclazuril standard solution, 50 µg/ml

Transfer 5,00 ml of the stock standard solution (3.8.1) into a 50 ml graduated flask, make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9. Internal standard substance: 2,6 dichloro-α-(4-chlorophenyl)-4-(4,5 dihydro-3,5-dioxo-1,2,4-triazine-2 (3H) — yl) α-methylbenzene-acetonitrile

3.9.1. Internal stock standard solution, 500 µg/ml

Weigh to the nearest 0,1 mg 25 mg of internal standard substance (3.9) in a 50 ml graduated flask. Dissolve in DMF (3.6), make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9.2. Internal standard solution, 50 µg/ml

Transfer 5,00 ml of the internal stock standard solution (3.9.1) into a 50 ml graduated flask, make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9.3. Internal standard solution for premixtures, p/1000 mg/ml

(p = nominal content of diclazuril in the premixture in mg/kg)

Weigh to the nearest 0,1 mg p/10 mg of the internal standard substance in a 100 ml graduated flask, dissolve in DMF (3.6) in a ultrasonic bath (4.6), make up to the mark with DMF and mix. Wrap the flask with aluminium foil or use amber flask and store in a refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.10. Calibration solution, 2 µg/ml.

Pipet 2,00 ml diclazuril standard solution (3.8.2) and 2,00 ml internal standard solution (3.9.2) into a 50 ml graduated flask. Add 16 ml DMF (3.6), make up to the mark with water and mix. This solution must be prepared freshly before use.

3.11. C_{18} solid phase extraction cartridge, e.g. Bond Elut, size: 1 cc, sorbent weight: 100 mg.

3.12. Extraction solvent: acidified methanol.

Pipet 5,0 ml hydrochloric acid (3.7) into 1 000 ml of methanol (3.5), and mix.

3.13. Mobile phase for HPLC

3.13.1. Eluent A: ammonium acetate — tetrabutylammonium hydrogen sulphate solution.

Dissolve 5 g ammonium acetate (3.2) and 3,4 g TBHS (3.3) in 1 000 ml water (3.1) and mix.

3.13.2. Eluent B: acetonitrile (3.4).

3.13.3. Eluent C: methanol (3.5).

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4. **Apparatus**

4.1. Mechanical shaker

4.2. Equipment for ternary gradient HPLC

4.2.1. Liquid chromatographic column, Hypersil ODS, 3 μm packing, 100 mm x 4,6 mm, or equivalent

4.2.2. UV detector with variable wavelength adjustment or diode array detector

4.3. Rotary film evaporator

4.4. Membrane filter, 0,45 μm

4.5. Vacuum manifold

4.6. Ultrasonic bath

5. **Procedure**

5.1. ***General***

5.1.1. Blank feed

A blank feed shall be analysed to check that neither diclazuril nor interfering substances are present. The blank feed shall be similar in type to that of the sample and on analysis diclazuril or interfering substances shall not be detected.

5.1.2. Recovery test

A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of diclazuril similar to that present in the sample. To fortify at a level of 1 mg/kg add 0,1 ml of the stock standard solution (3.8.1) to 50 g of a blank feed, mix thoroughly and leave for 10 min. mixing again several times before proceeding (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample to be analysed is fortified with a quantity of diclazuril, similar to that already present in the sample. This sample is analysed, together with the unfortified sample and the recovery can be calculated by subtraction.

5.2. ***Extraction***

5.2.1. Feed

Weigh to the nearest 0,01 g approximately 50 g of the sample. Transfer to a 500 ml conical flask, add 1,00 ml internal standard solution (3.9.2), 200 ml extraction solvent (3.12) and stopper the flask. Shake the mixture on the shaker (4.1) overnight. Allow to settle for 10 minutes. Transfer a 20 ml aliquot of the supernatant to a suitable glass container and dilute with 20 ml water. Transfer this solution on an extraction cartridge (3.11), and pass through by applying vacuum (4.5). Wash the cartridge with 25 ml of a mixture of extraction solvent (3.12) and water, 65 + 35 (V + V). Discard the collected fractions and elute the compounds with 25 ml of a mixture of extraction solvent (3.12) and water, 80 + 20 (V + V). Evaporate this fraction until it had just reached dryness by means of the rotary evaporator (4.3) at 60 ^{o}C. Dissolve the residue in 1,0 ml DMF (3.6), add 1,5 ml of water (3.1) and mix. Filter through a membrane filter (4.4). Proceed to the HPLC determination (5.3).

5.2.2. Premixtures

Weigh to the nearest 0,001 g approximately 1 g of the sample. Transfer to a 500 ml conical flask, add 1,00 ml internal standard solution (3.9.3), 200 ml extraction solvent (3.12) and stopper the flask. Shake the mixture overnight on the shaker (4.1). Allow to settle for 10 minutes. Transfer an

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aliquot of 10 000/p ml (p = nominal content of diclazuril in the premix in mg/kg) of the supernatant to a round bottomed flask of suitable size. Evaporate until it had just reached dryness, under reduced pressure at 60 °C by means of the rotary evaporator (4.3). Redissolve the residue in 10,0ml DMF (3.6), add 15,0 ml water (3.1) and mix. Proceed to the HPLC determination (5.3).

5.3. ***HPLC determination***

5.3.1. Parameters

The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results.

Liquid chromatographic column (4.2.1)	100 mm × 4,6 mm, Hypersil ODS, 3 µm packing, or equivalent	
Mobile phase:	Eluent A (3.13.1):	Aqueous solution of ammonium acetate and tetrabutyl-ammonium hydrogen sulphate
	Eluent B (3.13.2):	acetonitrile
	Eluent C (3.13.3):	methanol
Elution mode:	— linear gradient — initial conditions: A + B + C = 60 + 20 + 20 (V + V + V) — after 10 min. gradient elution during 30 min. to: A + B + C = 45 + 20 + 35 (V + V + V) Flush with B during 10 min.	
Flow rate:	1,5-2 ml/min.	
Injection volume:	20 µl	
Detector wavelength:	280 nm.	

Check the stability of the chromatographic system, injecting several times the calibration solution (3.10), containing 2 µg/ml, until constant peak heights and retention times are achieved.

5.3.2. Calibration solution

Inject 20 µl of the calibration solution (3.10) several times and determine the mean peak height (area) of the diclazuril and internal standard peaks.

5.3.3. Sample solution

Inject 20 µl of the sample solution (5.2.1 or 5.2.2) several times and determine the mean peak height (area) of the diclazuril and internal standard peaks.

6. **Calculation of the results**

6.1. ***Feeds***

The diclazuril content w (mg/kg) in the sample is given by the following formula:

$$w = \frac{h_{d,s} \times h_{i,c}}{h_{i,s} \times h_{d,c}} \times \frac{c_{d,c} \times 10\ V}{m}\ [mg/kg]$$

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where:

$h_{d,s}$ = peak height (area) of diclazuril in the sample solution (5.2.1)
$h_{i,s}$ = peak height (area) of the internal standard in the sample solution (5.2.1)
$h_{d,c}$ = peak height (area) of diclazuril in the calibration solution (3.10)
$h_{i,c}$ = peak height (area) of the internal standard in the calibration solution (3.10)
$c_{d,c}$ = diclazuril concentration in the calibration solution in µg/ml (3.10)
m = weight of the test portion in g
V = volume of the sample extract according to 5.2.1 (i.e. 2,5 ml)

6.2. ***Premixtures***

The diclazuril content w (mg/kg) in the sample is given by the following formula:

$$w = \frac{h_{d,s} \times h_{i,c}}{h_{i,s} \times h_{d,c}} \times \frac{c_{d,c} \times 0{,}02\ V \times p}{m}\ [mg/kg]$$

where:

$h_{d,c}$ = peak height (area) of diclazuril in the calibration solution (3.10)
$h_{i,c}$ = peak height (area) of the internal standard in the calibration solution (3.10)
$h_{d,s}$ = peak height (area) of diclazuril in the sample solution (5.2.2)
$h_{i,s}$ = peak height (area) of the internal standard in the sample solution (5.2.2)
$c_{d,c}$ = diclazuril concentration in the calibration solution in µg/ml (3.10)
m = weight of the test portion in g
V = volume of the sample extract according to 5.2.2 (i.e. 25 ml)
p = nominal content of diclazuril in mg/kg in the premixture

7. **Validation of the results**

7.1. ***Identity***

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract (5.2.1 or 5.2.2) and the calibration solution (3.10) are compared.

7.1.1. Co-chromatography

A sample extract (5.2.1 or 5.2.2) is fortified by addition of an appropriate amount of calibration solution (3.10). The amount of added diclazuril must be similar to the amount of diclazuril found in the sample extract.

Only the height of the diclazuril peak and the internal standard peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its height, must be within ± 10 % of the original width of the diclazuril peak or the internal standard peak of the unfortified sample extract.

7.1.2. Diode-array detection

The results are evaluated according to the following criteria:

(a) The wavelength of maximum absorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be the same within a margin determined by the resolving power of the detection system. For diode-array detection this is typically within ± 2 nm.

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(b) Between 230 and 320 nm, the sample and standard spectra recorded at the peak apex of the chromatogram, must not be different for those parts of the spectrum within the range 10 % 100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte.

(c) Between 230 and 320 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 % 100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the peak apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

— 30 % relative, to the higher value for diclazuril contents from 0,5 mg/kg to 2,5 mg/kg,

— 0,75 mg/kg for diclazuril contents between 2,5 mg/kg and 5 mg/kg,

— 15 % relative to the higher value for diclazuril contents of more than 5 mg/kg.

7.3. ***Recovery***

For a fortified (blank) sample the recovery shall be at least 80 %.

8. **Results of a collaborative study**

A collaborative study was arranged in which 5 samples were analysed by 11 laboratories. These samples consisted of two premixtures; one was mixed with an organic matrix (O 100) and the other with an inorganic matrix (A 100). The theoretical content is 100 mg diclazuril per kg. The three mixed feeds for poultry were made by 3 different producers (NL) (L1/Z1/K1). The theoretical content is 1 mg diclazuril per kg. The laboratories were instructed to analyse each of the samples once or in duplicate. (More detailed information on this collaborative study can be found in the ***Journal of AOAC International, Volume 77, No 6, 1994, p. 1359-1361)***. The results are given in the following table.

	Sample 1 A 100	Sample 2 O 100	Sample 3 L1	Sample 4 Z1	Sample 5 K1
L	11	11	11	11	6
n	19	18	19	19	12
Mean	100,8	103,5	0,89	1,15	0,89
S_r (mg/kg)	5,88	7,64	0,15	0,02	0,03
CV_r (%)	5,83	7,38	17,32	1,92	3,34
S_R (mg/kg)	7,59	7,64	0,17	0,11	0,12
CV_R (%)	7,53	7,38	18,61	9,67	13,65
Nominal content (mg/kg)	100	100	1	1	1

L = number of laboratories
n = number of single values
S_r = standard deviation of repeatability
CV_r = coefficient of variation of repeatability

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S_R = standard deviation of reproducibility

CV_R = coefficient of variation of reproducibility

9. **Observations**

The diclazuril response must have been previously demonstrated to be linear over the range of concentrations being measured.

G. DETERMINATION OF LASALOCID SODIUM

Sodium salt of a polyether monocarboxylic acid produced by Streptomyces lasaliensis

1. **Purpose and scope**

The method makes it possible to determine the level of lasalocid sodium in feed and premixtures. The limit of detection is 5 mg/kg, the limit of quantification is 10 mg/kg.

2. **Principle**

Lasalocid sodium is extracted from the sample into acidified methanol and determined by reversed-phase high performance liquid chromatography (HPLC) using a spectrofluorometric detector.

3. **Reagents**

3.1. Potassium dihydrogen phosphate (KH_2PO_4).

3.2. Orthophosphoric acid, w (w/w) = 85 %.

3.3. Orthophosphoric acid solution, c = 20 %.

Dilute 23,5 ml of orthophosphoric acid (3.2) to 100 ml with water.

3.4. 6-Methyl-2-heptylamine (1,5-dimethylhexylamine), w (w/w) = 99 %.

3.5. Methanol, equivalent to HPLC grade.

3.6. Hydrochloric acid, density = 1,19 g/ml.

3.7. Phosphate buffer solution, c = 0,01 mol/l.

Dissolve 1,36 g of KH_2PO_4 (3.1) in 500 ml of water (3.11), add 3,5 ml of orthophosphoric acid (3.2) and 10,0 ml of 6-methyl-2-heptylamine (3.4). Adjust the pH to 4,0 with orthophosphoric acid solution (3.3) and dilute to 1 000 ml with water (3.11).

3.8. Acidified methanol.

Transfer 5,0 ml of hydrochloric acid (3.6) into a 1 000 ml graduated flask, make up to the mark with methanol (3.5) and mix. This solution must be prepared freshly before use.

3.9. HPLC mobile phase, phosphate buffer-methanol solution 5 + 95 (V + V).

Mix 5 ml of phosphate buffer solution (3.7) with 95 ml of methanol (3.5).

3.10. Lasalocid sodium standard substance with guaranteed purity, $C_{34}H_{53}O_8Na$ (sodium salt of a polyether monocarboxylic acid produced by ***Streptomyces lasaliensis***), E763.

3.10.1. Lasalocid sodium stock standard solution, 500 µg/ml

Weigh to the nearest 0,1 mg, 50 mg of lasalocid sodium (3.10) into a 100 ml graduated flask, dissolve in acidified methanol (3.8), make up to the mark with the same solvent and mix. This solution must be freshly prepared before use.

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3.10.2. Lasalocid sodium intermediate standard solution, 50 µg/ml

Pipette 10,0 ml of stock standard solution (3.10.1) into a 100 ml graduated flask, make up to the mark with acidified methanol (3.8) and mix. This solution must be prepared freshly before use.

3.10.3. Calibration solutions

Into a series of 50 ml graduated flasks transfer 1,0, 2,0, 4,0, 5,0 and 10,0 ml of the intermediate standard solution (3.10.2). Make up to the mark with acidified methanol (3.8) and mix. These solutions correspond to 1,0, 2,0, 4,0, 5,0 and 10,0 µg of lasalocid sodium per ml respectively. These solutions must be prepared freshly before use.

3.11. Water, equivalent to HPLC grade.

4. **Apparatus**

4.1. Ultrasonic bath (or shaking water-bath) with temperature control.

4.2. Membrane filters, 0,45 µm.

4.3. HPLC equipment with injection system, suitable for injecting volumes of 20 µl.

4.3.1. Liquid chromatographic column 125 mm x 4 mm, reversed-phase C_{18}, 5 µm packing or equivalent.

4.3.2. Spectrofluorometer with variable wavelength adjustment of excitation and emission wavelengths.

5. **Procedure**

5.1. ***General***

5.1.1. Blank feed

For the performance of the recovery test (5.1.2) a blank feed shall be analysed to check that neither lasalocid sodium nor interfering substances are present. The blank feed shall be similar in type to that of the sample and lasalocid sodium or interfering substances shall not be detected.

5.1.2. Recovery test

A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of lasalocid sodium, similar to that present in the sample. To fortify at a level of 100 mg/kg, transfer 10,0 ml of the stock standard (3.10.1) to a 250 ml conical flask and evaporate the solution to approximately 0,5 ml. Add 50 g of the blank feed, mix thoroughly and leave for 10 minutes mixing again several times before proceeding with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case the sample to be analysed is fortified with a quantity of lasalocid sodium similar to that already present in the sample. This sample is analysed together with the unfortified sample and the recovery calculated by subtraction.

5.2. ***Extraction***

5.2.1. Feed

Weigh to the nearest 0,01 g, from 5 g to 10 g of the sample into a 250 ml conical flask with stopper. Add 100,0 ml of acidified methanol (3.8) by pipette. Stopper loosely and swirl to disperse. Place the flask in an ultrasonic bath (4.1) at approximately 40 °C for 20 minutes, then remove and cool to room temperature. Allow to stand for about 1 hour until the

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suspended matter has settled, then filter an aliquot portion through a 0,45 μm membrane filter (4.2) into a suitable vessel. Proceed to the HPLC determination (5.3).

5.2.2. Premixtures

Weigh to the nearest 0,001 g about 2 g of the unground premix into a 250 ml graduated flask. Add 100,0 ml of acidified methanol (3.8) and swirl to disperse. Place the flask and contents in an ultrasonic bath (4.1) at approximately 40 °C for 20 minutes, then remove and cool to room temperature. Dilute to the mark with acidified methanol (3.8) and mix thoroughly. Allow to stand for 1 hour until the suspended matter has settled, then filter an aliquot portion through a 0,45 μm membrane filter (4.2). Dilute an appropriate volume of the clear filtrate with acidified methanol (3.8) to produce a final test solution containing about 4 μg/ml of lasalocid sodium. Proceed to the HPLC determination (5.3).

5.3. ***HPLC determination***

5.3.1. Parameters

The following conditions are offered for guidance; other conditions may be used, provided they yield equivalent results:

Liquid chromatographic column (4.3.1):	125 mm × 4 mm, reversed-phase C_{18}, 5 μm packing or equivalent
Mobile phase (3.9):	Mixture of phosphate buffer solution (3.7) and methanol (3.5), 5+95 (V+V)
Flow rate:	1,2 ml/min.
Detection wavelengths:	
Excitation:	310 nm
Emission:	419 nm
Injection volume:	20 μl

Check the stability of the chromatographic system, injecting the calibration solution (3.10.3) containing 4,0 μg/ml several times, until constant peak heights (or areas) and retention times are achieved.

5.3.2. Calibration graph

Inject each calibration solution (3.10.3) several times and determine the mean peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights (areas) as the ordinates and the corresponding concentrations in μg/ml as the abscissae.

5.3.3. Sample solution

Inject the sample extracts obtained in 5.2.1 or 5.2.2 several times, using the same volume as taken for the calibration solution and determine the mean peak heights (areas) of the lasalocid sodium peaks.

6. **Calculation of results**

From the mean peak height (area) produced by injection of the sample solution (5.3.3) determine the concentration of lasalocid sodium (μg/ml) by reference to the calibration graph.

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6.1. ***Feed***

The lasalocid sodium content, w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times V_1}{m} \text{ [mg/kg]}$$

where:

c = lasalocid sodium concentration of the sample solution (5.2.1) in µg/ml
V_1 = volume of the sample extract according to 5.2.1 in ml (i.e. 100)
m = weight of the test portion in g

6.2. ***Premixtures***

The lasalocid sodium content, w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times V_2 \times f}{m} \text{ [mg/kg]}$$

where:

c = lasalocid sodium concentration of the sample solution (5.2.2) in µg/ml
V_2 = volume of the sample extract according to 5.2.2 in ml (i.e. 250)
f = dilution factor according to 5.2.2
m = weight of the test portion in g

7. **Validation of the results**

7.1. ***Identity***

Methods based on spectrofluorometry are less subject to interference than those in which UV detection is used. The identity of the analyte can be confirmed by co-chromatography.

7.1.1. Co-chromatography

A sample extract (5.2.1 or 5.2.2) is fortified by the addition of an appropriate amount of a calibration solution (3.10.3). The amount of added lasalocid sodium must be similar to the amount of lasalocid sodium found in the sample extract. Only the height of the lasalocid sodium peak shall be enhanced after taking into account the amount of lasalocid sodium added and the dilution of the extract. The peak width, at half height, must be within ± 10 % of the original peak width produced by the unfortified sample extract.

7.2. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

— 15 % relative to the higher value for lasalocid sodium contents from 30 mg/kg to 100 mg/kg,

— 15 mg/kg for lasalocid sodium contents from 100 mg/kg to 200 mg/kg,

— 7,5 % relative to the higher value for lasalocid sodium contents of more than 200 mg/kg.

7.3. ***Recovery***

For the fortified (blank) feed sample, the recovery shall be at least 80 %. For the fortified premixture samples, the recovery shall be at least 90 %.

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8. **Results of a collaborative study**

A collaborative study (*) was arranged in which 2 premixtures (samples 1 and 2) and 5 feeds (samples 3-7) were analysed by 12 laboratories. Duplicate analyses were performed on each sample. The results are given in the following table:

	Sample 1 Chicken premix	Sample 2 Turkey premix	Sample 3 Turkey pellets	Sample 4 Chicken crumbs	Sample 5 Turkey Feed	Sample 6 Poultry Feed A	Sample 7 Poultry Feed B
L	12	12	12	12	12	12	12
n	23	23	23	23	23	23	23
Mean [mg/kg]	5 050	16 200	76,5	78,4	92,9	48,3	32,6
s_r [mg/kg]	107	408	1,71	2,23	2,27	1,93	1,75
CV_r [%]	2,12	2,52	2,24	2,84	2,44	4,00	5,37
s_R [mg/kg]	286	883	3,85	7,32	5,29	3,47	3,49
CV_R [%]	5,66	5,45	5,03	9,34	5,69	7,18	10,70
Nominal content [mg/kg]	5 000 (*)	16 000 (*)	80 (*)	105 (*)	120 (*)	50 (**)	35 (**)

(*) Content declared by manufacturer.
(**) Feed prepared in the laboratory.

L = number of laboratories
n = number of single results
s_r = standard deviation of repeatability
s_R = standard deviation of reproducibility
CV_r = coefficient of variation of repeatability, %
CV_R = coefficient of variation of reproducibility, %.

(*) ***Analyst***, 1995, 120, 2175-2180.

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ANNEX V

METHODS OF ANALYSIS TO CONTROL UNDESIRABLE SUBSTANCES IN FEED

A. DETERMINATION OF FREE AND TOTAL GOSSYPOL

1. **Purpose and scope**

This method makes it possible to determine the levels of free gossypol, total gossypol and chemically related substances in cottonseed, cottonseed meal and cottonseed cake and in compound feed containing these feed materials where more than 20 mg/kg of free gossypol, total gossypol and chemically related substances are present.

2. **Principle**

The gossypol is extracted in the presence of 3-aminopropan-1-ol, either with a mixture of propan-2-ol and hexane, for the determination of free gossypol, or with dimethylformamide, for the determination of total gossypol. The gossypol is converted by aniline into gossypol-dianiline, the optical density of which is measured at 440 nm.

3. **Reagents**

3.1. Propan-2-ol-hexane mixture: mix 60 parts by volume of propan-2-ol with 40 parts by volume of ***n***-hexane.

3.2. Solvent A: Place in a 1 litre graduated flask approximately 500 ml of propan-2-ol-hexane mixture (3.1), 2 ml of 3-aminopropan-1-ol, 8 ml of glacial acetic acid and 50 ml of water. Make up to volume with the propan-2-ol-hexane mixture (3.1). This reagent is stable for one week.

3.3. Solvent B: Pipette 2 ml of 3-aminopropan-1-ol and 10 ml of glacial acetic acid into a 100 ml graduated flask. Cool to room temperature and make up to volume with N, N-dimethylformamide. This reagent is stable for one week.

3.4. Aniline: ***If the optical density in the blank test exceeds 0,022***, distil the aniline over zinc dust, discarding the first and last 10 % fractions of the distillate. Refrigerated and stored in a brown, stoppered glass flask, this reagent will keep for several months.

3.5. Standard gossypol solution A: Place 27,9 mg of gossypol acetate in a 250 ml graduated flask. Dissolve and make up to volume with solvent A (3.2). Pipette 50 ml of this solution into a 250 ml graduated flask and make up to volume with solvent A. The gossypol concentration of this solution is 0,02 mg/ml. Leave to stand for one hour at room temperature before use.

3.6. Standard gossypol solution B: Place 27,9 mg of gossypol acetate in a 50 ml graduated flask, Dissolve and make up to volume with solvent B (3.3). The gossypol concentration of this solution is 0,5 mg/ml.

Standard gossypol solutions A and B will remain stable for 24 hours if protected from the light.

4. **Apparatus**

4.1. Mixer (tumbler): approximately 35 r.p.m.

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4.2. Spectrophotometer.

5. **Procedure**

5.1. ***Test sample***

The amount of test sample used depends on the presumed gossypol content of the sample. It is preferable to work with a small test sample and a relatively large aliquot part of the filtrate, so as to obtain sufficient gossypol for precise photometric measurement to be possible. ***For the determination of free gossypol*** in cottonseed, cottonseed meal and cottonseed cake, the test sample shall not exceed 1 g; for compound feed, it may be as much as 5 g. A 10 ml aliquot part of filtrate is suitable in most cases; it shall contain 50 to 100 μg of gossypol. ***For the determination of total gossypol,*** the test sample shall be between 0,5 and 5 g, that a 2 ml aliquot part of filtrate will contain 40 to 200 μg of gossypol.

The analysis shall be carried out at a room temperature of about 20 °C.

5.2. ***Determination of free gossypol***

Place the test sample in a ground-necked 250 ml flask, the bottom of the flask having been covered with crushed glass. Using a pipette, add 50 ml of solvent A (3.2), stopper the flask and mix for one hour in the mixer. Filter through a dry filter and collect the filtrate in a small ground-necked flask. During filtration, cover the funnel with a watch glass.

Pipette identical aliquot parts of filtrate containing 50 to 100 μg of gossypol into each of two 25 ml graduated flasks (A and B). If necessary, make up the volume to 10 ml with solvent A (3.2). Then make the contents of flask (A) up to volume with the propan-2-ol-hexane mixture (3.1). This solution will be used as a reference solution against which to measure the sample solution.

Pipette 10 ml of solvent A (3.2) into each of two other 25 ml graduated flasks (C and D). Make the contents of flask (C) up to volume with the propan-2-ol-hexane mixture (3.1). This solution will be used as a reference solution against which to measure the blank test solution.

Add 2 ml of aniline (3.4) to each of flasks (D) and (B). Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenise and leave to stand for one hour.

Determine the optical density of the blank test solution (D) by comparison with the reference solution (C), and the optical density of the sample solution (B) by comparison with the reference solution (A), in the spectrophotometer at 440 nm using 1 cm glass cells.

Subtract the optical density of the blank test solution from that of the sample solution (= corrected optical density). From this value calculate the free gossypol content as indicated in 6.

5.3. ***Determination of total gossypol***

Place a test sample containing 1 to 5 mg of gossypol in a 50 ml graduated flask and add 10 ml of solvent B (3.3). At the same time, prepare a blank test, placing 10 ml of solvent B (3.3) in another 50 ml graduated flask. Heat the two flasks for 30 minutes over a boiling water bath. Cool to room temperature and make the contents of each flask up

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to volume with the propan-2-ol-hexane mixture (3.1). Homogenise and leave to settle for 10 to 15 minutes, then filter and collect the filtrates in ground-necked flasks.

Pipette 2 ml of the sample filtrate into each of two 25 ml graduated flasks, and 2 ml of the blank test filtrate into each of two other 25 ml flasks. Make the contents of one flask from each series up to 25 ml with the propan-2-ol-hexane mixture (3.1). These solutions will be used as reference solutions.

Add 2 ml of aniline (3.4) to each of the other two flasks. Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to 25 ml with the propan-2-ol-hexane mixture (3.1), homogenise and leave to stand for one hour.

Determine the optical density as indicated in 5.2 for free gossypol. From this value calculate the total gossypol content as indicated in 6.

6. **Calculation of results**

Results may be calculated either from the specific optical density (6.1), or by reference to a calibration curve (6.2).

6.1. ***From the specific optical density***

The specific optical densities, under the conditions described, will be the following:

Free gossypol: $E\,\frac{1\,\%}{1\ \text{cm}} = 625$

Total gossypol: $E\,\frac{1\,\%}{1\ \text{cm}} = 600$

The free or total gossypol content of the sample is calculated by using the following formula:

$$\%\ \text{gossypol} : \frac{E \times 1\,250}{E\,\frac{1\,\%}{1\text{cm}} \times p \times a}$$

where:

E = corrected optical density, determined as indicated in 5.2,
p = test sample in g,
a = aliquot part of the filtrate in ml.

6.2. ***From a calibration curve***

6.2.1. Free gossypol

Prepare 2 series of five 25 ml graduated flasks. Pipette aliquots of 2,0, 4,0, 6,0, 8,0 and 10,0 ml of standard gossypol solution A (3.5) into each series of flasks. Make up the volumes to 10 ml with solvent A (3.2). Complete each series with a 25 ml graduated flask containing only 10 ml of solvent A (3.2) (blank test).

Make the volume of the flasks in the first series (including the flask for the blank test) up to 25 ml with the propan-2-ol-hexane mixture (3.1) (reference series).

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Add 2 ml of aniline (3.4) to each flask in the second series (including the flask for the blank test). Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenise and leave to stand for one hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series by comparison with the corresponding solutions in the reference series. Trace the calibration curve by plotting the optical densities against the quantities of gossypol (in µg).

6.2.2. Total gossypol

Prepare six 50 ml graduated flasks. In the first flask place 10 ml of solvent B (3.3), and in the others 2,0, 4,0, 6,0, 8,0 and 10,0 ml of standard gossypol solution B (3.6) respectively. Make the contents of each flask up to 10 ml with solvent B (3.3). Heat for 30 minutes over a boiling water bath. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1) and homogenise.

Place 2,0 ml of these solutions in each of two series of six 25 ml graduated flasks. Make the contents of the flasks in the first series up to 25 ml with the propan-2-ol-hexane mixture (3.1) (reference series).

Add 2 ml of aniline (3.4) to each flask in the second series. Heat for 30 minutes over a boiling water bath. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenise and leave to stand for one hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series by comparison with the corresponding solutions in the reference series. Trace the calibration curve by plotting the optical densities against the quantities of gossypol (in µg).

6.3. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

— 15 %, in relative value to the higher level, for gossypol contents of less than 500 ppm,

— 75 ppm, in absolute value, for contents of not less than 500 ppm and not more than 750 ppm,

— 10 %, in relative value to the higher value, for contents of more than 750 ppm.

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B. DETERMINATION OF THE LEVELS OF DIOXINS (PCDD/PCDF) AND PCBs

CHAPTER I

Methods of sampling and interpretation of analytical results

1. **Scope and definitions**

The samples intended for the official control of the levels of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyls (PCBs) ([1]) and non dioxin-like PCBs in feed shall be taken in accordance with the provisions of Annex I. The quantitative requirements in relation to the control of substances or products uniformly distributed throughout the feed as provided for in point 5.1. of Annex I shall be applied. Aggregate samples thus obtained shall be considered representative for the lots or sublots from which they are taken. Compliance with maximum levels laid down by Directive 2002/32/EC shall be established on the basis of the levels determined in the laboratory samples.

For the purposes of this Part B, the definitions laid down in Annex I to Commission Decision 2002/657/EC ([2]) shall apply.

([1]) Table of TEF (= toxic equivalency factors) for PCDDs, PCDFs and dioxin-like PCBs: WHO-TEFs for human risk assessment based on the conclusions of the World Health Organization (WHO) — International Programme on Chemical Safety (IPCS) expert meeting which was held in Geneva in June 2005 (Martin van den Berg et al., The 2005 World Health Organization Re-evaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-like Compounds. Toxicological Sciences 93(2), 223–241 (2006)).

Congener	TEF value	Congener	TEF value
Dibenzo-p-dioxins (‘PCDDs’) and Dibenzo-p-furans '(‘PCDFs’)		‘Dioxin-like’ PCBs Non-ortho PCBs + Mono-ortho PCBs	
2,3,7,8-TCDD	1		
1,2,3,7,8-PeCDD	1	Non-ortho PCBs	
1,2,3,4,7,8-HxCDD	0,1	PCB 77	0,0001
1,2,3,6,7,8-HxCDD	0,1	PCB 81	0,0003
1,2,3,7,8,9-HxCDD	0,1	PCB 126	0,1
1,2,3,4,6,7,8-HpCDD	0,01	PCB 169	0,03
OCDD	0,0003	Mono-ortho PCBs	
2,3,7,8-TCDF	0,1	PCB 105	0,00003
1,2,3,7,8-PeCDF	0,03	PCB 114	0,00003
2,3,4,7,8-PeCDF	0,3	PCB 118	0,00003
1,2,3,4,7,8-HxCDF	0,1	PCB 123	0,00003
1,2,3,6,7,8-HxCDF	0,1	PCB 156	0,00003
1,2,3,7,8,9-HxCDF	0,1	PCB 157	0,00003
2,3,4,6,7,8-HxCDF	0,1	PCB 167	0,00003
1,2,3,4,6,7,8-HpCDF	0,01	PCB 189	0,00003
1,2,3,4,7,8,9-HpCDF	0,01		
OCDF	0,0003		

Abbreviations used: ‘T’ = tetra; ‘Pe’ = penta; ‘Hx’ = hexa; ‘Hp’ = hepta; ‘O’ = octa; ‘CDD’ = chlorodibenzodioxin; ‘CDF’ = chlorodibenzofuran; ‘CB’ = chlorobiphenyl.

([2]) Commission Decision 2002/657/EC of 14 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and interpretation of results (OJ L 221, 17.8.2002, p. 8).

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In addition to those definitions, the following definitions shall apply for the purpose of this Part B:

'Screening methods' means methods used for selection of those samples with levels of PCDD/Fs and dioxin-like PCBs that exceed the maximum levels or the action thresholds. They shall allow a cost-effective high sample-throughput, thus increasing the chance to discover new incidents with high exposure and health risks to consumers. Screening methods shall be based on bioanalytical or GC-MS methods. Results from samples exceeding the cut-off value used to check compliance with the maximum level shall be verified by a full re-analysis from the original sample using a confirmatory method.

'Confirmatory methods' means methods that provide full or complementary information enabling the PCDD/Fs and dioxin-like PCBs to be identified and quantified unequivocally at the maximum or in case of need at the action threshold. Such methods utilize gas chromatography/high resolution mass spectrometry (GC-HRMS) or gas chromatography/tandem mass spectrometry (GC-MS/MS).

2. **Compliance of the lot or sublot with the maximum level**

2.1. ***As regards non-dioxin-like PCBs***

The lot or sublot complies with the maximum level if the analytical result for the sum of PCB 28, PCB 52, PCB 101, PCB 138, PCB 153 and PCB 180 (hereafter referred to as non-dioxin-like PCBs) does not exceed the maximum level laid down by Directive 2002/32/EC, taking into account the expanded measurement uncertainty ([1]). The lot or sublot does not comply with the maximum level as laid down by Directive 2002/32/EC, if the mean of two upper-bound ([2]) analytical results obtained from duplicate analysis ([3]), taking into account the expanded measurement uncertainty, exceeds the maximum level beyond reasonable doubt, i.e. the analysed concentration after deduction of the expanded measurement uncertainty is used to assess compliance.

The expanded measurement uncertainty is calculated using a coverage factor of 2 which gives a level of confidence of approximately 95 %. A lot or sublot is non-compliant if the mean of the measured values minus the expanded uncertainty of the mean is above the maximum level.

([1]) The principles described in the 'Guidance Document on Measurement Uncertainty for Laboratories performing PCDD/F and PCB Analysis using Isotope Dilution Mass Spectrometry' (http://ec.europa.eu/food/safety/animal-feed_en) shall be followed when applicable.

([2]) The concept of 'upper-bound' requires using the limit of quantification for the contribution of each non-quantified congener. The concept of 'lower-bound' requires using zero for the contribution of each non-quantified congener. The concept of 'medium-bound' requires using half of the limit of quantification calculating the contribution of each non-quantified congener.

([3]) Duplicate analysis: Separate analysis of the analytes of interest using a second aliquot of the same homogenized sample. In general, the requirements for duplicate analysis as provided for in Annex II, Chapter C, point 3 apply. However, for methods with the use of ^{13}C-labelled internal standard for the relevant analytes, the duplicate analysis is only necessary if the result of the first determination is not compliant. The duplicate analysis is necessary to exclude the possibility of internal cross-contamination or an accidental mix-up of samples. In case the analysis is performed in the course of a contamination incident, confirmation by duplicate analysis may be omitted in case the samples selected for analysis are through traceability linked to the contamination incident and the level found is significantly above the maximum level.

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The rules, mentioned in the paragraphs above under this point, shall apply for the analytical result obtained on the sample for official control. In case of analysis for defence or reference purposes, the national rules shall apply.

2.2. ***As regards PCDD/Fs and dioxin-like PCBs***

The lot or sublot complies with the maximum level if the result of a single analysis

— performed by a screening method with a false-compliant rate below 5 %, indicates that the level does not exceed the respective maximum level of PCDD/Fs and the sum of PCDD/Fs and dioxin-like PCBs laid down by Directive 2002/32/EC,

— performed by a confirmatory method, does not exceed the respective maximum level of PCDD/Fs and the sum of PCDD/Fs and dioxin-like PCBs laid down by Directive 2002/32/EC, taking into account the expanded measurement uncertainty.

For screening assays a cut-off value shall be established for decisions on sample compliance with the respective maximum levels set for either PCDD/Fs, or for the sum of PCDD/Fs and dioxin-like PCBs.

The lot or sublot does not comply with the maximum level as laid down by Directive 2002/32/EC if the mean of two upper-bound ([1]) analytical results obtained from duplicate analysis ([2]) using a confirmatory method, taking into account the expanded measurement uncertainty, exceeds the maximum level beyond reasonable doubt, i.e. the analysed concentration after deduction of the expanded measurement uncertainty is used to assess compliance.

The expanded measurement uncertainty is calculated using a coverage factor of 2 which gives a level of confidence of approximately 95 %. A lot or sublot is non-compliant if the mean of the measured values minus the expanded uncertainty of the mean is above the maximum level.

The sum of the estimated expanded uncertainties of the separate analytical results of PCDD/Fs and dioxin-like PCBs shall be used for the sum of PCDD/Fs and dioxin-like PCBs.

The rules, mentioned in the paragraphs above under this point, shall apply for the analytical result obtained on the sample for official control. In case of analysis for defence or reference purposes, the national rules shall apply.

([1]) The concept of ‘upper-bound’ requires using the limit of quantification for the contribution of each non-quantified congener to the Toxic Equivalent (TEQ). The concept of ‘lower-bound’ requires using zero for the contribution of each non-quantified congener to the TEQ. The concept of ‘medium-bound’ requires using half of the limit of quantification calculating the contribution of each non-quantified congener to the TEQ.

([2]) In general, the requirements for duplicate analysis as provided for in Annex II, Chapter C, point 2 apply. However, for confirmatory methods with the use of ^{13}C-labelled internal standard for the relevant analytes, the duplicate analysis is only necessary if the result of the first determination is not compliant. The duplicate analysis is necessary to exclude the possibility of internal cross-contamination or an accidental mix-up of samples. In case the analysis is performed in the course of a contamination incident, confirmation by duplicate analysis may be omitted in case the samples selected for analysis are through traceability linked to the contamination incident and the level found is significantly above the maximum level.

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3. **Results exceeding action thresholds as laid down in Annex II to Directive 2002/32/EC**

Action thresholds serve as a tool for the selection of samples in those cases where it is necessary to identify a source of contamination and to take measures for its reduction or elimination. Screening methods shall establish the appropriate cut-off values for selection of those samples. Where significant efforts are necessary to identify a source and to reduce or eliminate the contamination, it is appropriate to confirm exceedance of the action thresholds by duplicate analysis using a confirmatory method and taking into account the expanded measurement uncertainty ([1]).

CHAPTER II

Sample preparation and requirements for methods of analysis used in offical control of the levels of dioxins (PCDD/Fs) and dioxin-like PCBs in feed

1. **Field of application**

The requirements set out in this Chapter shall be applied where feed is analysed for the official control of the levels of 2,3,7,8-substituted PCDD/Fs and dioxin-like PCBs and as regards sample preparation and analytical requirements for other regulatory purposes, which includes the controls performed by the feed business operator to ensure compliance with the provisions of Regulation (EC) No 183/2005 of the European Parliament and of the Council ([2]).

Monitoring for the presence of PCDD/Fs and dioxin-like PCBs in feed may be performed with two different types of analytical methods:

(a) ***Screening methods***

The goal of screening methods is to select those samples with levels of PCDD/Fs and dioxin-like PCBs that exceed the maximum levels or the action thresholds. Screening methods shall ensure cost-effective high sample-throughput, thus increasing the chance to discover new incidents with high exposure and health risks of consumers. Their application shall aim to avoid false-compliant results. They may comprise bioanalytical and GC-MS methods.

Screening methods compare the analytical result with a cut-off value, providing a yes/no-decision over the possible exceedance of the maximum level or action threshold. The concentration of PCDD/Fs and the sum of PCDD/Fs and dioxin-like PCBs in samples suspected to be non-compliant with the maximum level shall be determined or confirmed by a confirmatory method.

In addition, screening methods may give an indication of the levels of PCDD/Fs and dioxin-like PCBs present in the sample. In case of application of bioanalytical screening methods the result is expressed as Bioanalytical Equivalents (BEQ), whereas in case of application of physico-chemical GC-MS methods it is expressed as Toxic Equivalents (TEQ). The numerically indicated results of screening methods are suitable for demonstrating compliance or suspected

([1]) Identical explanation and requirements for duplicate analysis for control of action thresholds as in footnote 2 above for maximum levels.

([2]) Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene (OJ L 35, 8.2.2005, p. 1).

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noncompliance or exceedance of action thresholds and give an indication of the range of levels in case of follow-up by confirmatory methods. They are not suitable for purposes such as evaluation of background levels, estimation of intake, following of time trends in levels or re-evaluation of action thresholds and maximum levels.

(b) ***Confirmatory methods***

Confirmatory methods allow the unequivocal identification and quantification of PCDD/Fs and dioxin-like PCBs present in a sample and provide full information on congener level. Therefore, those methods allow the control of maximum levels and action thresholds, including the confirmation of results obtained by screening methods. Furthermore, results may be used for other purposes such as determination of low background levels in feed monitoring, following of time trends, exposure assessment and building of a database for possible re-evaluation of action thresholds and maximum levels. They are also important for establishing congener patterns in order to identify the source of a possible contamination. Such methods utilise GC-HRMS. For confirming compliance or non-compliance with the maximum level, also GC-MS/MS can be used.

2. **Background**

For calculation of TEQ concentrations, the concentrations of the individual substances in a given sample shall be multiplied by their respective Toxic Equivalency Factor (TEF) (see footnote 1 of Chapter I) and subsequently summed to give the total concentration of dioxin-like compounds expressed as TEQs.

For the purposes of this Part B, the accepted specific limit of quantification of an individual congener means the lowest content of the analyte that can be measured with reasonable statistical certainty, fulfilling the identification criteria as described in internationally recognised standards, for example, in standard EN 16215:2012 (Animal feed — Determination of dioxins and dioxin-like PCBs by GC-HRMS and of indicator PCBs by GC-HRMS) and/or in EPA methods 1613 and 1668 as revised.

The limit of quantification of an individual congener may be identified as

(a) the concentration of an analyte in the extract of a sample which produces an instrumental response at two different ions to be monitored with a S/N (signal/noise) ratio of 3:1 for the less intensive raw data signal; or

(b) if for technical reasons the signal-to-noise calculation does not provide reliable results, the lowest concentration point on a calibration curve that gives an acceptable (≤ 30 %) and consistent (measured at least at the start and at the end of an analytical series of samples) deviation to the average relative response factor calculated for all points on the calibration curve in each series of samples. The limit of quantification (LOQ) is calculated from the lowest concentration point taking into account the recovery of internal standards and sample intake.

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Bioanalytical screening methods will not give results at the congener level but merely an indication ([1]) of the TEQ level, expressed in BEQ to acknowledge the fact that not all compounds present in a sample extract that produce a response in the test may fulfill or meet all requirements of the TEQ-principle.

Screening and confirmatory methods may only be applied for control of a certain matrix if the methods are sensitive enough to detect levels reliably at the action threshold or maximum level.

3. **Quality assurance requirements**

3.1. Measures shall be taken to avoid cross-contamination at each stage of the sampling and analysis procedure.

3.2. The samples shall be stored and transported in glass, aluminum, polypropylene or polyethylene containers suitable for storage without any influence on the levels of PCDD/Fs and dioxin-like PCBs in the samples. Traces of paper dust shall be removed from the sample container.

3.3. The sample storage and transportation shall be performed in a way that maintains the integrity of the feed sample.

3.4. Insofar as relevant, each laboratory sample shall be finely grinded and mixed thoroughly using a process that has been demonstrated to achieve complete homogenisation (for example, ground to pass a 1 mm sieve). Samples shall be dried before grinding if the moisture content is too high.

3.5. Control of reagents, glassware and equipment for possible influence of TEQ- or BEQ-based results shall be carried out.

3.6. A blank analysis shall be performed by carrying out the entire analytical procedure omitting only the sample.

3.7. For bioanalytical methods, all glassware and solvents used in analysis shall be tested to be free of compounds that interfere with the detection of target compounds in the working range. Glassware shall be rinsed with solvents or heated at temperatures suitable to remove traces of PCDD/Fs, dioxin-like compounds and interfering compounds from its surface.

3.8. Sample quantity used for the extraction shall be sufficient to fulfill the requirements with respect to a sufficiently low working range including the concentrations of maximum levels or action threshold.

3.9. The specific sample preparation procedures used for the products under consideration shall follow internationally accepted guidelines.

([1]) Bioanalytical methods are not specific to those congeners included in the TEF-scheme. Other structurally related AhR-active compounds may be present in the sample extract which contribute to the overall response. Therefore, bioanalytical results cannot be an estimate but rather an indication of the TEQ level in the sample.

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4. **Requirements for laboratories**

4.1. In accordance with the provisions of Regulation (EC) No 882/2004, laboratories shall be accredited by a recognised body operating in accordance with ISO Guide 58 to ensure that they are applying analytical quality assurance. Laboratories shall be accredited following the EN ISO/IEC 17025 standard. The principles as described in the Technical Guidelines for the estimation of measurement uncertainty and limits of quantification for PCDD/F and PCB analysis shall be followed when applicable ([1])

4.2. Laboratory proficiency shall be proven by the continuous successful participation in inter-laboratory studies for the determination of PCDD/Fs and dioxin-like PCBs in relevant feed matrices and concentration ranges.

4.3. Laboratories applying screening methods for the routine control of samples shall establish a close cooperation with laboratories applying the confirmatory method, both for quality control and confirmation of the analytical result of suspected samples.

5. **Basic requirements to be met by analytical procedure for dioxins (PCDD/Fs) and dioxin-like PCBs**

5.1. ***Low working range and limits of quantification***

For PCDD/Fs, detectable quantities shall be in the upper femtogram (10^{-15}g) range because of extreme toxicity of some of these compounds. For most PCB congeners a limit of quantification in the nanogram (10^{-9}g) range is already sufficient. For the measurement of the more toxic dioxin-like PCB congeners (in particular non-ortho-substituted congeners), the lower end of the working range shall reach the low picogram (10^{-12}g) levels. For all other PCB congeners a limit of quantification in the nanogram (10^{-9}g) range is sufficient.

5.2. ***High selectivity (specificity)***

5.2.1. A distinction is required between PCDD/Fs and dioxin-like PCBs and a multitude of other, coextracted and possibly interfering compounds present at concentrations up to several orders of magnitude higher than those of the analytes of interest. For GC-MS methods, a differentiation among various congeners is required, such as between toxic (for example, the seventeen 2,3,7,8-substituted PCDD/Fs, and twelve dioxin-like PCBs) and other congeners.

5.2.2. Bioanalytical methods shall be able to detect the target compounds as the sum of PCDD/Fs, and/or dioxin-like PCBs. Sample clean-up shall aim at removing compounds causing false non-compliant results or compounds that may decrease the response, causing false compliant results.

5.3. ***High accuracy (trueness and precision, bioassay apparent recovery)***

5.3.1. For GC-MS methods, the determination shall provide a valid estimate of the true concentration in a sample. High accuracy is required to avoid the rejection of a sample analysis result on the basis of poor reliability of

([1]) 'Guidance Document on Measurement Uncertainty for Laboratories performing PCDD/F and PCB Analysis using Isotope Dilution Mass Spectrometry' (http://ec.europa.eu/food/safety/animal-feed_en), 'Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food' (http://ec.europa.eu/food/safety/animal-feed_en).

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the determined TEQ level. Accuracy is expressed as ***trueness*** (difference between the mean value measured for an analyte in a certified material and its certified value, expressed as a percentage of this value) and ***precision*** (RSD_R relative standard deviation calculated from results generated under reproducibility conditions).

5.3.2. For bioanalytical methods, the bioassay apparent recovery shall be determined. Bioassay apparent recovery means the BEQ level calculated from the TCDD or PCB 126 calibration curve corrected for the blank and then divided by the TEQ level determined by the confirmatory method. It aims at correcting factors like the loss of PCDD/Fs and dioxin-like compounds during the extraction and clean-up steps, co-extracted compounds increasing or decreasing the response (agonistic and antagonistic effects), the quality of the curve fit, or differences between the TEF values and the Relative Potency (REP) values. The bioassay apparent recovery is calculated from suitable reference samples with representative congener patterns around the level of interest.

5.4. ***Validation in the range of maximum level and general quality control measures***

5.4.1. Laboratories shall demonstrate the performance of a method in the range of the maximum level, for example, 0,5x, 1x and 2x the maximum level with an acceptable coefficient of variation for repeated analysis, during the validation procedure and during routine analysis.

5.4.2. Regular blank controls and spiking experiments or analysis of control samples (preferably, if available, certified reference material) shall be performed as internal quality control measures. Quality control charts for blank controls, spiking experiments or analysis of control samples shall be recorded and checked to make sure the analytical performance is in accordance with the requirements.

5.5. ***Limit of quantification***

5.5.1. For a bioanalytical screening method, the establishment of the limit of quantification (LOQ) is not an indispensable requirement but the method shall prove that it can differentiate between the blank and the cut-off value. When providing a BEQ level, a reporting level shall be established to deal with samples showing a response below this level. The reporting level shall be demonstrated to be different from procedure blank samples at least by a factor of three, with a response below the working range. It shall therefore be calculated from samples containing the target compounds around the required minimum level, and not from an S/N ratio or an assay blank.

5.5.2. The LOQ for a confirmatory method shall be about one fifth of the maximum level.

5.6. ***Analytical criteria***

For reliable results from confirmatory or screening methods, the following criteria shall be met in the range of the maximum level for the TEQ or BEQ value, respectively, whether determined as total TEQ or total BEQ (as the sum of PCDD/Fs and dioxin-like PCBs) or separately for PCDD/Fs and dioxin-like PCBs:

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	Screening with bioanalytical or physico-chemical methods	Confirmatory methods
False-compliant rate ([1])	< 5 %	
Trueness		– 20 % to + 20 %
Repeatability (RSD_r)	< 20 %	
Intermediate precision (RSD_R)	< 25 %	< 15 %

([1]) With respect to the maximum levels.

5.7. ***Specific requirements for screening methods***

5.7.1. Both GC-MS and bioanalytical methods may be used for screening. For GC-MS methods the requirements laid down in point 6 shall be met. For cell based bioanalytical methods specific requirements are laid down in point 7.

5.7.2. Laboratories applying screening methods for the routine control of samples shall establish a close cooperation with laboratories applying the confirmatory method.

5.7.3. Performance verification of the screening method is required during routine analysis, by analytical quality control and on-going method validation. There shall be a continuous programme for the control of compliant results.

5.7.4. Check on possible suppression of the cell response and cytotoxicity:

20 % of the sample extracts shall be measured in routine screening without and with 2,3,7,8-TCDD added corresponding to the maximum level or action threshold, to check if the response is possibly suppressed by interfering substances present in the sample extract. The measured concentration of the spiked sample shall be compared to the sum of the concentration of the unspiked extract plus the spiking concentration. If this measured concentration is more than 25 % lower than the calculated (sum) concentration, this is an indication of potential signal suppression and the respective sample shall be submitted to GC-HRMS confirmatory analysis. Results shall be monitored in quality control charts.

5.7.5. Quality control on compliant samples:

Approximately 2 to 10 % of the compliant samples, depending on sample matrix and laboratory experience, shall be confirmed by GC/HRMS.

5.7.6. Determination of false-compliant rates from quality control data:

The rate of false-compliant results from screening of samples below and above the maximum level or the action threshold shall be determined. Actual false-compliant rates shall be below 5 %. When a minimum of 20 confirmed results per matrix/matrix group is available from the quality control of compliant samples, conclusions on the false

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compliant rate shall be drawn from this database. The results from samples analysed in ring trials or during contamination incidents, covering a concentration range up to for example 2x the maximum level (ML), may also be included in the minimum of 20 results for evaluation of the false-compliant rate. The samples shall cover most frequent congener patterns, representing various sources.

Although screening assays shall preferentially aim to detect samples exceeding the action threshold, the criterion for determining false-compliant rates is the maximum level, taking into account the expanded measurement uncertainty of the confirmatory method.

5.7.7. Potential non-compliant samples from screening shall always be verified by a full re-analysis of the original sample by a confirmatory method of analysis. These samples may also be used to evaluate the rate of false non-compliant results. For screening methods, the rate of false non-compliant results shall be the fraction of results confirmed to be compliant from confirmatory analysis, while in previous screening the sample has been declared to be potentially non-compliant. Evaluation of the advantages of the screening method shall be based on comparison of false-non-compliant samples with the total number of samples checked. This rate shall be low enough to make the use of a screening tool advantageous.

5.7.8. Under validation conditions, bioanalytical methods shall provide a valid indication of the TEQ level, calculated and expressed as BEQ.

Also for bioanalytical methods carried out under repeated conditions, the intra-laboratory RSD_r would typically be smaller than under reproducibility conditions(RSD_R)

6. **SPECIFIC requirements for GC-MS methods to be complied with for screening or confirmatory purposes**

6.1. ***Acceptable differences between upper-bound and lower-bound WHO-TEQ results***

The difference between upper-bound level and lower-bound level shall not exceed 20 % for confirmation of exceedance of maximum level or in case of need of action thresholds.

6.2. ***Control of recoveries***

6.2.1. Addition of ^{13}C-labelled 2,3,7,8-chlorine-substituted internal PCDD/F standards and of ^{13}C-labelled internal dioxin-like PCB standards shall be carried out at the very beginning of the analytical method e.g. prior to extraction in order to validate the analytical procedure. At least one congener for each of the tetra- to octa-chlorinated homologous groups for PCDD/Fs and at least one congener for each of the homologous groups for dioxin-like PCBs shall be added (alternatively, at least one congener for each mass spectrometric selected ion recording function used for monitoring PCDD/Fs and dioxin-like PCBs). In the case of confirmatory methods, all 17 ^{13}C-labelled 2,3,7,8-substituted internal PCDD/F standards and all 12 ^{13}C-labelled internal dioxin-like PCB standards shall be used.

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6.2.2. Relative response factors shall also be determined for those congeners for which no ^{13}C-labelled analogue is added by using appropriate calibration solutions.

6.2.3. For feed of plant origin and feed of animal origin containing less than 10 % fat, the addition of the internal standards shall be mandatory prior to extraction. For feed of animal origin containing more than 10 % fat, the internal standards shall be added either before or after fat extraction. An appropriate validation of the extraction efficiency shall be carried out, depending on the stage at which internal standards are introduced.

6.2.4. Prior to GC-MS analysis, 1 or 2 recovery (surrogate) standard(s) shall be added.

6.2.5. Control of recovery is required. For confirmatory methods, the recoveries of the individual internal standards shall be in the range of 60 to 120 %. Lower or higher recoveries for individual congeners, in particular for some hepta- and octa- chlorinated dibenzo-p-dioxins and dibenzofurans, shall be acceptable on the condition that their contribution to the TEQ value does not exceed 10 % of the total TEQ value (based on sum of PCDD/F and dioxin-like PCBs). For GC-MS screening methods, the recoveries shall be in the range of 30 to 140 %.

6.3. ***Removal of interfering substances***

— Separation of PCDD/Fs from interfering chlorinated compounds such as non-dioxin-like PCBs and chlorinated diphenyl ethers shall be carried out by suitable chromatographic techniques (preferably with a florisil, alumina and/or carbon column).

— Gas-chromatographic separation of isomers shall be < 25 % peak to peak between 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF.

6.4. ***Calibration with standard curve***

The range of the calibration curve shall cover the relevant range of maximum level or action thresholds.

6.5. ***Specific criteria for confirmatory methods***

— For GC-HRMS:

In HRMS, the resolution shall typically be greater than or equal to 10 000 for the entire mass range at 10 % valley.

Fulfilment of further identification and confirmation criteria as described in internationally recognised standards, for example, in standard EN 16215:2012 (Animal feed — Determination of dioxins and dioxin-like PCBs by GC-HRMS and of indicator PCBs by GC-HRMS) and/or in EPA methods 1613 and 1668 as revised.

— For GC-MS/MS:

Monitoring of at least 2 specific precursor ions, each with one specific corresponding transition product ion for all labelled and unlabelled analytes in the scope of analysis.

Maximum permitted tolerance of relative ion intensities of ± 15 % for selected transition product ions in comparison to calculated or measured values (average from calibration standards), applying identical MS/MS conditions, in particular collision energy and collision gas pressure, for each transition of an analyte.

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Resolution for each quadrupole to be set equal to or better than unit mass resolution (unit mass resolution: sufficient resolution to separate two peaks one mass unit apart) in order to minimise possible interferences on the analytes of interest.

Fulfilment of the further criteria as described in internationally recognised standards, for example, in standard EN 16215:2012 (Animal feed — Determination of dioxins and dioxin-like PCBs by GC-HRMS and of indicator PCBs by GC-HRMS) and/or in EPA methods 1613 and 1668 as revised, except the obligation to use GC-HRMS.

7. **Specific requirements for bioanalytical methods**

Bioanalytical methods are methods based on the use of biological principles like cell-based assays, receptor-assays or immunoassays. This point 7 establishes requirements for bioanalytical methods in general.

A screening method in principle classifies a sample as compliant or suspected to be non-compliant. For this, the calculated BEQ level is compared to the cut-off value (see point 7.3). Samples below the cut-off value are declared compliant, samples equal or above the cut-off value are suspected to be non-compliant, requiring analysis by a confirmatory method. In practice, a BEQ level corresponding to two-thirds of the maximum level may serve as cut-off value provided that a false-compliant rate below 5 % and an acceptable rate for false non-compliant results are ensured. With separate maximum levels for PCDD/Fs and for the sum of PCDD/Fs and dioxin-like PCBs, checking compliance of samples without fractionation requires appropriate bioassay cut-off values for PCDD/Fs. For checking of samples exceeding the action thresholds, an appropriate percentage of the respective action threshold shall suit as cut-off value.

If an indicative level is expressed in BEQs, sample results shall be in the working range and shall exceed the reporting limit (see points 7.1.1 and 7.1.6).

7.1. ***Evaluation of the test response***

7.1.1. General requirements

— When calculating the concentrations from a TCDD calibration curve, values at the higher end of the curve will show a high variation (high coefficient of variation (CV)). The working range is the area where this CV is smaller than 15 %. The lower end of the working range (reporting limit) shall be set at least by a factor of three above the procedure blanks. The upper end of the working range is usually represented by the EC_{70} value (70 % of maximal effective concentration), but lower if the CV is higher than 15 % in this range. The working range shall be established during validation. Cut-off values (see point 7.3) shall be well within the working range.

— Standard solutions and sample extracts shall be tested in triplicate or at least in duplicate. When using duplicates, a standard solution or a control extract tested in four to six wells divided over the plate shall produce a response or concentration (only possible in the working range) based on a CV < 15 %.

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7.1.2. Calibration

7.1.2.1. Calibration with standard curve

— Levels in samples shall be estimated by comparison of the test response with a calibration curve of TCDD (or PCB 126 or a PCDD/PCDF/dioxin-like PCB standard mixture) to calculate the BEQ level in the extract and subsequently in the sample.

— Calibration curves shall contain 8 to 12 concentrations (at least in duplicates), with enough concentrations in the lower part of the curve (working range). Special attention shall be paid to the quality of the curve-fit in the working range. As such, the R^2 value is of little or no value in estimating the goodness of fit in non-linear regression. A better fit shall be achieved by minimising the difference between calculated and observed levels in the working range of the curve, for example by minimising the sum of squared residuals.

— The estimated level in the sample extract shall be subsequently corrected for the BEQ level calculated for a matrix or solvent blank sample (to account for impurities from solvents and chemicals used), and the apparent recovery (calculated from the BEQ level of suitable reference samples with representative congener patterns around the maximum level or action threshold). To perform a recovery correction, the apparent recovery shall be within the required range (see point 7.1.4). Reference samples used for recovery correction shall comply with the requirements laid down in point 7.2.

7.1.2.2. Calibration with reference samples

Alternatively, a calibration curve prepared from at least four reference samples (see point 7.2.4): one matrix blank, plus three reference samples at 0,5x, 1x and 2x the maximum level or action threshold may be used, eliminating the need to correct for blank and recovery if matrix properties of the reference samples match those of the unknown samples. In this case, the test response corresponding to two-thirds of the maximum level (see point 7.3) may be calculated directly from these samples and used as cut-off value. For checking of samples exceeding the action thresholds, an appropriate percentage of these action thresholds shall suit as cut-off value.

7.1.3. Separate determination of PCDD/Fs and dioxin-like PCBs

Extracts may be split into fractions containing PCDD/Fs and dioxin-like PCBs, allowing a separate indication of PCDD/Fs and dioxin-like PCB TEQ levels (in BEQ). A PCB 126 standard calibration curve shall preferentially be used to evaluate results for the fraction containing dioxin-like PCBs.

7.1.4. Bioassay apparent recoveries

The ‘bioassay apparent recovery’ shall be calculated from suitable reference samples with representative congener patterns around the maximum level or action threshold and expressed as percentage of the BEQ level in comparison to the TEQ level. Depending on the type of assay and TEFs ([1]) used, the differences between TEF and REP factors for dioxin-like PCBs can cause low apparent recoveries for dioxin-like PCBs in comparison to PCDD/Fs. Therefore, if a separate determination

([1]) Current requirements are based on the TEFs published in: M. Van den Berg et al, Toxicol Sci 93 (2), 223–241 (2006).

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of PCDD/Fs and dioxin-like PCBs is performed, bioassay apparent recoveries shall be: for dioxin-like PCBs 20 % to 60 %, for PCDD/Fs 50 % to 130 % (ranges apply for the TCDD calibration curve). As the contribution of dioxin-like PCBs to the sum of PCDD/Fs and dioxin-like PCBs can vary between different matrices and samples, bioassay apparent recoveries for the sum of PCDD/Fs and dioxin-like PCBs reflect these ranges and shall be between 30 % and 130 %. Any implication of substantially revised TEF values for the Union legislation for PCDD/Fs and dioxin-like PCBs requires the revision of these ranges.

7.1.5. Control of recoveries for clean-up

The loss of compounds during the clean-up shall be checked during validation. A blank sample spiked with a mixture of the different congeners shall be submitted to clean-up (at least n = 3) and the recovery and variability checked by a confirmatory method. The recovery shall be within 60 % to 120 % especially for congeners contributing more than 10 % to the TEQ-level in various mixtures.

7.1.6. Reporting limit

When reporting BEQ levels, a reporting limit shall be determined from relevant matrix samples involving typical congener patterns, but not from the calibration curve of the standards due to low precision in the lower range of the curve. Effects from extraction and clean-up shall be taken into account. The reporting limit shall be set at least by a factor of three above the procedure blanks.

7.2. ***Use of reference samples***

7.2.1. Reference samples shall represent sample matrix, congener patterns and concentration ranges for PCDD/Fs and dioxin-like PCBs around the maximum level or action threshold.

7.2.2. A matrix blank, and where it is not possible, a procedure blank, and a reference sample at the maximum level or action threshold shall be included in each test series. These samples shall be extracted and tested at the same time under identical conditions. The reference sample shall show a clearly elevated response in comparison to the blank sample, thus ensuring the suitability of the test. Those samples may be used for blank and recovery corrections.

7.2.3. Reference samples chosen to perform a recovery correction shall be representative for the test samples, meaning that congener patterns may not lead to an underestimation of levels.

7.2.4. Extra reference samples at e.g. 0,5x and 2x the maximum level or action threshold may be included to demonstrate the proper performance of the test in the range of interest for the control of the maximum level or action threshold. Combined, these samples may be used for calculating the BEQ levels in test samples (see point 7.1.2.2).

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7.3. ***Determination of cut-off values***

The relationship between bioanalytical results in BEQ and results from the confirmatory method in TEQ shall be established, for example by matrix-matched calibration experiments, involving reference samples spiked at 0, 0,5x, 1x and 2x the ML, with 6 repetitions on each level (n = 24). Correction factors (blank and recovery) may be estimated from this relationship but shall be checked in accordance with point 7.2.2.

Cut-off values shall be established for decisions over sample compliance with maximum levels or for the control of action thresholds, if relevant, with the respective maximum levels or action threshold set for either PCDD/Fs and dioxin-like PCBs alone, or for the sum of PCDD/Fs and dioxin-like PCBs. They are represented by the ***lower*** end-point of the distribution of bioanalytical results (corrected for blank and recovery) corresponding to the decision limit of the confirmatory method based on a 95 % level of confidence, implying a false-compliant rate < 5 %, and on a RSD_R < 25 %. The decision limit of the confirmatory method is the maximum level, taking into account the expanded measurement uncertainty.

The cut-off value (in BEQ) may be calculated in accordance with one of the approaches set out in points 7.3.1, 7.3.2 and 7.3.3. (see Figure 1).

7.3.1. Use of the ***lower*** band of the 95 % prediction interval at the decision limit of the confirmatory method:

$$\text{Cut} - \text{off value} = \text{BEQ}_{DL} - s_{y,x} \times t_{\alpha,f=m-2}\sqrt{1/\boldsymbol{n} + 1/\boldsymbol{m} + (\boldsymbol{x_i} - \bar{\boldsymbol{x}})^2/\boldsymbol{Q_{xx}}}$$

with:

BEQ_{DL} BEQ corresponding to the decision limit of the confirmatory method, being the maximum level taking into account the expanded measurement uncertainty

$s_{y,x}$ residual standard deviation

$t_{\alpha,f = m - 2}$ student factor (α = 5 %, f = degrees of freedom, single-sided)

m total number of calibration points (index j)

n number of repetitions on each level

x_i sample concentration (in TEQ) of calibration point i determined by a confirmatory method

$\bar{x}$ mean of the concentrations (in TEQ) of all calibration samples

$Q_{xx} = \sum_{\boldsymbol{j}=1}^{\boldsymbol{m}} (\boldsymbol{x_i} - \bar{\boldsymbol{x}})^2$ square sum parameter, i = index for calibration pointi

7.3.2. Calculation from bioanalytical results (corrected for blank and recovery) of multiple analyses of samples (n ≥ 6) contaminated at the decision limit of the confirmatory method, as the ***lower*** endpoint of the data distribution at the corresponding mean BEQ value:

$$\text{Cut-off value} = \text{BEQ}_{DL} - 1{,}64 \times SD_R$$

with:

SD_R standard deviation of bioassay results at BEQ_{DL}, measured under within-laboratory reproducibility conditions

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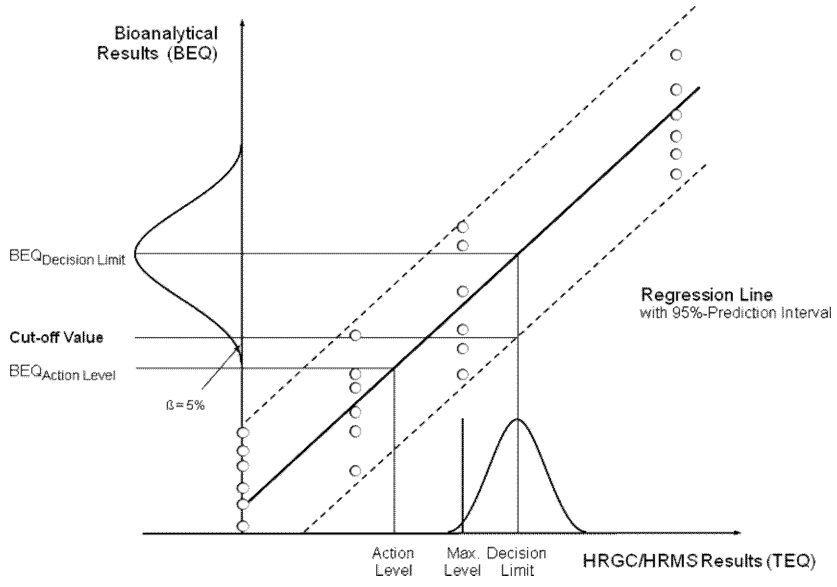
7.3.3. Calculation as mean value of bioanalytical results (in BEQ, corrected for blank and recovery) from multiple analysis of samples ($n \geq 6$) contaminated at two-thirds of the maximum level or action threshold, based on the observation that this level will be around the cut-off value determined under point 7.3.1 or point 7.3.2:

Calculation of cut-off values based on a 95 % level of confidence implying a false-compliant rate < 5 %, and a RSD_R < 25 %:

(1) from the ***lower*** band of the 95 % prediction interval at the decision limit of the confirmatory method.

(2) from multiple analysis of samples ($n \geq 6$) contaminated at the decision limit of the confirmatory method as the ***lower*** end-point of the data distribution (represented in the figure by a bell-shaped curve) at the corresponding mean BEQ value.

Figure 1



7.3.4. Restrictions to cut-off values

BEQ-based cut-off values calculated from the RSD_R achieved during validation using a limited number of samples with different matrix/congener patterns may be higher than the TEQ-based maximum levels or action thresholds due to a better precision than attainable in routine when an unknown spectrum of possible congener patterns has to be controlled. In such cases, cut-off values shall be calculated from an RSD_R = 25 %, or two-thirds of the maximum level or action threshold shall be preferred.

7.4. ***Performance characteristics***

7.4.1. Since no internal standards can be used in bioanalytical methods, tests on the repeatability of bioanalytical methods shall be carried out to obtain information on the standard deviation within and between test series. Repeatability shall be below 20 % and intra-laboratory reproducibility shall be below 25 %. This shall be based on the calculated levels in BEQ after blank and recovery correction.

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7.4.2. As part of the validation process, the test shall be shown to discriminate between a blank sample and a level at the cut-off value, allowing the identification of samples above the corresponding cut-off value (see point 7.1.2).

7.4.3. Target compounds, possible interferences and maximum tolerable blank levels shall be defined.

7.4.4. The percent standard deviation in the response or concentration calculated from the response (only possible in working range) of a triplicate determination of a sample extract may not be above 15 %.

7.4.5. The uncorrected results of the reference sample(s) expressed in BEQ (blank and at the maximum level or action threshold) shall be used for evaluation of the performance of the bioanalytical method over a constant time period.

7.4.6. Quality control charts for procedure blanks and each type of reference sample shall be recorded and checked to make sure the analytical performance is in accordance with the requirements, in particular for the procedure blanks with regard to the requested minimum difference to the lower end of the working range and for the reference samples with regard to within-laboratory reproducibility. Procedure blanks shall be controlled in a manner to avoid false-compliant results when subtracted.

7.4.7. The results from the confirmatory methods of suspected samples and 2 to 10 % of the compliant samples (minimum of 20 samples per matrix) shall be collected and used to evaluate the performance of the screening method and the relationship between BEQ and TEQ. This database may be used for the re-evaluation of cut-off values applicable to routine samples for the validated matrices.

7.4.8. Successful method performance may also be demonstrated by participation in ring trials. The results from samples analysed in ring trials, covering a concentration range up to e.g. 2 × maximum level, may be included in the evaluation of the false-compliant rate, if a laboratory is able to demonstrate its successful performance. The samples shall cover most frequent congener patterns, representing various sources.

7.4.9. During incidents, the cut-off values may be re-evaluated, reflecting the specific matrix and congener patterns of this single incident.

8. **Reporting of the results**

8.1. ***Confirmatory methods***

8.1.1. The analytical results shall contain the levels of the individual PCDD/F and dioxin-like PCB congeners and TEQ-values shall be reported as lower-bound, upper-bound and medium-bound in order to include a maximum of information in the reporting of the results and thereby enabling the interpretation of the results according to specific requirements.

8.1.2. The report shall include the method used for extraction of PCDD/Fs and dioxin-like PCBs.

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8.1.3. The recoveries of the individual internal standards shall be made available in case the recoveries are outside the range referred to in point 6.2.5, in case the maximum level is exceeded (in this case, the recoveries for one of the two duplicate analysis) and in other cases upon request.

8.1.4. As the expanded measurement uncertainty is to be taken into account when deciding about the compliance of a sample, this parameter shall be made available. Thus, analytical results shall be reported as x +/– U whereby x is the analytical result and U is the expanded measurement uncertainty using a coverage factor of 2 which gives a level of confidence of approximately 95 %. In the case of a separate determination of PCDD/Fs and dioxin-like-PCBs, the sum of the estimated expanded uncertainty of the separate analytical results of PCDD/Fs and dioxin-like PCBs shall be used for the sum of PCDD/Fs and dioxin-like PCBs.

8.1.5. The results shall be expressed in the same units and with at least the same number of significant figures as the maximum levels laid down by Directive 2002/32/EC

8.2. ***Bioanalytical screening methods***

8.2.1. The result of the screening shall be expressed as ‘compliant’ or ‘suspected to be non-compliant’ (‘suspected’).

8.2.2. In addition, an indicative result for PCDD/Fs and/or dioxin-like PCBs expressed in BEQ, and not TEQ, may be given.

8.2.3. Samples with a response below the reporting limit shall be expressed as ‘lower than the reporting limit’. Samples with a response above the working range shall be reported as ‘exceeding the working range’ and the level corresponding to the upper end of the working range shall be given in BEQ.

8.2.4. For each type of sample matrix, the report shall mention the maximum level or action threshold on which the evaluation is based.

8.2.5. The report shall mention the type of the test applied, the basic test principle and the kind of calibration.

8.2.6. The report shall include the method used for extraction of PCDD/Fs and dioxin-like PCBs.

8.2.7. In case of samples suspected to be non-compliant, the report needs to include a note on the action to be taken. The concentration of PCDD/Fs and the sum of PCDD/Fs and dioxin-like PCBs in those samples with elevated levels has to be determined/confirmed by a confirmatory method.

8.2.8. Non-compliant results shall only be reported from confirmatory analysis.

8.3. ***Physico-chemical screening methods***

8.3.1. The result of the screening shall be expressed as ‘compliant’ or ‘suspected to be non-compliant’ (‘suspected’).

8.3.2. For each type of sample matrix, the report shall mention the maximum level or action threshold on which the evaluation is based.

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8.3.3. In addition, levels for individual PCDD/F and/or dioxin-like PCB congeners and TEQ-values reported as lower-bound, upper-bound and medium-bound may be given. The results shall be expressed in the same units and with at least the same number of significant figures as the maximum levels laid down by Directive 2002/32/EC.

8.3.4. The recoveries of the individual internal standards shall be made available in case the recoveries are outside the range referred to in point 6.2.5, in case the maximum level is exceeded (in this case, the recoveries for one of the two duplicate analysis) and in other cases upon request.

8.3.5. The report shall mention the GC-MS method applied.

8.3.6. The report shall include the method used for extraction of PCDD/Fs and dioxin-like PCBs.

8.3.7. In case of samples suspected to be non-compliant, the report needs to include a note on the action to be taken. The concentration of PCDD/Fs and the sum of PCDD/Fs and dioxin-like PCBs in those samples with elevated levels has to be determined/confirmed by a confirmatory method.

8.3.8. Non-compliance can only be decided after confirmatory analysis.

CHAPTER III

Sample preparation and requirements for methods of analysis used in offical control of the levels of non dioxin-like PCBs in feed

1. **Field of application**

The requirements set out in this Chapter shall be applied where feed is analysed for the official control of the levels of non-dioxin-like PCBs and as regards sample preparation and analytical requirements for other regulatory purposes, which includes the controls performed by the feed business operator to ensure compliance with the provisions of Regulation (EC) No 183/2005.

2. **Applicable detection methods**

Gas chromatography/Electron Capture Detection (GC-ECD), GC-LRMS, GC-MS/MS, GC-HRMS or equivalent methods.

3. **Identification and confirmation of analytes of interest**

3.1. Relative retention time in relation to internal standards or reference standards (acceptable deviation of +/– 0,25 %).

3.2. Gas chromatographic separation of the non-dioxin-like PCBs from interfering substances, especially co-eluting PCBs, in particular if levels of samples are in the range of legal limits and non-compliance is to be confirmed ([1]).

3.3. Requirements for GC-MS techniques

Monitoring of at least the following number of molecular ions or characteristic ions from the molecular cluster:

(a) two specific ions for HRMS;

([1]) Congeners often found to co-elute are for example PCB 28/31, PCB 52/69 and PCB 138/163/164. For GC-MS also possible interferences from fragments of higher chlorinated congeners shall be considered.

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(b) three specific ions for LRMS;

(c) two specific precursor ions, each with one specific corresponding transition product ion for for MS-MS.

Maximum permitted tolerances for abundance ratios for selected mass fragments:

Relative deviation of abundance ratio of selected mass fragments from theoretical abundance or calibration standard for target ion (most abundant ion monitored) and qualifier ion(s): ± 15 %

3.4. Requirements for GC-ECD techniques

Results exceeding the maximum level shall be confirmed with two GC columns with stationary phases of different polarity.

4. **Demonstration of performance of method**

The performance of the method shall be validated in the range of the maximum level (0,5 to 2 times the maximum level) with an acceptable coefficient of variation for repeated analysis (see requirements for intermediate precision in point (9).

5. **Limit of quantification**

The sum of the LOQs ([1]) of non-dioxin-like PCBs shall not be higher than one-third of the maximum level ([2]).

6. **Quality control**

Regular blank controls, analysis of spiked samples, quality control samples, participation in inter-laboratory studies on relevant matrices.

7. **Control of recoveries**

7.1. Suitable internal standards with physico-chemical properties comparable to analytes of interest shall be used.

7.2. Addition of internal standards:

Addition to products (before extraction and clean-up process).

7.3. Requirements for methods using all six isotope-labelled non-dioxin-like PCB congeners

(a) results shall be corrected for recoveries of internal standards;

(b) recoveries of isotope-labelled internal standards shall be between 60 and 120 %;

(c) lower or higher recoveries for individual congeners with a contribution to the sum of non-dioxin-like PCBs below 10 % are acceptable.

7.4. Requirements for methods using not all six isotope-labelled internal standards or other internal standards:

(a) recovery of internal standard(s) shall be controlled for every sample;

([1]) The principles as described in the ‘Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food’ (http://ec.europa.eu/food/safety/animal-feed_en) shall be followed when applicable.

([2]) It is highly recommendable to have a lower contribution of the reagent blank level to the level of a contaminant in a sample. It is in the responsibility of the laboratory to control the variation of blank levels, in particular, if the blank levels are subtracted.

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(b) recoveries of internal standard(s) shall be between 60 and 120 %;

(c) results shall be corrected for recoveries of internal standards.

7.5. The recoveries of unlabelled congeners shall be checked by spiked samples or quality control samples with concentrations in the range of the maximum level. Recoveries for these congeners shall be considered acceptable, if they are between 60 and 120 %.

8. **Requirements for laboratories**

In accordance with the provisions of Regulation (EC) No 882/2004, laboratories shall be accredited by a recognised body operating in accordance with ISO Guide 58 to ensure that they are applying analytical quality assurance. Laboratories shall be accredited following the EN ISO/IEC 17025 standard. In addition, the principles as described in Technical Guidelines for the estimation of measurement uncertainty and limits of quantification for PCB analysis shall be followed when applicable ([1]).

9. **Performance characteristics: criteria for the sum of non-dioxin-like PCBs at the maximum level**

	Isotope dilution mass spectrometry ([1])	Other techniques
Trueness	– 20 to + 20 %	– 30 to + 30 %
Intermediate precision (RSD %)	≤ 15 %	≤ 20 %
Difference between upper and lower-bound calculation	≤ 20 %	≤ 20 %

([1]) Use of all six ^{13}C-labelled analogues as internal standards required.

10. **Reporting of the results**

10.1. The analytical results shall contain the levels of the individual non-dioxin-like PCBs and the sum of those PCB congenersreported as lower-bound, upper-bound and medium-bound in order to include a maximum of information in the reporting of the results and thereby enabling the interpretation of the results according to specific requirements.

10.2. The report shall include the method used for the extraction of PCBs.

10.3. The recoveries of the individual internal standards shall be made available in case the recoveries are outside the range referred to in point 7, in case the maximum level is exceeded and in other cases upon request.

10.4. As the expanded measurement uncertainty is to be taken into account when deciding about the compliance of a sample, that parameter shall also be made available. Thus, analytical results shall be reported as x +/– U whereby x is the analytical result and U is the expanded measurement uncertainty using a coverage factor of 2 which gives a level of confidence of approximately 95 %.

10.5. The results shall be expressed in the same units and with at least the same number of significant figures as the maximum levels laid down by Directive 2002/32/EC.

([1]) Current requirements are based on the TEFs published in: M. Van den Berg et al, Toxicol Sci 93(2), 223–241 (2006).

▼M2

ANNEX VI

METHODS OF ANALYSIS FOR THE DETERMINATION OF CONSTITUENTS OF ANIMAL ORIGIN FOR THE OFFICIAL CONTROL OF FEED

1. PURPOSE AND SCOPE

The determination of constituents of animal origin in feed shall be performed by light microscopy or polymerase chain reaction (PCR) in accordance with the provisions laid down in this Annex.

These two methods make it possible to detect the presence of constituents of animal origin in feed materials and compound feed. However, they do not make it possible to calculate the amount of such constituents in feed materials and compound feed. Both methods have a limit of detection below 0,1 % (w/w).

The PCR method makes it possible to identify the taxonomic group of constituents of animal origin present in feed materials and compound feed.

These methods shall apply for the control of the application of the prohibitions laid down in Article 7(1) and Annex IV to Regulation (EC) No 999/2001 and in Article 11(1) of Regulation (EC) No 1069/2009.

Depending on the type of feed being tested, these methods may be used, within one single operational protocol, either on their own or combined together in accordance with the standard operating procedures (SOP) established by the EU reference laboratory for animal proteins in feedingstuffs (EURL-AP) and published on its website ([1]).

2. METHODS

2.1. **Light microscopy**

2.1.1. ►M7 ***Principle***

The constituents of animal origin which may be present in feed materials and compound feed sent for analysis are identified on the basis of typical and microscopically identifiable characteristics like muscle fibres and other meat particles, cartilage, bones, horn, hair, bristles, blood, milk globules, lactose crystals, feathers, egg shells, fish bones and scales. ◄

2.1.2. ***Reagents and equipment***

2.1.2.1. Reagents

2.1.2.1.1. Concentrating agent

2.1.2.1.1.1. Tetrachloroethylene (specific gravity 1,62)

2.1.2.1.2. Staining reagent

2.1.2.1.2.1. Alizarin Red solution (dilute 2,5 ml 1M hydrochloric acid in 100 ml water and add 200 mg Alizarin Red to this solution)

2.1.2.1.3. Mounting media

2.1.2.1.3.1. Lye (NaOH 2,5 % w/v or KOH 2,5 % w/v)

([1]) http://eurl.craw.eu/

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2.1.2.1.3.2. ►M7 Glycerol (undiluted, viscosity: 1 490 cP) or a mounting medium with equivalent properties for non-permanent slide preparation ◄

2.1.2.1.3.3. Norland ® Optical Adhesive 65 (viscosity: 1 200 cP) or a resin with equivalent properties for permanent slide preparation

2.1.2.1.4. Mounting media with staining properties

2.1.2.1.4.1. Lugol solution (dissolve 2 g potassium iodide in 100 ml water and add 1 g iodine while frequently shaking)

2.1.2.1.4.2. Cystine reagent (2 g lead acetate, 10 g NaOH/100 ml water)

2.1.2.1.4.3. Fehling's reagent (prepared before use from equals parts (1/1) of two stock solutions A and B. Solution A: dissolve 6,9 g copper (II) sulphate pentahydrate in 100 ml water. Solution B: dissolve 34,6 g potassium sodium tartrate tetrahydrate and 12 g NaOH in 100 ml water)

2.1.2.1.4.4. Tetramethylbenzidine/Hydrogen peroxide. (dissolve 1 g 3,3',5,5' tetramethylbenzidine (TMB) in 100 ml glacial acetic acid and 150 ml water. Before use, mix 4 parts of this TMB solution with 1 part 3 % hydrogen peroxide)

2.1.2.1.5. Rinsing agents

2.1.2.1.5.1. Ethanol ≥ 96 % (technical grade)

2.1.2.1.5.2. Acetone (technical grade)

2.1.2.1.6. Bleaching reagent

2.1.2.1.6.1. Commercial sodium hypochlorite solution (9 - 14 % active chlorine)

2.1.2.2. Equipment

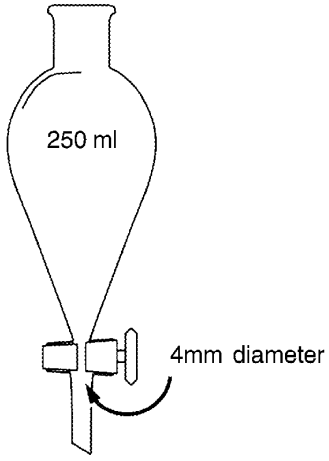
2.1.2.2.1. Analytical balance with an accuracy of 0,001 g

2.1.2.2.2. ►M7 Grinding equipment: knife or rotor mill. If a rotor mill is used, mill sieves ≤ 0,5 mm shall be prohibited ◄

2.1.2.2.3. ►M7 Sieves with square meshes of 0,25 mm and 1 mm width. With the exception of sample pre-sieving, the diameter of the sieves should not exceed 10 cm to avoid loss of materials. Calibration of sieves is not required ◄

2.1.2.2.4. Conical glass separation funnel with a content of 250 ml with Teflon or ground glass stopcock at the base of the cone. Stopcock opening diameter shall be ≥ 4mm. Alternatively, a conical bottomed settling beaker may be used provided the laboratory has demonstrated that detection levels are equivalent to that obtained using the conical glass separation funnel.

Separation funnel



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2.1.2.2.5. Stereomicroscope covering at least a 6,5× to 40× final magnification range

2.1.2.2.6. Compound microscope covering at least a 100× to 400× final magnification range with transmitted light bright field. Polarised light and differential interferential contrast can additionally be used

2.1.2.2.7. Standard laboratory glassware

2.1.2.2.8. Equipment for slide preparation: classical microscope slides, hollow slides, coverslips (20 × 20 mm), tweezers, fine spatula

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2.1.2.2.9. Laboratory oven

2.1.2.2.10. Centrifuge

2.1.2.2.11. Filter paper: qualitative cellulose filter (pore size 4-11 μm)

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2.1.3. ***Sampling and sample preparation***

2.1.3.1. ►M7 Sampling

A representative sample, taken in accordance with the provisions laid down in Annex I to this Regulation shall be used. ◄

2.1.3.2. Precautions to be taken

In order to avoid laboratory cross-contamination, all reusable equipment shall be carefully cleaned before use. Separation funnel pieces shall be disassembled before cleaning. Separation funnel pieces and glassware shall be pre-washed manually and then washed in a washing machine. Sieves shall be cleaned by using a brush with stiff synthetic hairs. A final cleaning of sieves with acetone and compressed air is recommended after sieving of fatty material like fishmeal.

2.1.3.3. Preparation of samples other than fat or oil

2.1.3.3.1. ►M7 Sample drying: samples with a moisture content > 14 % shall be dried prior to handling according to Annex III to this Regulation. ◄

2.1.3.3.2. ►M7 Sample pre-sieving: in order to collect information on possible environmental contamination of the feed, it is recommended to pre-sieve at 1 mm pelleted feeds and kernels and to subsequently prepare, analyse, and report separately on the two resulting fractions, which must be considered as distinct samples. ◄

2.1.3.3.3. Sub-sampling and grinding: at least 50 g of the sample shall be sub-sampled for analysis and subsequently ground.

2.1.3.3.4. Extraction and preparation of the sediment: a portion of 10 g (accurate to 0,01 g) of the ground sub-sample shall be transferred into the separation funnel or conical bottomed settling beaker and 50 ml of tetrachloroethylene shall be added. The portion transferred into the funnel shall be limited to 3 g in case of fishmeal or other pure animal products, mineral ingredients or premixes which generate more than 10 % of sediment. The mixture shall be vigorously shaken for at least 30 s and at least 50 ml more of tetrachloroethylene shall be added cautiously while washing down the inside surface of the funnel to remove any adhering particles. The resulting mixture shall be left to stand for at least 5 minutes before the sediment is separated off by opening the stopcock.

If a conical bottomed settling beaker is used then the mixture shall be vigorously stirred for at least 15 s and any particles adhering to the side of the beaker shall be carefully washed down the inside surface with at least 10 ml of clean tetrachloroethylene. The mixture shall be left to stand for 3 minutes and then stirred again for 15 seconds and any particles adhering to the side of the beaker shall be carefully washed down the inside surface with at least 10 ml of clean tetrachloroethylene. The resulting mixture shall be left to stand for at least 5 minutes and then the liquid fraction is removed and discarded by careful decanting, taking care not to lose any of the sediment.

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The sediment shall be collected on a filter paper placed into a funnel to allow the separation of the remaining TCE while avoiding fat deposition into the sediment. The sediment shall be dried. It is recommended to subsequently weigh the sediment (accurate to 0,001 g) to control the sedimentation step. Lastly, the sediment shall be sieved at 0,25 mm and the two resulting fractions shall be examined, unless sieving is not deemed necessary.

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2.1.3.3.5. Extraction and preparation of the flotate: after recovery of the sediment with the method described above, two phases should remain in the separation funnel: a liquid one consisting of tetrachloroethylene and a solid one made of floating material. This solid phase is the flotate and shall be recovered by pouring off completely tetrachloroethylene from the funnel by opening the stopcock. By inverting the separation funnel, the flotate shall be transferred into a large Petri dish and air dried in a fumehood. If more than 5 % of the flotate consists of particles > 0,50 mm, it shall be sieved at 0,25 mm and the two resulting fractions shall be examined.

2.1.3.3.6. Preparation of raw material: a portion of at least 5 g of the ground sub-sample shall be prepared. If more than 5 % of the material consists of particles > 0,50 mm, it shall be sieved at 0,25 mm and the two resulting fractions shall be examined.

2.1.3.4. Preparation of samples consisting of fat or oil

The following protocol shall be followed for the preparation of samples consisting of fat or oil:

— if the fat is solid, it shall be warmed in a oven until it is liquid.

— by using a pipette, 40 ml of fat or oil shall be transferred from the bottom of the sample to a centrifugation tube.

— centrifuge during 10 minutes at 4 000 r.p.m.

— if the fat is solid after centrifugation, it shall be warmed in an oven until it is liquid.

— repeat the centrifugation during 5 minutes at 4 000 r.p.m.

— by using a small spoon or a spatula, one half of the decanted impurities shall be transferred to microscopic slides for examination, Glycerol is recommended as mounting medium.

— the remaining impurities shall be used for preparing the sediment as described in point 2.1.3.3.

2.1.3.5. Use of staining reagents

In order to facilitate the correct identification of the constituents of animal origin, the operator may use staining reagents during the sample preparation in accordance with guidelines issued by the EURL-AP and published on its website.

In case Alizarin Red solution is used to colour the sediment, the following protocol shall apply:

— the dried sediment shall be transferred into a glass test tube and rinsed twice with approximately 5 ml of ethanol (each time a vortex of 30 s shall be used, the solvent shall be let settle about 1 min 30 s and poured off).

— the sediment shall be bleached by adding at least 1 ml sodium hypochlorite solution. The reaction shall be allowed to continue for 10 min. The tube shall be filled with water, the sediment shall be let settle 2-3 min, and the water and the suspended particles shall be poured off gently.

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— the sediment shall be rinsed twice more with about 10 ml of water (a vortex shall be used for 30 s, let settle, and pour off the water each time).

— 2 to 10 drops of the Alizarin Red solution shall be added and the mixture shall be vortexed. The reaction shall be let occur for 30 s and the coloured sediment shall be rinsed twice with approximately 5 ml ethanol followed by one rinse with acetone (each time a vortex of 30 s shall be used, the solvent shall be let settle about 1 min and poured off).

— the coloured sediment shall be dried.

2.1.4. ***Microscopic examination***

2.1.4.1. Slide preparation

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Microscopic slides shall be prepared from the sediment and, depending on the operator's choice, from either the flotate or the raw material.

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A sufficient number of slides shall be prepared in order to ensure that a complete examination protocol as laid down in point 2.1.4.2 can be carried-out.

Microscopic slides shall be mounted with the adequate mounting medium in accordance with the SOP established by the EURL-AP and published on its website. The slides shall be covered with coverslips.

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2.1.4.2. Observation flowchart for the detection of animal particles in compound feed and feed material

The prepared microscopic slides shall be observed in accordance with the observation flowcharts laid down in diagrams 1 and 2.

The microscopic observations shall be conducted using the compound microscope on the sediment and, depending on the operator's choice, either on the flotate or on the raw material. The stereomicroscope may be used in addition to the compound microscope for the coarse fractions. Each slide shall be screened entirely at various magnifications. Precise explanations on how to use the observation flowcharts are detailed by a SOP established by the EURL-AP and published on its website.

The minimum numbers of slides to be observed at each step of the observation flowcharts shall be strictly respected, unless the entire fraction material does not permit to reach the stipulated slide number, for instance when no sediment is obtained. No more than 6 slides per determination shall be used for recording of the number of particles.

When additional slides are prepared on the flotate or the raw material using a more specific mounting medium with staining properties, as laid down in point 2.1.2.1.4, to further characterise structures (e.g. feathers, hairs, muscle or blood particles) which have been detected on slides prepared by other mounting media, as laid down in point 2.1.2.1.3, the number of particles shall be counted based on a number of slides per determination not exceeding 6, including the additional slides with a more specific mounting medium.

In order to facilitate the identification of the particles' nature and origin, the operator may use support tools like decision support systems, image libraries and reference samples.

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Diagram 1

Observation flowchart for the detection of animal particles in compound feed and feed material for the first determination.

(D1 and D2 refer to the first and second determinations; *: terrestrial vertebrate, fish)

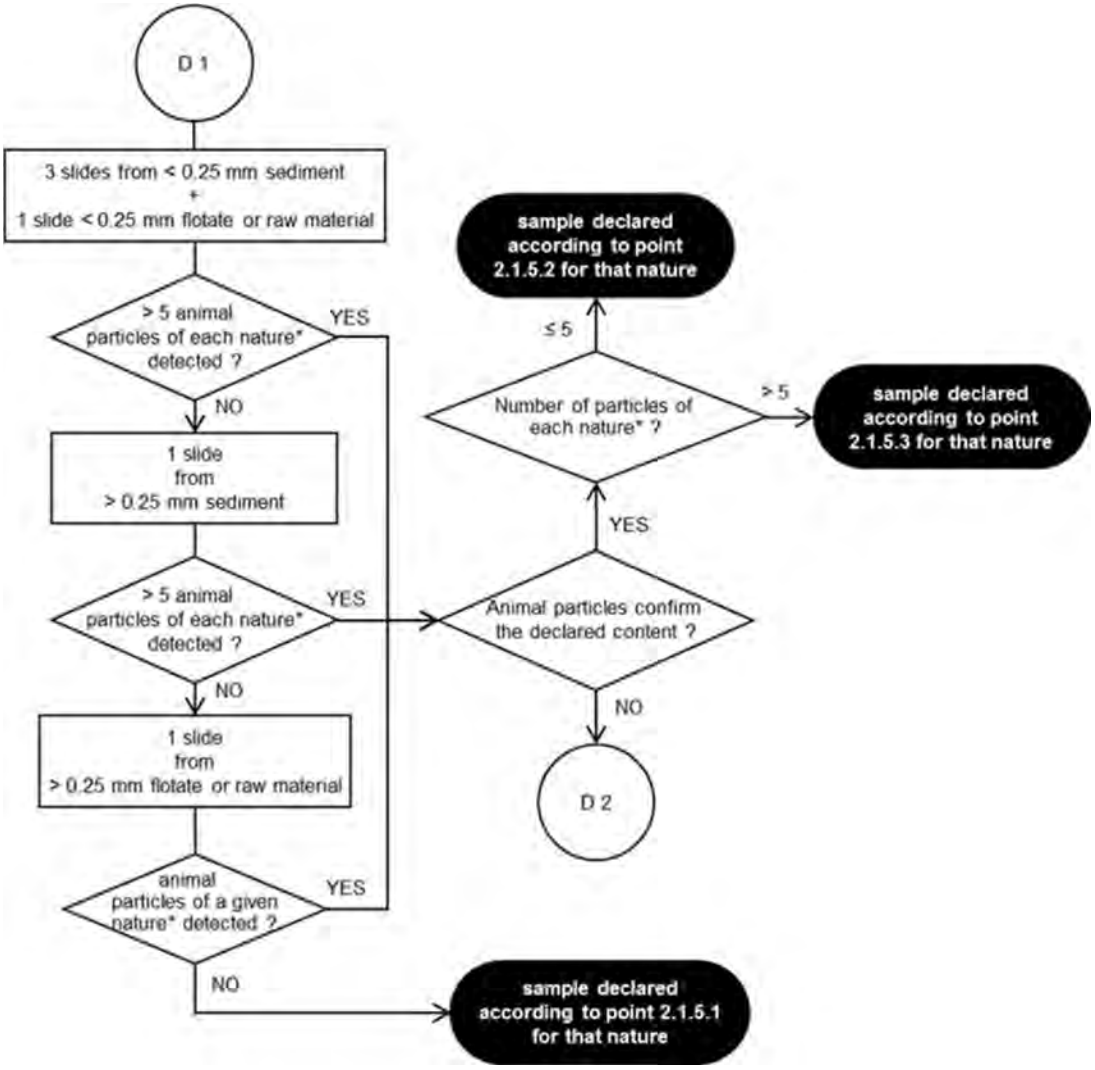

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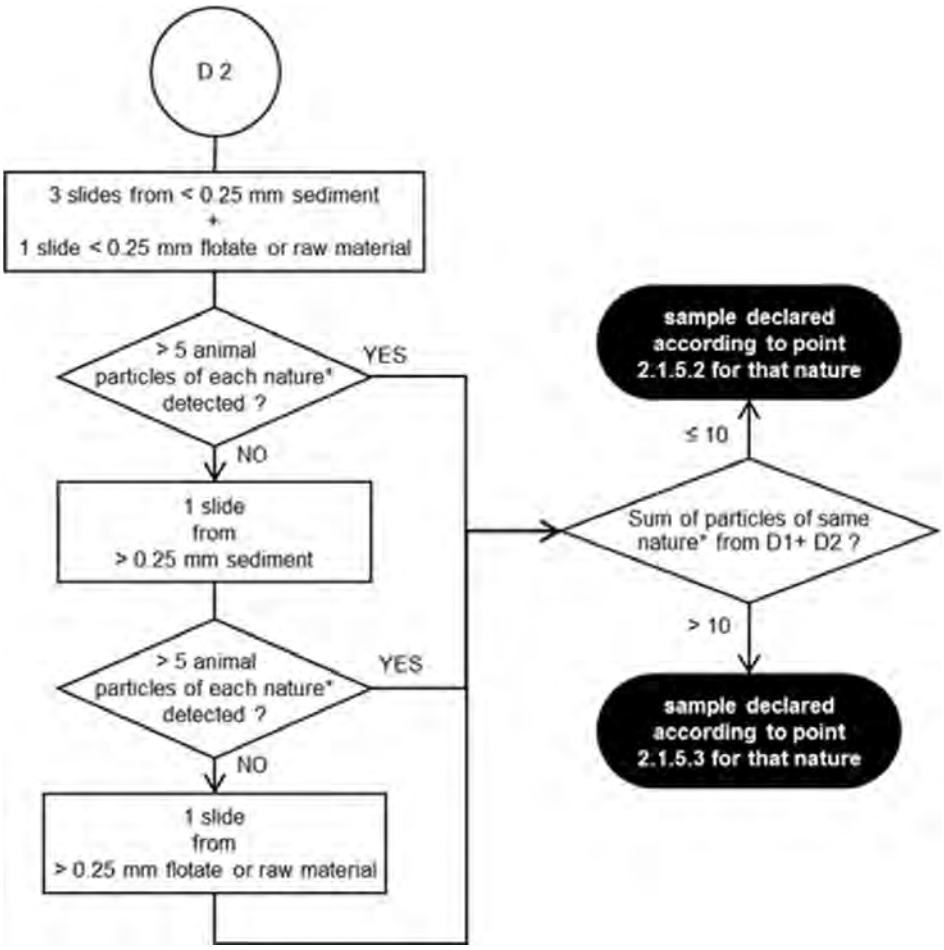
Diagram 2

Observation flowchart for the detection of animal particles in compound feed and feed material for the second determination

(D1 and D2 refer to the first and second determinations; *: terrestrial vertebrate, fish)



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2.1.4.3. ►M7 Number of determinations

Determinations shall be performed on different sub-samples of 50 g each.

If following the first determination carried out in accordance with the observation flowchart laid down in diagram 1, no animal particles are detected, no additional determination is necessary and the result of the analysis shall be reported using the terminology laid down in point 2.1.5.1.

If, following the first determination carried out in accordance with the observation flowchart laid down in diagram 1, one or more animal particles of a given nature (i.e. terrestrial vertebrate or fish) are detected, and the nature of the particles found confirms the declared content of the sample, no second determination is necessary. If the number of the animal particles of a given nature detected during this first determination is higher than 5, the result of the analysis shall be reported per animal nature using the terminology laid down in point 2.1.5.3. Otherwise, the result of the analysis shall be reported per animal nature using the terminology laid down in point 2.1.5.2.

In other cases, including when no declaration of content has been provided to the laboratory a second determination shall be carried out from a new sub-sample.

If, following the second determination carried out in accordance with the observation flowchart laid down in diagram 2, the sum of the animal particles of a given nature detected over the two determinations is higher than 10, the result of the analysis shall be reported per animal nature using the terminology laid down in point 2.1.5.3. Otherwise, the result of the analysis shall be reported per animal nature using the terminology laid down in point 2.1.5.2. ◄

2.1.5. ►M7 ***Expression of the results***

When reporting the results, the laboratory shall indicate on which type of material the analysis has been carried-out (sediment, flotate or raw material). The reporting shall clearly indicate how many determinations have been carried-out and if sieving of the fractions prior to slide preparation, in accordance with the last paragraph of point 2.1.3.3.4., was not performed.

The laboratory report shall at least contain information on the presence of constituents derived from terrestrial vertebrates and from fish.

The different situations shall be reported in the following ways.

2.1.5.1. No animal particle of a given nature detected:

— ‘As far as was discernible using a light microscope, no particle derived from terrestrial vertebrates was detected in the submitted sample.’

— ‘As far as was discernible using a light microscope, no particle derived from fish was detected in the submitted sample.’

▼M2

2.1.5.2. Between 1 and 5 animal particles of a given nature detected when only one determination has been performed, or between 1 and 10 particles of a given nature detected in case of two determinations (the number of detected particles is below the decision limit established in the standard operating procedures (SOP) of the EU reference laboratory for animal proteins in feedingstuffs (EURL-AP) and published on its website ([1])):

When only one determination has been performed:

— 'As far as was discernible using a light microscope, no more than 5 particles derived from terrestrial vertebrates were detected in the submitted sample. The particles were identified as … [bone, cartilage, muscle, hair, horn…]. This low level presence is below the decision limit established for this microscopic method.'

— 'As far as was discernible using a light microscope, no more than 5 particles derived from fish were detected in the submitted sample. The particles were identified as … [fishbone, fish scale, cartilage, muscle, otolith, gill…]. This low level presence, is below the decision limit established for this microscopic method.'

When two determinations have been performed:

— 'As far as was discernible using a light microscope, no more than 10 particles derived from terrestrial vertebrates were detected over the two determinations in the submitted sample. The particles were identified as … [bone, cartilage, muscle, hair, horn…]. This low level presence is below the decision limit established for this microscopic method.'

— 'As far as was discernible using a light microscope, no more than 10 particles derived from fish were detected over the two determinations in the submitted sample. The particles were identified as … [fishbone, fish scale, cartilage, muscle, otolith, gill…]. This low level presence is below the decision limit established for this microscopic method.'

Additionally:

— In case of sample pre-sieving, the laboratory report shall mention in which fraction (sieved fraction, pelleted fraction or kernels) the animal particles have been detected insofar as the detection of animal particles only in the sieved fraction may be the sign of an environmental contamination.

— When only animal particles which cannot be categorised as either terrestrial vertebrates or fish are detected (e.g. muscle fibres), the report shall mention that only such animal particles were detected and that it cannot be excluded that they originate from terrestrial vertebrates

2.1.5.3. More than 5 animal particles of a given nature detected when only one determination has been performed, or more than 10 particles of a given nature detected in case of two determinations:

([1]) http://eurl.craw.eu/

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When only one determination has been performed:

— ‘As far as was discernible using a light microscope, more than 5 particles derived from terrestrial vertebrates were detected in the submitted sample. The particles were identified as … [bone, cartilage, muscle, hair, horn…].’

— ‘As far as was discernible using a light microscope, more than 5 particles derived from fish were detected in the submitted sample. The particles were identified as … [fishbone, fish scale, cartilage, muscle, otolith, gill…].’

When two determinations have been performed:

— ‘As far as was discernible using a light microscope, more than 10 particles derived from terrestrial vertebrates were detected over the two determinations in the submitted sample. The particles were identified as … [bone, cartilage, muscle, hair, horn…].’

— ‘As far as was discernible using a light microscope, more than 10 particles derived from fish were detected over the two determinations in the submitted sample. The particles were identified as … [fishbone, fish scale, cartilage, muscle, otolith, gill…].’

Additionally:

— In case of sample pre-sieving, the laboratory report shall mention in which fraction (sieved fraction, pelleted fraction or kernels) the animal particles have been detected insofar as the detection of animal particles only in the sieved fraction may be the sign of an environmental contamination.

— When only animal particles which cannot be categorised as either terrestrial vertebrates or fish are detected (e.g. muscle fibres), the report shall mention that only such animal particles were detected and that it cannot be excluded that they originate from terrestrial vertebrates. ◄

2.2. **PCR**

2.2.1. ***Principle***

Deoxyribonucleic acid (DNA) fragments of animal origin which may be present in feed materials and compound feed are detected by a genetic amplification technique through PCR, targeting species-specific DNA sequences.

The PCR method first requires a DNA extraction step. The amplification step shall be applied afterwards to the so-obtained DNA extract, in order to detect the animal species targeted by the assay.

2.2.2. ***Reagents and equipment***

2.2.2.1. Reagents

2.2.2.1.1. Reagents for DNA extraction step

Only reagents approved by the EURL-AP and published on its website shall be used.

2.2.2.1.2. Reagents for genetic amplification step

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2.2.2.1.2.1. Primers and probes

Only primers and probes with sequences of oligonucleotides validated by the EURL-AP shall be used ([1]).

2.2.2.1.2.2. Master Mix

Only Master Mix solutions which do not contain reagents susceptible to lead to false results due to presence of animal DNA shall be used ([2]).

2.2.2.1.2.3. Decontamination reagents

2.2.2.1.2.3.1. Hydrochloric acid solution (0,1 N)

2.2.2.1.2.3.2. Bleach (solution of sodium hypochlorite at 0,15 % of active chlorine)

2.2.2.1.2.3.3. Non-corrosive reagents for decontaminating costly devices like analytical balances (e.g. DNA Erase™ of MP Biomedicals)

2.2.2.2. Equipment

2.2.2.2.1. Analytical balance with an accuracy of 0,001 g

2.2.2.2.2. Grinding equipment

2.2.2.2.3. Thermocycler enabling real-time PCR

2.2.2.2.4. Microcentrifuge for microfuge tubes

2.2.2.2.5. Set of micropipettes allowing to pipet from 1 µl up to 1 000 µl

2.2.2.2.6. Standard molecular biology plastic-ware: microfuge tubes, filtered plastic tips for micropipettes, plates suitable for the thermocycler.

2.2.2.2.7. Freezers to store samples and reagents

2.2.3. ***Sampling and sample preparation***

2.2.3.1. Sampling

A representative sample, taken in accordance with the provisions laid down in Annex I, shall be used.

2.2.3.2. Sample preparation

The preparation of laboratory samples up to DNA extraction shall comply with the requirements set out in Annex II. At least 50 g of the sample shall be sub-sampled for analysis and subsequently ground.

The sample preparation shall be performed in a room different from the ones dedicated to DNA extraction and to genetic amplification reactions as described by ISO 24276.

Two test portions of at least 100 mg each shall be prepared.

2.2.4. ***DNA extraction***

The DNA extraction shall be performed on each test portion prepared using the SOP established by the EURL-AP and published on its website.

Two extraction controls shall be prepared for each extraction series as described by ISO 24276.

— an extraction blank control,

— a positive DNA extraction control.

([1]) The list of these primers and probes for each animal species targeted by the assay is available on the EURL-AP website.

([2]) Examples of Master Mixes that are functional are available on the EURL-AP website.

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2.2.5. ***Genetic amplification***

The genetic amplification shall be performed using the methods validated for each species requiring identification. These methods are laid down in the SOP established by the EURL-AP and published on its website. Each DNA extract shall be analysed at least at two different dilutions in order to evaluate inhibition.

Two amplification controls shall be prepared per species target as described by ISO 24276.

— a positive DNA target control shall be used for each plate or series of PCR assays,

— an amplification reagent control (also called no template control) shall be used for each plate or series of PCR assays.

2.2.6. ***Interpretation and expression of results***

When reporting the results, the laboratory shall indicate at least the weight of the test portions used, the extraction technique used, the number of determinations carried-out and the limit of detection of the method.

Results shall not be interpreted and reported if the positive DNA extraction control and the positive DNA target controls do not provide positive results for the target under assay while the amplification reagent control is negative.

In case results from the two test portions are not consistent, at least the genetic amplification step shall be repeated. If the laboratory suspects that the DNA extracts can be the cause of the inconsistency, a new DNA extraction and a subsequent genetic amplification shall be performed before interpreting the results.

The final expression of the results shall be based on the integration and the interpretation of the results of the two test portions in accordance with the SOP established by the EURL-AP and published on its website.

2.2.6.1. Negative result

A negative result shall be reported as follows:

No DNA from X was detected in the submitted sample (with X being the animal species or group of animal species that is targeted by the assay).

2.2.6.2. Positive result

A positive result shall be reported as follows:

DNA from X was detected in the submitted sample (with X being the animal species or group of animal species that is targeted by the assay).

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ANNEX VII

METHOD OF CALCULATING THE ENERGY VALUE OF POULTRYFEED

1. **Method of calculation and expression of energy value**

   The energy value of compound poultry feed must be calculated in accordance with the formula set out below on the basis of the percentages of certain analytical components of the feed. This value is to be expressed in megajoules (MJ) of metabolisable energy (ME), corrected for nitrogen, per kilogram of compound feed:

   MJ/kg of ME = 0,1551 × % crude protein + 0,3431 × % crude fat + 0,1669 × % starch + 0,1301 × % total sugar (expressed as sucrose).

2. **Tolerances applicable to declared values**

   If the official inspection reveals a discrepancy (increased or reduced energy value of the feed) between the result of the inspection and the declared energy value, a minimum tolerance of 0,4 MJ/kg of ME shall be permitted.

3. **Expression of result**

   After application of the above formula, the result obtained must be given to one decimal place.

4. **Sampling and analysis methods**

   Sampling of the compound feed and determination of the content of analytical components indicated in the method of calculation must be performed in accordance with the Community sampling methods and analysis methods for the official control of feed respectively.

   The following are to be applied:

   — for determining the crude fat content: procedure B of the method for the determination of crude oils and fats, laid down in Part H of Annex III.

   — for determining the starch content: the polarimetric method, laid down in Part L of Annex III.

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ANNEX VIII

METHODS OF ANALYSIS TO CONTROL ILLEGAL PRESENCE OF NO LONGER AUTHORISED ADDITIVES IN FEED

Important notes:

More sensitive methods of analysis than the methods of analysis mentioned in this Annex can be used to detect the illegal presence of no longer authorised additives in feed.

The methods of analysis mentioned in this Annex shall be used for confirmatory purposes.

A. DETERMINATION OF METHYL BENZOQUATE

7-benzyloxy-6-butyl-3-methoxycarbonyl-4-quinolone

1. **Purpose and scope**

This method makes it possible to determine the level of methyl benzoquate in feed. The limit of quantification is 1 mg/kg.

2. **Principle**

Methyl benzoquate is extracted from the sample with methanolic methanesulfonic acid solution. The extract is purified with dichloromethane, by ion-exchange chromatography and then again with dichloromethane. The methyl benzoquate content is determined by reversed-phase high-performance liquid chromatography (HPLC) with an UV detector.

3. **Reagents**

3.1. Dichloromethane

3.2. Methanol, equivalent to HPLC grade

3.3. HPLC mobile phase

Mixture of methanol (3.2) and water (equivalent to HPLC grade) 75 + 25 (v + v).

Filter through a 0,22 µm filter (4.5) and degas the solution (e.g. by ultrasonification for 10 minutes).

3.4. Methanesulfonic acid solution, c = 2 %

Dilute 20,0 ml methanesulfonic acid to 1 000 ml with methanol (3.2).

3.5. Hydrochloric acid solution, c = 10 %

Dilute 100 ml hydrochloric acid (ρ_{20}1,18 g/ml) to 1 000 ml with water.

3.6. Cation-exchange resin Amberlite CG-120 (Na), 100 to 200 mesh

The resin is pretreated before use. Slurry 100 g resin with 500 ml hydrochloric acid solution (3.5) and heat on a hot plate to boiling, stirring continuously. Allow to cool and decant off the acid. Filter through a filter paper under vacuum. Wash the resin twice with 500 ml portions of water and then with 250 ml of methanol (3.2). Rinse the resin with a further 250 ml portion of methanol and dry by passing air through the filter cake. Store the dried resin in a stoppered bottle.

3.7. Standard substance: pure methyl benzoquate (7-benzyloxy-6-butyl-3-methoxycarbonyl-4-quinolone)

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3.7.1. Methyl benzoquate stock standard solution, 500 µg/ml

Weigh to the nearest 0,1 mg, 50 mg of standard substance (3.7), dissolve in methanesulfonic acid solution (3.4) in a 100 ml graduated flask, make up to the mark and mix.

3.7.2. Methyl benzoquate intermediate standard solution, 50 µg/ml

Transfer 5,0 ml of methyl benzoquate stock standard solution (3.7.1) into a 50 ml graduated flask, make up to the mark with methanol (3.2) and mix.

3.7.3. Calibration solutions

Transfer 1,0, 2,0, 3,0, 4,0 and 5,0 ml of methyl benzoquate intermediate standard solution (3.7.2) into a series of 25 ml graduated flasks. Make up to the mark with the mobile phase (3.3) and mix. These solutions have concentrations of 2,0, 4,0, 6,0, 8,0 and 10,0 µg/ml methyl benzoquate respectively. These solutions must be freshly prepared before use.

4. **Apparatus**

4.1. Laboratory shaker

4.2. Rotary film evaporator

4.3. Glass column (250 mm × 15 mm) fitted with a stopcock and reservoir of approximately 200 ml capacity

4.4. HPLC equipment with variable wavelength ultraviolet detector or diode-array detector

4.4.1. Liquid chromatographic column: 300 mm × 4 mm, C_{18}, 10 µm packing or equivalent

4.5. Membrane filters, 0,22 µm

4.6. Membrane filters, 0,45 µm

5. **Procedure**

5.1. ***General***

5.1.1. A blank feed shall be analysed to check that neither methyl benzoquate nor interfering substances are present.

5.1.2. A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of methyl benzoquate, similar to that present in the sample. To fortify at a level of 15 mg/kg, add 600 µl of the stock standard solution (3.7.1) to 20 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Note for the purpose of this method, the blank feed shall be similar in type to that of the sample and on analysis methyl benzoquate must not be detected.

5.2. ***Extraction***

Weigh to the nearest 0,01 g, approximately 20 g of the prepared sample and transfer to a 250 ml conical flask. Add 100,0 ml of methanesulfonic acid solution (3.4) and shake mechanically (4.1) for 30 minutes. Filter the solution through a filter paper and retain the filtrate for the liquid-liquid partition step (5.3).

5.3. ***Liquid-liquid partition***

Transfer into a 500 ml separating funnel containing 100 ml of hydrochloric acid solution (3.5), 25,0 ml of the filtrate obtained in (5.2). Add 100 ml dichloromethane (3.1) to the funnel and shake for one minute. Allow the layers to separate and run off the lower (dichloromethane) layer into a 500 ml round-bottomed flask. Repeat the extraction of the aqueous phase with two further 40-ml portions of dichloromethane and

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combine these with the first extract in the round-bottomed flask. Evaporate the dichloromethane extract down to dryness on the rotary evaporator (4.2) operating under reduced pressure at 40 °C. Dissolve the residue in 20 to 25 ml methanol (3.2), stopper the flask and retain the whole of the extract for ion-exchange chromatography (5.4).

5.4. ***Ion-exchange chromatography***

5.4.1. Preparation of the cation-exchange column

Insert a plug of glass wool into the lower end of a glass column (4.3). Prepare a slurry of 5,0 g of the treated cation-exchange resin (3.6) with 50 ml of hydrochloric acid (3.5), pour into the glass column and allow to settle. Run out the excess acid to just above the resin surface and wash the column with water until the effluent is neutral to litmus. Transfer 50 ml methanol (3.2) onto the column and allow to drain down to the resin surface.

5.4.2. Column chromatography

By means of a pipette, carefully transfer the extract obtained in (5.3) onto the column. Rinse the round-bottomed flask with two portions of 5 to 10 ml methanol (3.2) and transfer these washings to the column. Run the extract down to the resin surface and wash the column with 50 ml methanol, ensuring that the flow rate does not exceed 5 ml per minute. Discard the effluent. Elute the methyl benzoquate from the column using 150 ml of methanesulfonic acid solution (3.4) and collect the column eluate in a 250 ml conical flask.

5.5. ***Liquid-liquid partition***

Transfer the eluate obtained in (5.4.2) into a 1 litre separating funnel. Rinse the conical flask with 5 to 10 ml methanol (3.2) and combine the washings with the contents of the separating funnel. Add 300 ml of hydrochloric acid solution (3.5) and 130 ml of dichloromethane (3.1). Shake for 1 minute and allow the phases to separate. Run off the lower (dichloromethane) layer into a 500 ml round bottomed flask. Repeat the extraction of the aqueous phase with two further 70 ml portions of dichloromethane and combine these extracts with the first in the round-bottomed flask.

Evaporate the dichloromethane extract down to dryness on the rotary evaporator (4.2) operating under reduced pressure at 40 °C. Dissolve the residue in the flask with approximately 5 ml of methanol (3.2) and transfer this solution quantitatively to a 10 ml graduated flask. Rinse the round-bottomed flask with a further two portions of 1 to 2 ml of methanol and transfer these to the graduated flask. Make up to the mark with methanol and mix. An aliquot portion is filtered through a membrane filter (4.6). Reserve this solution for HPLC-determination (5.6).

5.6. ***HPLC determination***

5.6.1. Parameters

The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results:

— liquid chromatographic column (4.4.1),

— HPLC mobile phase: methanol-water mixture (3.3),

— flow rate: 1 to 1,5 ml/minute,

— detection wavelength: 265 nm,

— Injection volume: 20 to 50 µl.

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Check the stability of the chromatographic system, injecting the calibration solution (3.7.3) containing 4 μg/ml several times, until constant peak heights or areas and retention times are achieved.

5.6.2. Calibration graph

Inject each calibration solution (3.7.3) several times and measure the peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights or areas of the calibration solutions as the ordinates and the corresponding concentrations in μg/ml as the abscissae.

5.6.3. Sample solution

Inject the sample extract (5.5) several times, using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the methyl benzoquate peaks.

6. **Calculation of results**

Determine the concentration of the sample solution in μg/ml from the mean height (area) of the methyl benzoquate peaks of the sample solution by reference to the calibration graph (5.6.2).

The content of methyl benzoquate w (mg/kg) of the sample is given by the following formula:

$$w = \frac{c \times 40}{m}$$

in which:

c = methyl benzoquate concentration of the sample solution in μg/ml,
m = weight of the test portion in grams.

7. **Validation of the results**

7.1. ***Identity***

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract and the calibration solution (3.7.3) containing 10 μg/ml are compared.

7.1.1. Co-chromatography

A sample extract is fortified by addition of an appropriate amount of the intermediate standard solution (3.7.2). The amount of added methyl benzoquate must be similar to the estimated amount of methyl benzoquate in the sample extract.

Only the height of the methyl-benzoquate peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within approximately 10 % of the original width.

7.1.2. Diode-array detection

The results are evaluated according to the following criteria:

(a) the wavelength of maximum absorption of the sample and of the standard spectra recorded at the peak apex on the chromatogram must be the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within approximately 2 nm;

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(b) between 220 and 350 nm, the sample and standard spectra recorded at the peak apex on the chromatogram must not be different for those parts of the spectrum within the range 10 % to 100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte;

(c) between 220 and 350 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 % to 100 % of relative absorbance. This criterion is met when the same maxima are present and when at no observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed: 10 % relative to the higher result for methyl benzoquate contents between 4 and 20 mg/kg.

7.3. ***Recovery***

For a fortified blank sample the recovery shall be at least 90 %.

8. **Results of a collaborative study**

Five samples were analysed by 10 laboratories. Duplicate analyses were performed on each sample.

	Blank	Meal 1	Pellet 1	Meal 2	Pellet2
Mean [mg/kg]	ND	4,50	4,50	8,90	8,70
s_r [mg/kg]	—	0,30	0,20	0,60	0,50
CV_r [%]	—	6,70	4,40	6,70	5,70
s_R [mg/kg]	—	0,40	0,50	0,90	1,00
CV_R [%]	—	8,90	11,10	10,10	11,50
Recovery [%]	—	92,00	93,00	92,00	89,00

ND = Not detected
s_r = standard deviation of repeatability
CV_r = coefficient of variation of repeatability, %
s_R = standard deviation of reproducibility
CV_R = coefficient of variation of reproducibility, %.

B. DETERMINATION OF OLAQUINDOX

2-[N-2'-(hydroxyethyl)carbamoyl]-3-methylquinoxaline-N^1,N^4-dioxide

1. **Purpose and scope**

This method makes it possible to determine the level olaquindox in feed. The limit of quantification is 5 mg/kg.

2. **Principle**

The sample is extracted by a water-methanol mixture. The content of olaquindox is determined by reversed-phase high-performance liquid chromatography (HPLC) using an UV detector.

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3. **Reagents**

3.1. Methanol.

3.2. Methanol, equivalent to HPLC grade.

3.3. Water, equivalent to HPLC grade.

3.4. Mobile phase for HPLC.

Water (3.3)-methanol (3.2) mixture, 900 +100 (V + V).

3.5. Standard substance: pure olaquindox 2-[N-2'-(hydroxyethyl)carbamoyl]-3-methylquinoxaline-N^1,N^4-dioxide, E 851.

3.5.1. Olaquindox stock standard solution, 250 µg/ml

Weigh to the nearest 0,1 mg 50 mg of olaquindox (3.5) in a 200 ml graduated flask and add ca. 190 ml water. Then place the flask for 20 min. into an ultrasonic bath (4.1). After ultrasonic treatment bring the solution to room temperature, make up to the mark with water and mix. Wrap the flask with aluminium foil and store in a refrigerator. This solution must be prepared fresh each month.

3.5.2. Olaquindox intermediate standard solution, 25 µg/ml

Transfer 10,0 ml of the stock standard solution (3.5.1) into a 100 ml graduated flask, make up to the mark with the mobile phase (3.4) and mix. Wrap the flask with aluminium foil and store in a refrigerator. This solution must be prepared fresh each day.

3.5.3. Calibration solutions

Into a series of 50 ml graduated flasks transfer 1,0, 2,0, 5,0, 10,0, 15,0 and 20,0 ml of the intermediate standard solution (3.5.2). Make up to the mark with the mobile phase (3.4) and mix. Wrap the flasks with aluminium foil. These solutions correspond to 0,5, 1,0, 2,5, 5,0, 7,5 and 10,0 µg of olaquindox per ml respectively.

These solutions must be prepared fresh each day.

4. **Apparatus**

4.1. Ultrasonic bath

4.2. Mechanical shaker

4.3. HPLC equipment with variable wavelength ultraviolet detector or diode array detector

4.3.1. Liquid chromatographic column, 250 mm × 4 mm, C_{18}, 10 µm packing, or equivalent

4.4. Membrane filters, 0,45 µm

5. **Procedure**

Note: Olaquindox is light sensitive. Carry out all procedures under subdued light or use amber glassware.

5.1. ***General***

5.1.1. A blank feed shall be analysed to check that neither olaquindox nor interfering substances are present.

5.1.2. A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of olaquindox, similar to that present in the sample. To fortify at a level of 50 mg/kg, transfer 10,0 ml of the stock standard solution (3.5.1) to a 250 ml conical flask and

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evaporate the solution to ca. 0,5 ml. Add 50 g of the blank feed, mix thoroughly and leave for 10 min. mixing again several times before proceeding with the extraction step (5.2).

Note: For the purpose of this method the blank feed shall be similar in type to that of the sample and olaquindox must not be detected.

5.2. ***Extraction***

Weigh to the nearest 0,01 g, approximately 50 g of the sample. Transfer to a 1 000 ml conical flask, add 100 ml of methanol (3.1) and place the flask for 5 min. in the ultrasonic bath (4.1). Add 410 ml water and leave in the ultrasonic bath for further 15 min. Remove the flask from the ultrasonic bath, shake it for 30 min. on the shaker (4.2) and filter through a folded filter. Transfer 10,0 ml of the filtrate into a 20 ml graduated flask, make up to the mark with water and mix. An aliquot is filtered through a membrane filter (4.4). (see 9. Observation) Proceed to the HPLC determination (5.3).

5.3. ***HPLC determination***

5.3.1. Parameters:

The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results.

Analytical column (4.3.1)	
Mobile Phase (3.4):	water (3.3)-methanol (3.2) mixture, 900 + 100 (V + V)
Flow rate:	1,5-2 ml/min.
Detection wavelength:	380 nm
Injection volume:	20 µl –100 µl

Check the stability of the chromatographic system, injecting several times the calibration solution (3.5.3) containing 2,5 µg/ml, until constant peak heights and retention times are achieved.

5.3.2. Calibration graph

Inject each calibration solution (3.5.3) several times and determine the mean peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights (areas) of the calibration solutions as the ordinates and the corresponding concentrations in µg/ml as the abscissae.

5.3.3. Sample solution

Inject the sample extract (5.2) several times using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the olaquindox peaks.

6. **Calculation of the results**

From the mean height (area) of the olaquindox peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.3.2).

The olaquindox content w in mg/kg of the sample is given by the following formula:

$$w = \frac{c \times 1000}{m}$$

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in which:

c = olaquindox concentration of the sample extract (5.2) in µg/ml
m = weight of the test portion in g (5.2).

7. **Validation of the results**

7.1. ***Identity***

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract (5.2) and the calibration solution (3.5.3) containing 5,0 µg/ml are compared.

7.1.1. Co-chromatography

A sample extract (5.2) is fortified by addition of an appropriate amount of calibration solution (3.5.3). The amount of added olaquindox must be similar to the amount of olaquindox found in the sample extract.

Only the height of the olaquindox peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its height, must be within ± 10 % of the original width of the olaquindox peak of the unfortified sample extract.

7.1.2. Diode array detection

The results are evaluated according to the following criteria:

(a) The wavelength of maximum absorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be the same within a margin determined by the resolving power of the detection system. For diode-array detection this is typically within ± 2 nm.

(b) Between 220 and 400 nm, the sample and standard spectra recorded at the peak apex of the chromatogram, must not be different for those parts of the spectrum within the range 10 %-100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte.

(c) Between 220 and 400 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 %-100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the peak apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed 15 % relative to the higher result for olaquindox contents between 10 and 200 mg/kg.

7.3. ***Recovery***

For a fortified blank sample the recovery shall be at least 90 %.

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8. **Results of a collaborative study**

An EC collaborative study was arranged in which four piglet feed samples including one blank feed were analysed by up to 13 laboratories. The results are given below:

	Sample 1	Sample 2	Sample 3	Sample 4
L	13	10	11	11
n	40	40	44	44
mean [mg/kg]	—	14,6	48,0	95,4
S_r [mg/kg]	—	0,82	2,05	6,36
S_R [mg/kg]	—	1,62	4,28	8,42
CV_r [%]	—	5,6	4,3	6,7
CV_R [%]	—	11,1	8,9	8,8
Nominal content [mg/kg]	—	15	50	100
recovery %	—	97,3	96,0	95,4

L = number of laboratories
n = number of single values
S_r = standard deviation of repeatability
S_R = standard deviation of reproducibility
CV_r = coefficient of variation of repeatability
CV_R = coefficient of variation of reproducibility.

9. **Observation**

Although the method has not been validated for feeds containing more than 100 mg/kg of olaquindox, it may be possible to obtain satisfactory results by taking a smaller sample weight and/or diluting the extract (5.2) to reach a concentration within the range of the calibration graph (5.3.2).

C. DETERMINATION OF AMPROLIUM

1-[(4-amino-2-propylpyrimidin-5-yl)methyl]-2-methyl-pyridinium chloride hydrochloride

1. **Purpose and Scope**

This method makes it possible to determine the level of amprolium in feed and premixtures. The detection limit is 1 mg/kg, the limit of quantification is 5 mg/kg.

2. **Principle**

The sample is extracted with a methanol-water mixture. After dilution with the mobile phase and membrane filtration the content of amprolium is determined by cation exchange high performance liquid chromatography (HPLC) using a UV detector.

3. **Reagents**

3.1. Methanol.

3.2. Acetonitrile, equivalent to HPLC grade.

3.3. Water, equivalent to HPLC grade.

3.4. Sodium dihydrogen phosphate solution, c = 0,1 mol/l.

Dissolve 13,80 g of sodium dihydrogen phosphate monohydrate in water (3.3) in a 1 000 ml graduated flask, make up to the mark with water (3.3) and mix.

3.5. Sodium perchlorate solution, c = 1,6 mol/l.

Dissolve 224,74 g of sodium perchlorate monohydrate in water (3.3) in a 1 000 ml graduated flask, make up to the mark with water (3.3) and mix.

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3.6. Mobile phase for HPLC (see observation 9.1).

Mixture of acetonitrile (3.2), sodium dihydrogen phosphate solution (3.4) and sodium perchlorate solution (3.5), 450+450+100 (v+v+v). Prior to use filter through a 0,22 µm membrane filter (4.3) and degas the solution (e.g. in the ultrasonic bath (4.4) for at least 15 minutes).

3.7. Standard substance: pure amprolium, 1-[(4-amino-2-propylpyrimidin-5-yl)methyl]-2-methyl-pyridinium chloride hydrochloride, E 750 (see 9.2).

3.7.1. Amprolium stock standard solution, 500 µg/ml

Weigh to the nearest 0,1 mg, 50 mg of amprolium (3.7) in a 100 ml graduated flask, dissolve in 80 ml methanol (3.1) and place the flask for 10 min. in an ultrasonic bath (4.4). After ultrasonic treatment bring the solution to room temperature, make up to the mark with water and mix. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.7.2. Amprolium intermediate standard solution, 50 µg/ml

Pipette 5,0 ml of the stock standard solution (3.7.1) into a 50 ml graduated flask, make up to the mark with the extraction solvent (3.8) and mix. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.7.3. Calibration solutions

Transfer 0,5, 1,0 and 2,0 ml of the intermediate standard solution (3.7.2) into a series of 50 ml graduated flasks. Make up to the mark with the mobile phase (3.6) and mix. These solutions correspond to 0,5, 1,0 and 2,0 µg of amprolium per ml respectively. These solutions must be prepared freshly before use.

3.8. Extraction solvent.

Methanol (3.1)-water mixture 2+1 (v+v).

4. **Apparatus**

4.1. HPLC equipment with injection system, suitable for injection volumes of 100 µl.

4.1.1. Liquid chromatographic column 125 mm × 4 mm, cation exchange Nucleosil 10 SA, 5 or 10 µm packing, or equivalent.

4.1.2. UV detector with variable wavelength adjustment or diode array detector.

4.2. Membrane filter, PTFE material, 0,45 µm.

4.3. Membrane filter, 0,22 µm.

4.4. Ultrasonic bath.

4.5. Mechanical shaker or magnetic stirrer.

5. **Procedure**

5.1. ***General***

5.1.1. Blank feed

For the performance of the recovery test (5.1.2) a blank feed shall be analysed to check that neither amprolium nor interfering substances are present. The blank feed shall be similar in type to that of the sample and amprolium or interfering substances must not be detected.

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5.1.2. Recovery test

A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of amprolium, similar to that present in the sample. To fortify at a level of 100 mg/kg, transfer 10,0 ml of the stock standard solution (3.7.1) to a 250 ml conical flask and evaporate the solution to approximately 0,5 ml. Add 50 g of the blank feed, mix thoroughly and leave for 10 min. mixing again several times before proceeding with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample to be analysed is fortified with a quantity of amprolium similar to that already present in the sample. This sample is analysed together with the unfortified sample and the recovery can be calculated by subtraction.

5.2. ***Extraction***

5.2.1. Premixtures (content < 1 % amprolium) and feed

Weigh to the nearest 0,01 g, 5-40 g of the sample depending on the amprolium content into a 500 ml conical flask and add 200 ml extraction solvent (3.8). Place the flask in the ultrasonic bath (4.4) and leave for 15 minutes. Remove the flask from the ultrasonic bath and shake it for 1 h on the shaker or stir on the magnetic stirrer (4.5). Dilute an aliquot of the extract with the mobile phase (3.6) to an amprolium content of 0,5-2 µg/ml and mix (see observation 9.3). Filter 5-10 ml of this diluted solution on a membrane filter (4.2). Proceed to the HPLC determination (5.3).

5.2.2. Premixtures (content ≥ 1 % amprolium)

Weigh to the nearest 0,001 g, 1-4 g of the premixture depending on the amprolium content into a 500 ml conical flask and add 200 ml extraction solvent (3.8). Place the flask in the ultrasonic bath (4.4) and leave for 15 minutes. Remove the flask from the ultrasonic bath and shake it for 1 h on the shaker or stir on the magnetic stirrer (4.5). Dilute an aliquot of the extract with the mobile phase (3.6) to an amprolium content of 0,5-2 µg/ml and mix. Filter 5-10 ml of this diluted solution on a membrane filter (4.2). Proceed to the HPLC determination (5.3).

5.3. ***HPLC determination***

5.3.1. Parameters:

The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results.

Liquid chromatographic

column (4.1.1):	125 mm × 4 mm, cation exchange Nucleosil 10 SA, 5 or 10 µm packing, or equivalent
Mobile phase (3.6):	Mixture of acetonitrile (3.2), sodium dihydrogen phosphate solution (3.4) and sodium perchlorate solution (3.5), 450+450+100 (v+v+v).
Flow rate:	0,7-1 ml/min
Detection wavelength:	264 nm
Injection volume:	100 µl

▼B

Check the stability of the chromatographic system, injecting several times the calibration solution (3.7.3) containing 1,0 µg/ml, until constant peak heights and retention times are achieved.

5.3.2. Calibration graph

Inject each calibration solution (3.7.3) several times and determine the mean peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights (areas) of the calibration solutions as the ordinates and the corresponding concentrations in µg/ml as the abscissae.

5.3.3. Sample solution

Inject the sample extract (5.2) several times using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the amprolium peaks.

6. **Calculation of the results**

From the mean height (area) of the amprolium peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.3.2).

The amprolium content w in mg/kg of the sample is given by the following formula:

$$w = \frac{V \times c \times f}{m} \text{ [mg/kg]}$$

in which:

V = volume of the extraction solvent (3.8) in ml according to 5.2 (i.e. 200 ml)
c = amprolium concentration of the sample extract (5.2) in µg/ml
f = dilution factor according to 5.2
m = weight of the test portion in g.

7. **Validation of the results**

7.1. ***Identity***

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract (5.2) and the calibration solution (3.7.3) containing 2,0 µg/ml are compared.

7.1.1. Co-chromatography

A sample extract (5.2) is fortified by addition of an appropriate amount of calibration solution (3.7.3). The amount of added amprolium must be similar to the amount of amprolium found in the sample extract.

Only the height of the amprolium peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its height, must be within ± 10 % of the original width of the amprolium peak of the unfortified sample extract.

7.1.2. Diode array detection

The results are evaluated according to the following criteria:

(a) The wavelength of maximum absorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be the same within a margin determined by the resolving power of the detection system. For diode-array detection this is typically within ± 2 nm.

▼B

(b) Between 210 and 320 nm, the sample and standard spectra recorded at the peak apex of the chromatogram, must not be different for those parts of the spectrum within the range 10 %-100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte.

(c) Between 210 and 320 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 %-100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the peak apex.

If one of these criteria is not met, the presence of the analyte has not been confirmed.

7.2. ***Repeatability***

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

— 15 % relative to the higher value for amprolium contents from 25 mg/kg to 500 mg/kg,

— 75 mg/kg for amprolium contents between 500 mg/kg and 1 000 mg/kg,

— 7,5 % relative to the higher value for amprolium contents of more than 1 000 mg/kg.

7.3. ***Recovery***

For a fortified (blank) sample the recovery shall be at least 90 %.

8. **Results of a collaborative study**

A collaborative study was arranged in which three poultry feeds (sample 1-3), one mineral feed (sample 4) and one premix (sample 5) were analysed. The results are given in the following table:

	Sample 1 (blank feed)	Sample 2	Sample 3	Sample 4	Sample 5
L	14	14	14	14	15
n	56	56	56	56	60
mean [mg/kg]	—	45,5	188	5 129	25 140
s_r [mg/kg]	—	2,26	3,57	178	550
CVr [%]	—	4,95	1,90	3,46	2,20
s_R [mg/kg]	—	2,95	11,8	266	760
CV_R [%]	—	6,47	6,27	5,19	3,00
nominal content [mg/kg]	—	50	200	5 000	25 000

L = number of laboratories
n = number of single values
s_r = standard deviation of repeatability
CV_r = coefficient of variation of repeatability
s_R = standard deviation of reproducibility
CV_R = coefficient of variation of reproducibility.

▼B

9. **Observations**

9.1. If the sample contains thiamine, the thiamine peak in the chromatogram appears shortly before the amprolium peak. Following this method amprolium and thiamine must be separated. If the amprolium and thiamine are not separated by the column (4.1.1) used in this method, replace up to 50 % of the acetonitrile portion of the mobile phase (3.6) by methanol.

9.2. According to the British Pharmacopoeia, the spectrum of an amprolium solution (c = 0,02 mol/l) in hydrochloric acid (c = 0,1 mol/l) shows maxima at 246 nm and 262 nm. The absorbance shall amount to 0,84 at 246 nm and 0,80 at 262 nm.

9.3. The extract must always be diluted with the mobile phase, because otherwise the retention time of the amprolium peak may shift significantly, due to changes in the ionic strength.

D. DETERMINATION OF CARBADOX

Methyl 3-(2-quinoxalinylmethylene)carbazate N^1,N^4-dioxide

1. **Purpose and scope**

This method makes it possible to determine the level of carbadox in feed, premixtures and preparations. The detection limit is 1 mg/kg. The limit of quantification is 5 mg/kg.

2. **Principle**

The sample is equilibrated with water and extracted with methanol-acetonitrile. For feed, an aliquot portion of the filtered extract is subjected to clean-up on an aluminium oxide column. For premixtures and preparations an aliquot portion of the filtered extract is diluted to an appropriate concentration with water, methanol and acetonitrile. The content of carbadox is determined by reversed-phase high-performance liquid chromatography (HPLC) using a UV detector.

3. **Reagents**

3.1. Methanol.

3.2. Acetonitrile, equivalent to HPLC grade.

3.3. Acetic acid, w = 100 %.

3.4. Aluminium oxide: neutral, activity grade I.

3.5. Methanol-acetonitrile 1 + 1 (v + v).

Mix 500 ml of methanol (3.1) with 500 ml of acetonitrile (3.2).

3.6. Acetic acid, σ = 10 %.

Dilute 10 ml acetic acid (3.3) to 100 ml with water.

3.7. Sodium acetate.

3.8. Water, equivalent to HPLC grade.

3.9. Acetate buffer solution, c = 0,01 mol/l, pH = 6,0.

Dissolve 0,82 g of sodium acetate (3.7) in 700 ml of water (3.8) and adjust the pH to 6,0 with acetic acid (3.6). Transfer to a 1 000 ml graduated flask, make up to the mark with water (3.8) and mix.

3.10. Mobile phase for HPLC.

Mix 825 ml of acetate buffer solution (3.9) with 175 ml of acetonitrile (3.2).

Filter through a 0,22 μm filter (4.5) and degas the solution (e.g. by ultrasonification for 10 minutes).

▼B

3.11. Standard substance.

Pure carbadox: Methyl 3-(2-quinoxalinylmethylene)carbazate N^1,N^4-dioxide, E 850.

3.11.1. Carbadox stock standard solution, 100 µg/ml (see ***Note*** 5. Procedure):

Weigh to the nearest 0,1 mg, 25 mg of carbadox standard substance (3.11) into a 250 ml graduated flask. Dissolve in methanol-acetonitrile (3.5) by ultrasonification (4.7). After ultrasonic treatment bring the solution to room temperature, make up to the mark with methanol-acetonitrile (3.5) and mix. Wrap the flask with aluminium foil or use amber glassware and store in a refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.11.2. Calibration solutions

Transfer 2,0, 5,0, 10,0, and 20,0 ml of the stock standard solution (3.11.1) into a series of 100 ml calibrated flasks. Add 30 ml of water, make up to the mark with methanol-acetonitrile (3.5) and mix. Wrap the flasks with aluminium foil. These solutions correspond to 2,0, 5,0, 10,0 and 20,0 µg/ml of carbadox respectively.

Calibration solutions must be freshly prepared before use.

Note: For the determination of carbadox in feed containing less than 10 mg/kg, calibration solutions with a concentration below 2,0 µg/ml must be prepared.

3.12. Water-[methanol-acetonitrile] (3.5) mixture, 300 + 700 (v + v).

Mix 300 ml of water with 700 ml of the mixture of methanol-acetonitrile (3.5).

4. **Apparatus**

4.1. Laboratory shaker or magnetic stirrer.

4.2. Glass fibre filter paper (Whatman GF/A or equivalent).

4.3. Glass column (length 300 to 400 mm, internal diameter approximately 10 mm) with sintered glass frit and draw-off valve.

Note: a glass column fitted with a stopcock or a glass column with a tapered end may also be used; in this case, a small glass-wool plug is inserted into the lower end and it is tamped down using a glass rod.

4.4. HPLC equipment with injection system, suitable for injection volumes of 20 µl.

4.4.1. Liquid chromatographic column: 300 mm x 4 mm, C_{18}, 10 µm packing or equivalent.

4.4.2. UV detector with variable wavelength adjustment or diode array detector operating in the range of 225 to 400 nm.

4.5. Membrane filter, 0,22 µm.

4.6. Membrane filter, 0,45 µm.

4.7. Ultrasonic bath.

▼B

5. **Procedure**

Note: Carbadox is light-sensitive. Carry out all procedures under subdued light or use amber glassware or glassware wrapped in aluminium foil.

5.1. ***General***

5.1.1. Blank feed

For the performance of the recovery test (5.1.2) a blank feed shall be analysed to check that neither carbadox nor interfering substances are present. The blank feed shall be similar in type to that of the sample and on analysis carbadox or interfering substances must not be detected.

5.1.2. Recovery test

A recovery test shall be carried out by analysing the blank feed (5.1.1) which has been fortified by the addition of a quantity of carbadox, similar to that present in the sample. To fortify at a level of 50 mg/kg, transfer 5,0 ml of the stock standard solution (3.11.1) to a 200 ml conical flask. Evaporate the solution to approximately 0,5 ml in a stream of nitrogen. Add 10 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample is fortified with a quantity of carbadox, similar to that already present in the sample. This sample is analysed, together with the unfortified sample and the recovery can be calculated by subtraction.

5.2. ***Extraction***

5.2.1. Feed

Weigh to the nearest 0,01 g, 10 g of the sample and transfer to a 200 ml conical flask. Add 15,0 ml of water, mix, and equilibrate for 5 min. Add 35,0 ml of methanol-acetonitrile (3.5), stopper and shake for 30 min. on the shaker or stir on the magnetic stirrer (4.1). Filter the solution through a glass fibre filter paper (4.2). Retain this solution for the purification step (5.3).

5.2.2. Premixtures (0,1 %-2,0 %)

Weigh to the nearest 0,001 g, 1 g of the unground sample and transfer to a 200 ml conical flask. Add 15,0 ml of water, mix, and equilibrate for 5 min. Add 35,0 ml of methanol-acetonitrile (3.5), stopper and shake for 30 min. on the shaker or stir on the magnetic stirrer (4.1). Filter the solution through a glass fibre filter paper (4.2).

Pipet an aliquot of filtrate into a 50 ml calibrated flask. Add 15,0 ml of water, make up to the mark with methanol-acetonitrile (3.5) and mix. The carbadox concentration of the final solution shall be approximately 10 μg/ml. An aliquot is filtered through a 0,45 μm filter (4.6).

Proceed to the HPLC determination (5.4).

5.2.3. Preparations (> 2 %)

Weigh to the nearest 0,001 g, 0,2 g of the unground sample and transfer to a 250 ml conical flask. Add 45,0 ml of water, mix, and equilibrate for 5 min. Add 105,0 ml of methanol-acetonitrile (3.5), stopper and homogenise.

▼B

Sonicate (4.7) the sample for 15 min. followed by shaking or stirring for 15 min. (4.1). Filter the solution through a glass fibre filter paper (4.2).

Dilute an aliquot of filtrate with the mixture of water-methanol-acetonitrile (3.12) to a final carbadox concentration of 10-15 µg/ml (for a 10 % preparation, the dilution factor is 10). An aliquot is filtered through a 0,45 µm filter (4.6).

Proceed to the HPLC determination (5.4).

5.3. ***Purification***

5.3.1. Preparation of the aluminium oxide column

Weigh 4 g of aluminium oxide (3.4) and transfer it to the glass column (4.3).

5.3.2. Sample purification

Apply 15 ml of the filtered extract (5.2.1) to the aluminium oxide column and discard the first 2 ml of eluate. Collect the next 5 ml and filter an aliquot through a 0,45 µm filter (4.6).

Proceed to the HPLC determination (5.4).

5.4. ***HPLC determination***

5.4.1. Parameters

The following conditions are offered for guidance, other conditions may be used provided they yield equivalent results:

Liquid chromatographic column (4.4.1):	300 mm × 4 mm, C_{18}, 10 µmpacking or equivalent
Mobile phase (3.10):	Mixture of acetate buffer solution (3.9) and acetonitrile (3.2), 825 + 175 (v+v)
Flow rate:	1,5-2 ml/min.
Detection wavelength:	365 nm
Injection volume:	20 µl

Check the stability of the chromatographic system, injecting the calibration solution (3.11.2) containing 5,0 µg/ml several times, until constant peak heights (areas) and retention times are achieved.

5.4.2. Calibration graph

Inject each calibration solution (3.11.2) several times and measure the peak heights (areas) for each concentration. Plot a calibration curve using the mean peak heights or areas of the calibration solutions as the ordinates and corresponding concentrations in µg/ml as the abscissae.

5.4.3. Sample solution

Inject the sample extract [(5.3.2) for feed, (5.2.2) for premixtures and (5.2.3) for preparations] several times and determine the mean peak height (area) of the carbadox peaks.

▼B

6. **Calculation of the results**

From the mean height (area) of the carbadox peaks of the sample solution determine the carbadox concentration of the sample solution in μg/ml by reference to the calibration graph (5.4.2).

6.1. ***Feed***

The content of carbadox w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times V_1}{m} \text{ [mg/kg]}$$

in which:

c = carbadox concentration of the sample extract (5.3.2) in μg/ml
V_1 = extraction volume in ml (i.e. 50)
m = weight of the test portion in g.

6.2. ***Premixtures and preparations***

The content of carbadox w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times V_2 \times f}{m} \text{ [mg/kg]}$$

in which:

c = carbadox concentration of the sample extract (5.2.2 or 5.2.3) in μg/ml
V_2 = extraction volume in ml (i.è. 50 for premixtures; 150 for preparations)
f = dilution factor according to 5.2.2 (premixtures) or 5.2.3 (preparations)
m = weight of the test portion in g.

7. **Validation of the results**

7.1. ***Identity***

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract and the calibration solution (3.11.2) containing 10,0 μg/ml are compared.

7.1.1. Co-chromatography

A sample extract is fortified by addition of an appropriate amount of calibration solution (3.11.2). The amount of added carbadox must be similar to the estimated amount of carbadox found in the sample extract.

Only the height of the carbadox peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within approximately 10 % of the original width.

7.1.2. Diode-array detection

The results are evaluated according to the following criteria:

(a) the wavelength of maximum absorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within + 2 nm;

▼B

(b) between 225 and 400 nm, the sample and standard spectra recorded at the peak apex on the chromatogram, must not be different for those parts of the spectrum within the range 10 % to 100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte;

(c) between 225 and 400 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 % to 100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. ***Repeatability***

For contents of 10 mg/kg and higher, the difference between the results of two parallel determinations carried out on the same sample must not exceed 15 % relative to the higher result.

7.3. ***Recovery***

For a fortified (blank) sample the recovery shall be at least 90 %.

8. **Results of a collaborative study**

A collaborative study was arranged in which 6 feed, 4 premixtures and 3 preparations were analysed by 8 laboratories. Duplicate analyses were performed on each sample. (More detailed information on this collaborative study can be found in the ***Journal of the AOAC, Volume 71, 1988, p. 484-490***). The results (excluding outliers) are shown below:

Table 1

Results of the collaborative study for feed

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
L	8	8	8	8	8	8
n	15	14	15	15	15	15
Mean (mg/kg)	50,0	47,6	48,2	49,7	46,9	49,7
Sr (mg/kg)	2,90	2,69	1,38	1,55	1,52	2,12
CVr (%)	5,8	5,6	2,9	3,1	3,2	4,3
SR (mg/kg)	3,92	4,13	2,23	2,58	2,26	2,44
CVR (%)	7,8	8,7	4,6	5,2	4,8	4,9
Nominal content (mg/kg)	50,0	50,0	50,0	50,0	50,0	50,0

Table 2

Results of the collaborative study for premixtures and preparations

	Premixtures				Preparations		
	A	B	C	D	A	B	C
L	7	7	7	7	8	8	8
n	14	14	14	14	16	16	16
Mean (g/kg)	8,89	9,29	9,21	8,76	94,6	98,1	104
Sr (g/kg)	0,37	0,28	0,28	0,44	4,1	5,1	7,7
CVr (%)	4,2	3,0	3,0	5,0	4,3	5,2	7,4
SR (g/kg)	0,37	0,28	0,40	0,55	5,4	6,4	7,7

▼B

	Premixtures				Preparations		
	A	B	C	D	A	B	C
CVR (%)	4,2	3,0	4,3	6,3	5,7	6,5	7,4
Nominal content (g/kg)	10,0	10,0	10,0	10,0	100	100	100

L = number of laboratories
n = number of single values
Sr = standard deviation of repeatability
CVr = coefficient of variation of repeatability
SR = standard deviation of reproducibility
CVR = coefficient of variation of reproducibility.

▼B

ANNEX IX

CORRELATION TABLES REFERRED TO IN ARTICLE 6

1. **Directive 71/250/EEC**

Directive 71/250/EEC	This Regulation
Article 1 first subparagraph	Article 3
Article 1 second subparagraph	Article 2
Article 2	—
Article 3	—
Annex, part 1	Annex II
Annex, part 2	—
Annex, part 3	—
Annex, part 4	Annex III, part O
Annex, part 5	Annex III, part M
Annex, part 6	Annex III, part N
Annex, part 7	Annex III, part Q
Annex, part 9	Annex III, part K
Annex, part 10	—
Annex, part 11	—
Annex, part 12	Annex III, part J
Annex, part 14	Annex III, part D
Annex, part 16	—

2. **Directive 71/393/EEC**

Directive 71/393/EEC	This Regulation
Article 1	Article 3
Article 2	—
Article 3	—
Annex, part I	Annex III, part A
Annex, part II	Annex III, part E
Annex, part III	Annex III, part P
Annex, part IV	Annex III, part H

3. **Directive 72/199/EEC**

Directive 72/199/EEC	This Regulation
Article 1	Article 3
Article 2	—
Article 3	—
Article 4	—
Annex I, part 1	Annex III, part L
Annex I, part 2	Annex III, part C
Annex I, part 3	—
Annex I, part 4	—
Annex I, part 5	Annex V, part A
Annex II	—

4. **Directive 73/46/EEC**

Directive 73/46/EEC	This Regulation
Article 1	Article 3
Article 3	—
Article 4	—
Annex I, part 1	Annex III, part B
Annex I, part 2	—
Annex I, part 3	Annex III, part I

▼B

5. **Directive 76/371/EEC**

Directive 76/371/EEC	This Regulation
Article 1	Article 1
Article 2	—
Article 3	—
Annex	Annex I

6. **Directive 76/372/EEC**

Directive 76/372/EEC	This Regulation
Article 1	—
Article 2	—
Article 3	—
Annex	—

7. **Directive 78/633/EEC**

Directive 78/633/EEC	This Regulation
Article 1	Article 3
Article 2	—
Article 3	—
Annex, part 1	—
Annex, part 2	—
Annex, part 3	Annex IV, part C

8. **Directive 81/715/EEC**

Directive 81/715/EEC	This Regulation
Article 1	—
Article 2	—
Article 3	—
Annex	—

9. **Directive 84/425/EEC**

Directive 84/425/EEC	This Regulation
Article 1	—
Article 2	—
Article 3	—
Annex	—

10. **Directive 86/174/EEC**

Directive 86/174/EEC	This Regulation
Article 1	Article 4
Article 2	—
Article 3	—
Annex	Annex VII

11. **Directive 93/70/EEC**

Directive 93/70/EEC	This Regulation
Article 1	Article 3
Article 2	—
Article 3	—
Annex	Annex IV, part D

▼B

12. **Directive 93/117/EC**

Directive 93/117/EC	This Regulation
Article 1	Articles 3 and 5
Article 2	—
Article 3	—
Annex, part 1	Annex IV, part E
Annex, part 2	Annex VIII, part A

13. **Directive 98/64/EC**

Directive 98/64/EC	This Regulation
Article 1	Articles 3 and 5
Article 2	—
Article 3	—
Article 4	—
Annex, part A	Annex III, part F
Annex, part C	Annex VIII, part B

14. **Directive 1999/27/EC**

Directive 1999/27/EC	This Regulation
Article 1	Articles 3 and 5
Article 2	—
Article 3	—
Article 4	—
Article 5	—
Article 6	—
Article 7	—
Annex, part A	Annex VIII, part C
Annex, part B	Annex IV, part F
Annex, part C	Annex VIII, part D

15. **Directive 1999/76/EC**

Directive 1999/76/EC	This Regulation
Article 1	Article 3
Article 2	—
Article 3	—
Article 4	—
Annex	Annex IV, part G

16. **Directive 2000/45/EC**

Directive 2000/45/EC	This Regulation
Article 1	Article 3
Article 2	—
Article 3	—
Article 4	—
Annex, part A	Annex IV, part A
Annex, part B	Annex IV, part B
Annex, part C	Annex III, part G

▼B

17. **Directive 2002/70/EC**

Directive 2002/70/EC	This Regulation
Article 1	Article 1
Article 2	Articles 2 and 3
Article 3	—
Article 4	—
Article 5	—
Annex I	Annex I and Annex V part B(I)
Annex II	Annex II and Annex V part B(II)

18. **Directive 2003/126/EC**

Directive 2003/126/EC	This Regulation
Article 1	Article 3
Article 2	—
Article 3	—
Article 4	—
Article 5	—
Article 6	—
Annex	Annex VI

Synthetic Sodium Aluminosilicate in X-Zelit®

Composition: Theoretical Calculations

Synthetic Sodium Aluminosilicate	(b) (4)
Wheat	
Rapeseed Oil	

	Stoichiometry	MW (g/mol)	Content in Additive (g/mol)	Content in Additive (%)	Content in Formulation (%)	Formulation Specifications (%)
Na_2O	(b) (4)					
Al_2O_3						
SiO_2						
H_2O (Bound)						
Molecular Weight of $Na_{12}Al_{12}Si_{12}O_{48}\cdot 27H_2O$ = 2190 (g/mol)						

BRITISH STANDARD

BS 3762-4.2:
1986
ISO 697:1981

Analysis of formulated detergents —

Part 4: Physical test methods —

Section 4.2 Method for determination of apparent bulk density

[ISO title: Surface active agents — Washing powders — Determination of apparent density — Method by measuring the mass of a given volume]

NOTE It is recommended that this Section be read in conjunction with the information in the *"General introduction"*, published separately as BS 3762-0.

UDC 661.185:531.755.22

This British Standard, having been prepared under the direction of the Chemicals Standards Committee, was published under the authority of the Board of BSI and comes into effect on 31 December 1986

The committees responsible for this British Standard are shown in Part 0.
The following BSI references relate to the work on this standard:
Committee reference CIC/34
Draft for comment 80/52622 DC

ISBN 0 580 15582 X

Amendments issued since publication

Amd. No.	Date of issue	Comments

Contents

National foreword

This Section of BS 3762 has been prepared under the direction of the Chemicals Standards Committee. It is identical with ISO 697:1981 "*Surface active agents — Washing powders — Determination of apparent density — Method by measuring the mass of a given volume*", published by the International Organization for Standardization (ISO).

This method supersedes method 2 in Part 3 of BS 3762:1964 which is being deleted by amendment.

Terminology and conventions. The text of the International Standard has been approved as suitable for publication as a British Standard without deviation. Some terminology and certain conventions are not identical with those used in British Standards; attention is drawn especially to the following.

The comma has been used as a decimal marker. In British Standards it is current practice to use a full point on the baseline as the decimal marker.

In British Standards it is current practice to use the symbol "L" for litre (and in its submultiples) rather than "l".

Wherever the words "International Standard" appear, referring to this standard, they should be read as "Section of this British Standard".

Cross-references

International Standard	Corresponding British Standard
ISO 607:1980	BS 3762 *Analysis of formulated detergents* Part 1:1983 *Methods of sample division* (Identical)
ISO 3424:1975	BS 5688 *Methods of test for boric acid, boric oxide, disodium tetraborates, sodium perborates and crude sodium borates for industrial use* Part 21:1979 *Determination of bulk density of sodium perborates* (Identical)

Additional information. With reference to clause **2**, **6.1**, **6.4** and the Figure, it is common practice in the UK to use the apparatus having a funnel orifice of 40 mm for all powders.

A British Standard does not purport to include all the necessary provisions of a contract. Users of British Standards are responsible for their correct application.

Compliance with a British Standard does not of itself confer immunity from legal obligations.

Summary of pages

This document comprises a front cover, an inside front cover, pages i and ii, pages 1 to 4, an inside back cover and a back cover.

This standard has been updated (see copyright date) and may have had amendments incorporated. This will be indicated in the amendment table on the inside front cover.

0 Introduction

The apparent density of a powder can be evaluated either by measuring the mass which occupies a given volume, or by measuring the volume occupied by a given mass. In both cases, the procedure involves transfer of the powder from its original container to that used for the measurement. Owing to the friability of the product, to its flow or caking properties, to the varying geometry of particles of which it is composed, and to the unavoidable compaction resulting from pouring into the container for measurement, the apparent density determined will generally differ from that of the product in its original container or package.

The result of the determination, therefore, gives only a conventional value related to the method used.

1 Scope

This International Standard specifies a method for the determination of the apparent density of washing powders by measuring the mass of a given volume.

2 Field of application

The method is applicable to free flowing powders and, provided that an appropriate funnel is used, to powders which have a tendency to cake.

The method is suitable for other substances in the form of powder or granules.

In the case of powder containing lumps, the method is applicable only if these can be disintegrated readily without breaking down the particles of the powder.

3 References

ISO 607, *Surface active agents and detergents — Methods of sample division.*

ISO 3424, *Sodium perborates for industrial use — Determination of bulk density.*

4 Definition[1)]

apparent density

the mass, in grams, of powder which occupies a volume of one millilitre under standardized conditions

5 Principle

Determination of the mass of powder in a receiver of known dimensions, after filling with the sample from a funnel of specified shape under specified conditions.

6 Apparatus

6.1 *Funnel,* made of stainless steel, plastics, wood or other suitable material.

All surfaces in contact with the flowing powder shall be smooth and polished and shall not permit a build up of an electrostatic charge by the flow of the powder.

The internal diameter of the orifice shall be 40 mm for use with free flowing powders and 60 mm for use with powders showing a tendency to cake.

6.2 *Receiver*, of capacity 500 ml, constructed of materials similar to those of the funnel.

The receiver shall be calibrated as described in **8.1** and the volume may, for convenience, be adjusted to 500 ± 0,5 ml by machining the rim.

6.3 *Stand*, capable of holding the funnel and the receiver in fixed positions relative to each other. The funnel shall be held by locating pins passing through holes in the flange of the funnel and the top plate of the stand. The receiver shall be located centrally beneath the funnel by locating studs or other suitable means.

The stand may incorporate, if desired, a mechanism for mechanical operation of the closure plate.

6.4 *Closure plate*, 110 mm × 70 mm.

This apparatus (see the Figure) is identical with that described in ISO 3424, except for the 60 mm orifice funnel the other dimensions of which are: superior diameter 112 mm and height 100 mm.

6.5 *Straightedge*, of length approximately 150 mm.

6.6 *Glass plate*, 100 mm × 100 mm × 7 mm.

7 Sampling

The laboratory sample of washing powder shall be prepared and stored in accordance with ISO 607.

[1)] The gram per millilitre (g/ml) is the unit of density of the CGS system. The unit of density of the International System of Units (SI) is the kilogram per cubic metre (kg/m^3): 1 kg/m^3 = 10^{-3} g/ml.

8 Procedure

8.1 Calibration of the receiver

Calibrate the receiver (**6.1**) by establishing its volume in the following manner.

Weigh, to the nearest 0,1 g, the clean, empty receiver and place it on a horizontal surface. Fill it with recently boiled distilled water at 20 °C, and remove any bubbles, which collect during the filling, by gently tapping the walls. Place the weighed glass plate (**6.6**) horizontally against the edge of the upper rim of the receiver. Gently slide the plate across the water surface and, when it is nearly across, add 1 to 2 ml of distilled water to the receiver and completely cover the receiver with the plate. Dry the exposed underside of the plate and the sides of the receiver with filter paper and weigh to the nearest 0,1 g.

The volume, in millilitres, of the receiver is given by the formula

$$m_2 - (m_0 + m_1)$$

where

m_0 is the mass, in grams, of the empty receiver;

m_1 is the mass, in grams, of the glass plate;

m_2 is the mass, in grams, of the receiver full of water with the glass plate in position.

8.2 Preparation of test sample

Break down any lumps present in the laboratory sample by shaking and rotating the container.

Take care to avoid breaking down the particles of powder.

Render the laboratory sample homogeneous and reduce it by means of a conical divider as described in ISO 607.

8.3 Determination

Place the funnel (**6.1**) on the stand (**6.3**). Place the tared receiver (**6.2**) in position.

Cover the bottom opening of the funnel by means of the closure plate (**6.4**), holding the plate lightly against the funnel.

Fill the funnel with the sample up to its top rim, then quickly remove the closure plate, thus allowing the contents of the funnel to run into, and overflow from, the receiver.

Remove the receiver and place it on a level surface. Carefully level the powder surface by means of the straightedge (**6.5**) and clean the outside wall with a dry cloth. Weigh the receiver and its contents to the nearest 0,1 g.

Carry out at least two determinations on different portions of the laboratory sample.

9 Expression of results

9.1 Method of calculation

The apparent density of the powder, in grams per millilitre, is given by the formula

$$\frac{m_3 - m_0}{V}$$

where

m_0 is the mass, in grams, of the empty receiver;

m_3 is the mass, in grams, of the receiver and its contents;

V is the volume, in millilitres, of the receiver.

Take, as the result, the arithmetic mean of the two determinations provided that the requirement for repeatability (see **9.2**) is satisfied.

If it is not, repeat the determination.

Express the result to three significant figures as follows:

"Apparent density . . . g/ml".

9.2 Repeatability

The difference between the results of the two determinations, carried out in rapid succession by the same analyst, shall not exceed 5 % of the mean value.

10 Test report

The test report shall include the following particulars:

a) all details required for complete identification of the sample;

b) whether or not lumps were present in the laboratory sample;

c) the reference of the method used;

d) type of funnel used (orifice 40 or 60 mm);

e) the results and the method of expression used;

f) any unusual features noted during the determination;

g) any operations not included in this International Standard, or regarded as optional.

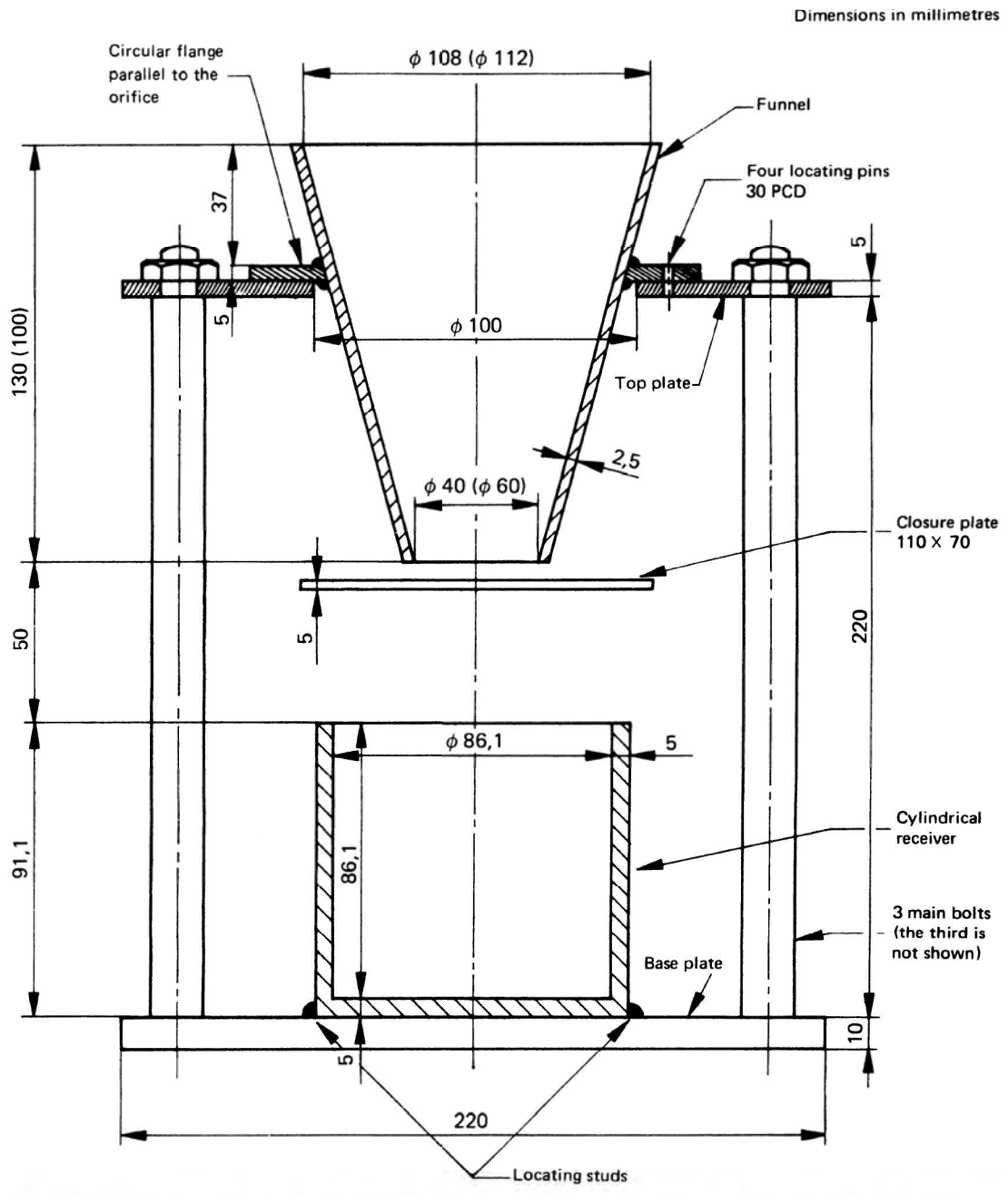

Figure — Apparatus for determination of apparent density of powders or granules

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Publications referred to

See national foreword.

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CERTIFICATE OF ANALYSIS

(b) (4)

(b) (4)

Date Received: 06-Feb-2018
Assay State: Dry

Date of Report: 09-Feb-2018

LMA Ref: M/336587
Client Ref: 18008001+17100001+17229001

Material Described As: Zeolit Type 4AMA

The results contained within this certificate are from test samples presented to L.M.A CV for analys s, and which, are of unknown provenance to L.M.A CV.

Test		Al2O3	Na2O	SiO2
Method		XRF	XRF	XRF
Unit				
1	18008001	(b) (4)		
2	17100001			
3	17229001			

(b) (4)

(b) (4)

Test Report

No. of test report:	1030/17-1
Customer:	
Order date:	2017-04-13
Object of analysis:	E554 Zeolithe
Objective of analysis:	Analysis of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/PCDF), polychlorinated biphenyls (PCB), heavy metals (As, Pb, Cd, Hg)
Sampling:	by customer
Arrival of sample:	2017-04-19
Procedure of analysis:	PCDD/PCDF, PCB: Commission Regulation (EC) No 152/2009 (amended by No 709/2014) HRGC/HRMS confirmatory analysis; As, Pb, Cd, Hg: DIN EN 15510
Time of analysis:	2017-04-19 to 04-28

Results:

Sample name:		**E554 Zeolithe** **Type: 4A MA** **Batch: 17100001 / 957681045**
Lab-code:		1030/17-1
Parameter	**Unit**	
PCDD/PCDF* (**WHO-TE$_{2005}$ LOQ incl.**)	**ng/kg 88% d.w.**	
Dioxin-like PCB* (**WHO-TE$_{2005}$ LOQ incl.**)	**ng/kg 88% d.w.**	
Sum of PCDD/PCDF and dioxin-like PCB* (**WHO-TE$_{2005}$ LOQ incl.**)	**ng/kg 88% d.w.**	
PCB 28*	µg/kg 88% d.w.	
PCB 52*	µg/kg 88% d.w.	
PCB 101*	µg/kg 88% d.w.	
PCB 138*	µg/kg 88% d.w.	
PCB 153*	µg/kg 88% d.w.	
PCB 180*	µg/kg 88% d.w.	
Sum of non dioxin-like PCB (LOQ incl.)*	**µg/kg 88% d.w.**	
Arsenic**	**mg/kg**	
Lead**	**mg/kg**	
Cadmium**	**mg/kg**	
Mercury**	**mg/kg**	

Remarks:

d.w.: dry weight LOQ: limit of quantification *processed by our laboratory site (b) (4) (b) (4) **analysis by accredited partner laboratory

The publication of this test report (even in parts) can be accomplished only by permission of (b) (4) The results refer exclusively to the tested samples.

(b) (4) 2017-04-28

(b) (4) (b) (4)

Unit Management

(This report is valid without signature if sent electronically)

Polychlorinated dibenzodioxins and dibenzofurans (PCDD/PCDF)

Sample name: E554 Zeolithe Type: 4A MA Batch: 17100001 / 957681045

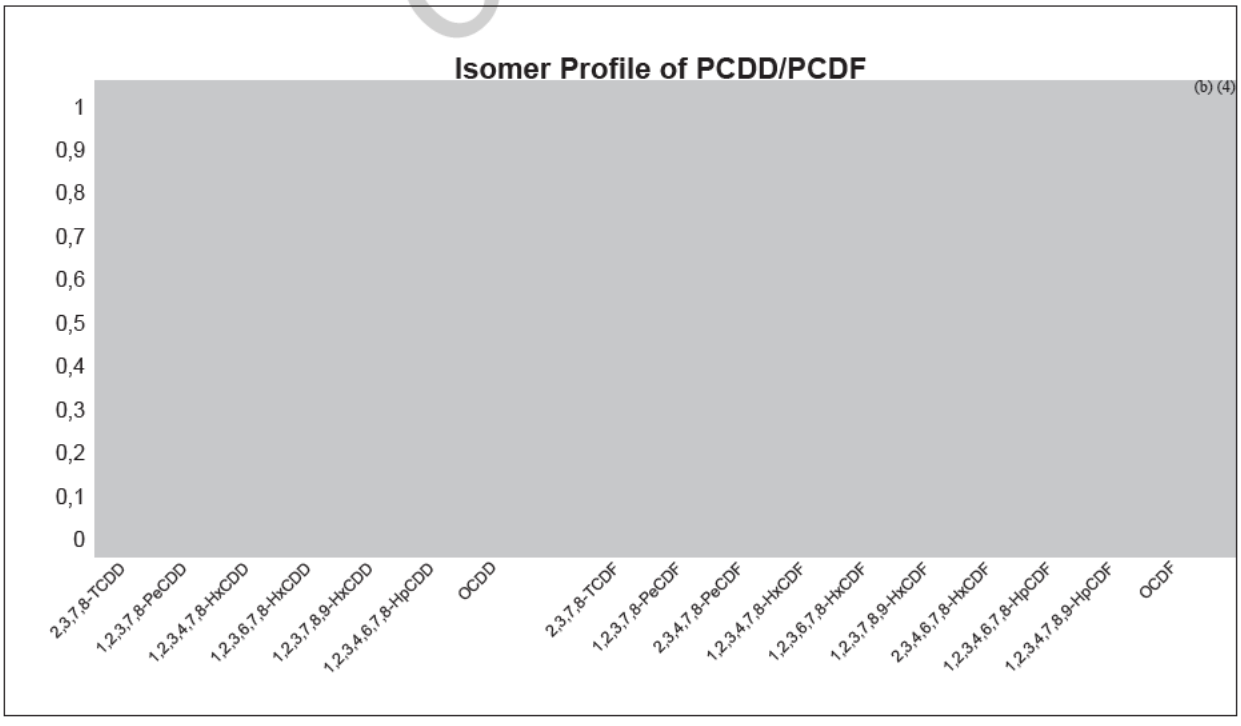
Lab-code: 1030/17-1
Unit: ng/kg 88% d.w.

	(b) (4)
2,3,7,8-TCDD	
1,2,3,7,8-PeCDD	
1,2,3,4,7,8-HxCDD	
1,2,3,6,7,8-HxCDD	
1,2,3,7,8,9-HxCDD	
1,2,3,4,6,7,8-HpCDD	
OCDD	
2,3,7,8-TCDF	
1,2,3,7,8-PeCDF	
2,3,4,7,8-PeCDF	
1,2,3,4,7,8-HxCDF	
1,2,3,6,7,8-HxCDF	
1,2,3,7,8,9-HxCDF	
2,3,4,6,7,8-HxCDF	
1,2,3,4,6,7,8-HpCDF	
1,2,3,4,7,8,9-HpCDF	
OCDF	

WHO-PCDD/F-TE 2005 (LOQ not included: 'lower bound'): (b) (4)

WHO-PCDD/F-TE 2005 (LOQ included: 'upper bound'):

(sum of PCDDs and PCDFs expressed in WHO toxic eq valents, u ng the WHO-TEFs, 2005)



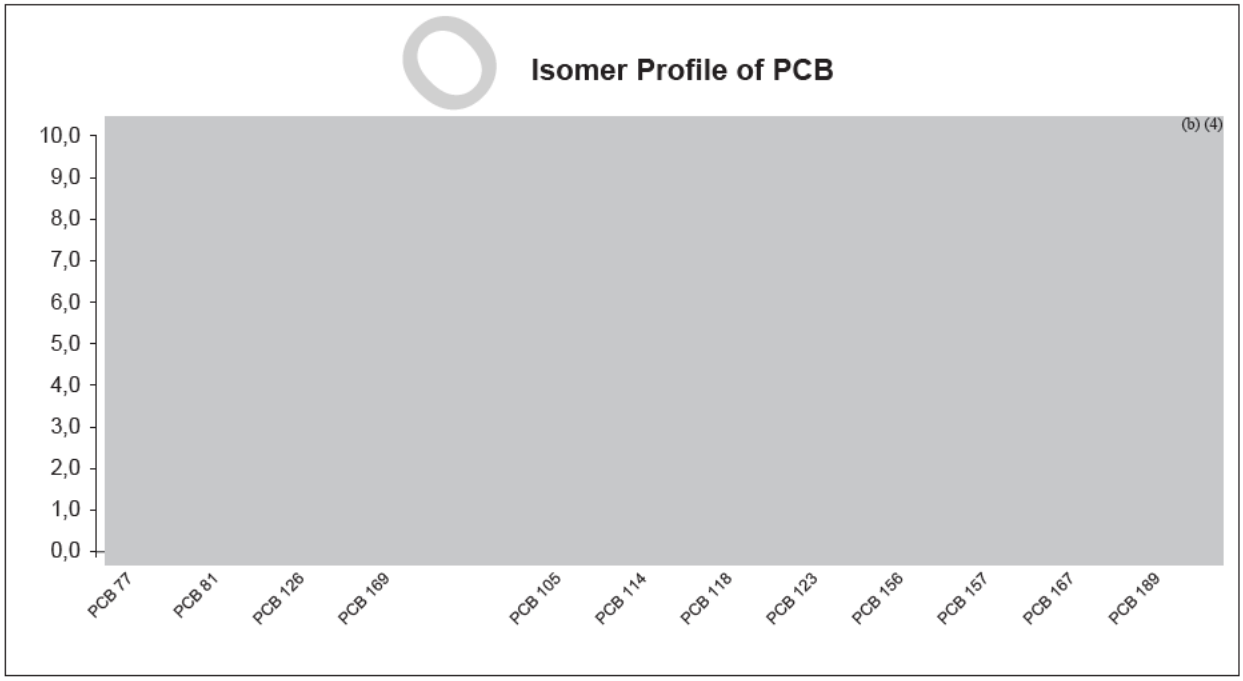
Dioxin-like polychlorinated biphenyls (PCB)

Sample name: E554 Zeolithe Type: 4A MA Batch: 17100001 / 957681045

Lab-code: 1030/17-1
Unit: ng/kg 88% d.w.

	(b) (4)
PCB 77	
PCB 81	
PCB 126	
PCB 169	
PCB 105	
PCB 114	
PCB 118	
PCB 123	
PCB 156	
PCB 157	
PCB 167	
PCB 189	

WHO-PCB-TE 2005 (LOQ not included: 'lower bound'):
WHO-PCB-TE 2005 (LOQ included: 'upper bound'):
(sum of dioxin-like PCBs expressed in WHO toxic equivalents, using the WHO-TEFs, 2005)



Test Report

No. of test report: 2237/17-1

Customer: 

Order date: 2017-08-28

Object of analysis: E554 Zeolite

Objective of analysis: Analysis of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/PCDF), polychlorinated biphenyls (PCB), heavy metals (As, Pb, Cd, Hg), Chromium, Aluminium

Sampling: by customer

Arrival of sample: 2017-08-31

Procedure of analysis: PCDD/PCDF, PCB: Commission Regulation (EC) No 152/2009 (amended by No 2017/771) HRGC/HRMS confirmatory analysis; As, Pb, Cd, Hg: DIN EN 15510; Chromium: LFGB L 00.00-19/3; Aluminium: DIN EN ISO 11885

Time of analysis: 2017-08-31 to 09-11

Results:

Sample name:		**E554 Zeolite** **Type: 4A MA** **Batch: 17229001**
Lab-code:		2237/17-1
Parameter	**Unit**	(b) (4)
PCDD/PCDF* (**WHO-TE$_{2005}$ LOQ incl.**)	**ng/kg 88% d.w.**	
Dioxin-like PCB* (**WHO-TE$_{2005}$ LOQ incl.**)	**ng/kg 88% d.w.**	
Sum of PCDD/PCDF and dioxin-like PCB* (**WHO-TE$_{2005}$ LOQ incl.**)	**ng/kg 88% d.w.**	
PCB 28*	µg/kg 88% d.w.	
PCB 52*	µg/kg 88% d.w.	
PCB 101*	µg/kg 88% d.w.	
PCB 138*	µg/kg 88% d.w.	
PCB 153*	µg/kg 88% d.w.	
PCB 180*	µg/kg 88% d.w.	
Sum of non dioxin-like PCB (LOQ incl.)*	**µg/kg 88% d.w.**	
Arsenic**	**mg/kg**	
Lead**	**mg/kg**	
Cadmium**	**mg/kg**	
Mercury**	**mg/kg**	
Chromium**	**mg/kg**	
Aluminium**	**mg/kg**	

Remarks:

d.w.: dry weight LOQ: limit of quantification *processed by our laboratory site (b) (4) (b) (4) analysis by accredited partner laboratory

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(b) (4) 2017-09-11

(b) (4)

Unit Management

(b) (4)

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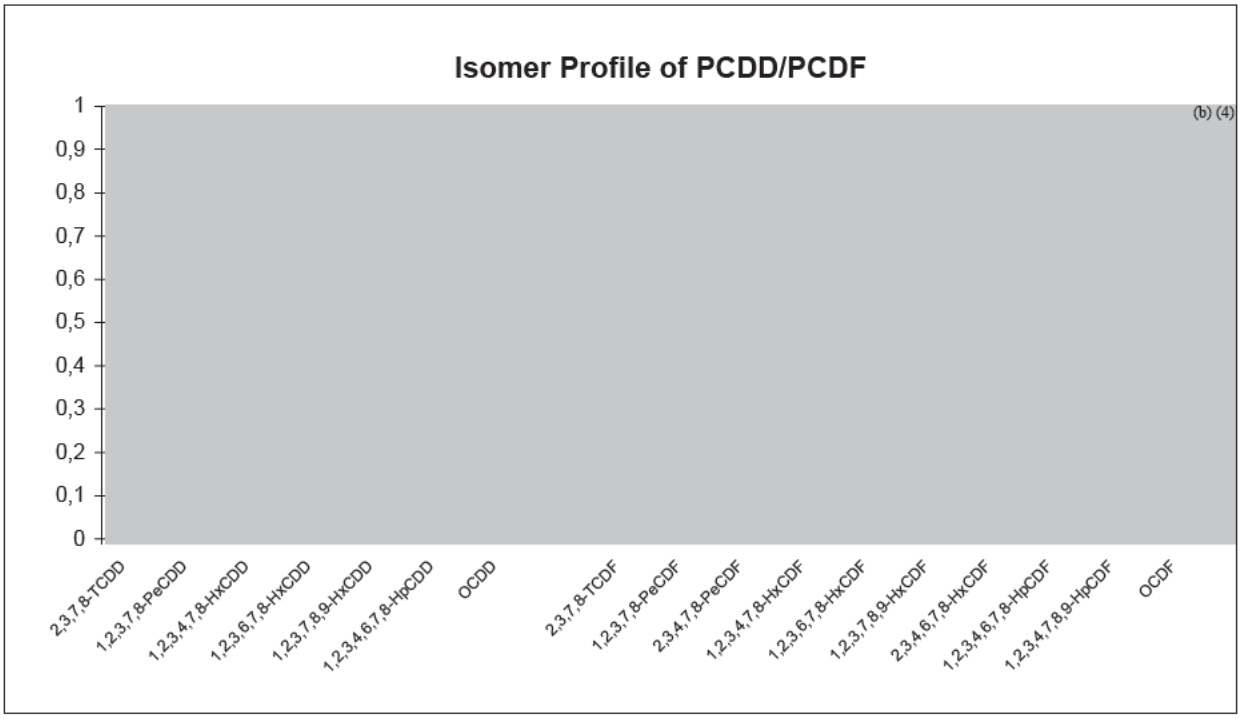
Polychlorinated dibenzodioxins and dibenzofurans (PCDD/PCDF)

Sample name: E554 Zeolite Type: 4A MA Batch: 17229001

Lab-code: 2237/17-1
Unit: ng/kg 88% d.w.

	(b) (4)
2,3,7,8-TCDD	
1,2,3,7,8-PeCDD	
1,2,3,4,7,8-HxCDD	
1,2,3,6,7,8-HxCDD	
1,2,3,7,8,9-HxCDD	
1,2,3,4,6,7,8-HpCDD	
OCDD	
2,3,7,8-TCDF	
1,2,3,7,8-PeCDF	
2,3,4,7,8-PeCDF	
1,2,3,4,7,8-HxCDF	
1,2,3,6,7,8-HxCDF	
1,2,3,7,8,9-HxCDF	
2,3,4,6,7,8-HxCDF	
1,2,3,4,6,7,8-HpCDF	
1,2,3,4,7,8,9-HpCDF	
OCDF	

WHO-PCDD/F-TE 2005 (LOQ not included: 'lower bound'):	
WHO-PCDD/F-TE 2005 (LOQ 1/2 included: 'medium bound'):	
WHO-PCDD/F-TE 2005 (LOQ included: 'upper bound'):	
(sum of PCDDs and PCDFs expressed in WHO toxic equivalents using the WHO-TEFs, 2005)	



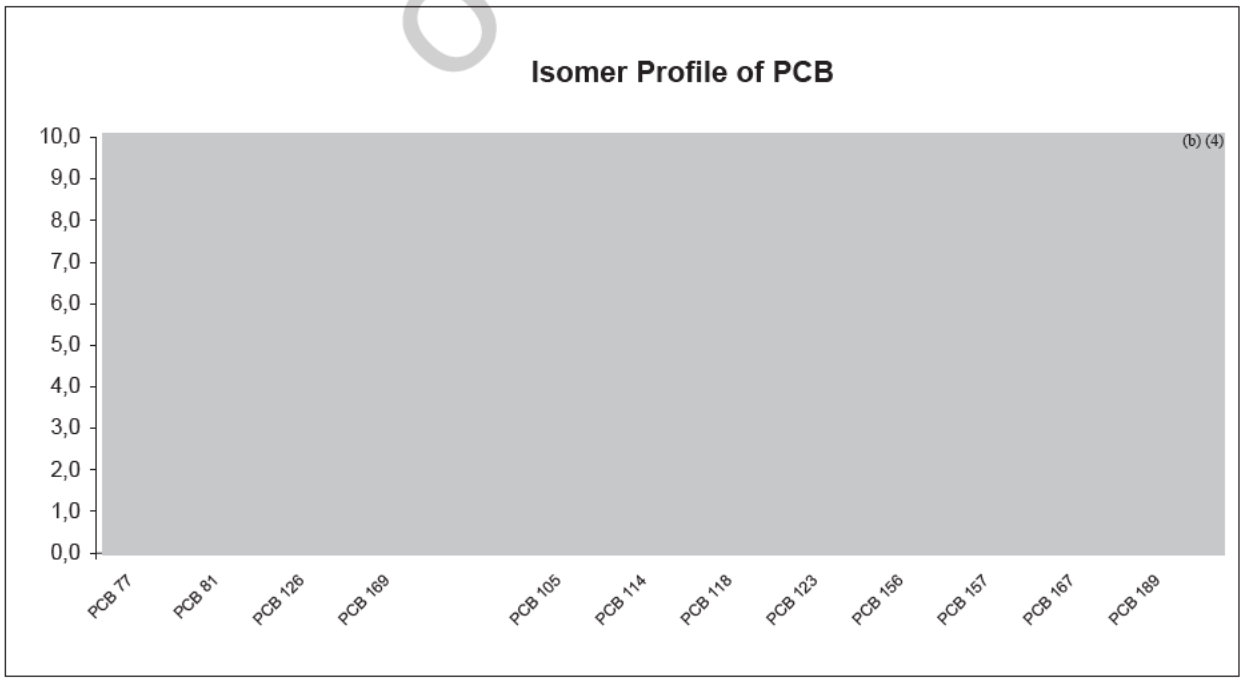
Dioxin-like polychlorinated biphenyls (PCB)

Sample name: E554 Zeolite Type: 4A MA Batch: 17229001

Lab-code: 2237/17-1
Unit: ng/kg 88% d.w.

PCB 77	(b) (4)
PCB 81	
PCB 126	
PCB 169	
PCB 105	
PCB 114	
PCB 118	
PCB 123	
PCB 156	
PCB 157	
PCB 167	
PCB 189	

WHO-PCB-TE 2005 (LOQ not included: 'lower bound'):
WHO-PCB-TE 2005 (LOQ 1/2 included: 'medium bound'):
WHO-PCB-TE 2005 (LOQ included: 'upper bound'):
(sum of dioxin-like PCBs expressed in WHO toxic equivalents, using the WHO-TEFs, 2005)



Test Report

No. of test report: 2237/17-2

Customer: 

Order date: 2017-08-28

Object of analysis: E554 Zeolite

Objective of analysis: Analysis of Chromium, Aluminium

Sampling: by customer

Arrival of sample: 2017-08-31

Procedure of analysis: Chromium: LFGB L 00.00-19/3; Aluminium: DIN EN ISO 11885

Time of analysis: 2017-08-31 to 09-07

Results:

Sample name:		**E554 Zeolite** **Type: 4A MA** **Batch: 17100001 (04/2017)**
Lab-code:		2237/17-2
Parameter	**Unit**	
Chromium*	**mg/kg**	(b) (4)
Aluminium*	**mg/kg**	

Remarks:
*analysis by accredited partner laboratory

(b) (4) 2017-09-07

(b) (4) (b) (4)

Unit Management
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(b) (4)

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Product specification sheet

Chemical properties	Unit	Specifications	Lab test results (b) (4)	Test methods
Na_2O	%	12.7 - 14.2		XRD
Al_2O_3	%	21 – 22.5		XRD
SiO_2	%	24 – 25.5		XRD
Physical properties	**Unit**	**Specifications**		
Colour		Grey		Internal/sensory
Product form		Granulate		Internal/sensory
Odour		neutral		Internal/sensory
Moisture (130°C)	%	Max 10 %		EC 152/2009
pH		10 – 12		Interna Potentiometry
Bulk density (tapped)	g/l	400 – 700		DIN ISO 697
Heavy metals	**Unit**	**Max. limit EU**		
Arsenic (As)	mg/kg	12		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Cadmium (Cd)	mg/kg	2		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Mercury (Hg)	mg/kg	0.2		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Lead (Pb)	mg/kg	15		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Dioxin	ng/kg	0.75		WHOPCDD/F-TEQ/kg
Sum of dioxin and dioxin, like PCBs	ng/kg	1		WHOPCDD/F-PCBTEQ/kg
Sum ikke-dioxin lign. PCB	µg/kg	10		(ICES-6, LOQ /kg

Composition:

75 – 80 % Zeolite 4A
17 – 20 % Wheat
1 – 3 % Rapeseed oil

Storage conditions/Shelf Li e

In case of stored under good s orage conditions (in a dry, good ventilated room), the product will be stable for at least 2 years after date of production.

Packaging:

15 kg – net bags

Transport:

Handle with care; avoid dust and any conditions which may damage the product quality.

Manufactured by:

(b) (4)

Patented. **GMP+ FSA assured.**

Net weight:	Production no.:	Product no.:	Production date:
15 kg	332775	254465	2020-01-03

Batch Qty: 8.055 kg

JIN-26-02-2020

(b) (4)

xzelit®

Product specification sheet

Chemical properties	Unit	Specifications	Lab test results (b) (4)	Test methods
Na_2O	%	12.7 - 14.2		XRD
Al_2O_3	%	21 – 22.5		XRD
SiO_2	%	24 – 25.5		XRD
Physical properties	**Unit**	**Specifications**		
Colour		Grey		Internal/sensory
Product form		Granulate		Internal/sensory
Odour		neutral		Internal/sensory
Moisture (130°C)	%	Max 10 %		EC 152/2009
pH		10 – 12		Interna Potentiometry
Bulk density (tapped)	g/l	400 – 700		DIN ISO 697
Heavy metals	**Unit**	**Max. limit EU**		
Arsenic (As)	mg/kg	12		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Cadmium (Cd)	mg/kg	2		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Mercury (Hg)	mg/kg	0.2		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Lead (Pb)	mg/kg	15		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Dioxin	ng/kg	0.75		WHOPCDD/F-TEQ/kg
Sum of dioxin and dioxin, like PCBs	ng/kg	1		WHOPCDD/F-PCBTEQ/kg
Sum ikke-dioxin lign. PCB	µg/kg	10		(ICES-6, LOQ /kg

Composition:

75 – 80 % Zeolite 4A
17 – 20 % Wheat
1 – 3 % Rapeseed oil

Storage conditions/Shelf Li e

In case of stored under good s orage conditions (in a dry, good ventilated room), the product will be stable for at least 2 years after date of production.

Packaging:

15 kg – net bags

Transport:

Handle with care; avoid dust and any conditions which may damage the product quality.

Manufactured bv:

(b) (4)

Patented. **GMP+ FSA assured.**

Net weight:	Production no.:	Product no.:	Production date:
15 kg	342384	254465	2020-01-24

Batch Qty: 7.020 kg

JIN-26-02-2020

(b) (4)

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Product specification sheet

Chemical properties	Unit	Specifications	Lab test results	Test methods
Na_2O	%	12.7 - 14.2	(b) (4)	XRD
Al_2O_3	%	21 – 22.5		XRD
SiO_2	%	24 – 25.5		XRD
Physical properties	**Unit**	**Specifications**		
Colour		Grey		Internal/sensory
Product form		Granulate		Internal/sensory
Odour		neutral		Internal/sensory
Moisture (130°C)	%	Max 10 %		EC 152/2009
pH		10 – 12		Interna Potentiometry
Bulk density (tapped)	g/l	400 – 700		DIN ISO 697
Heavy metals	**Unit**	**Max. limit EU**		
Arsenic (As)	mg/kg	12		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Cadmium (Cd)	mg/kg	2		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Mercury (Hg)	mg/kg	0.2		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Lead (Pb)	mg/kg	15		EN 13805m:2014, EN ISO 17294m:2016 / ICP-MS
Dioxin	ng/kg	0.75		WHOPCDD/F-TEQ/kg
Sum of dioxin and dioxin, like PCBs	ng/kg	1		WHOPCDD/F-PCBTEQ/kg
Sum ikke-dioxin lign. PCB	µg/kg	10		(ICES-6, LOQ /kg

Composition:

75 – 80 % Zeolite 4A
17 – 20 % Wheat
1 – 3 % Rapeseed oil

Storage conditions/Shelf Li e

In case of stored under good s orage conditions (in a dry, good ventilated room), the product will be stable for at least 2 years after date of production.

Packaging:

15 kg – net bags

Transport:

Handle with care; avoid dust and any conditions which may damage the product quality.

(b) (4)

Patented. **GMP+ FSA assured.**

Net weight:	Production no.:	Product no.:	Production date:
15 kg	347568	254465	2020-02-04

Batch Qty: 7.665 kg

JIN-26-02-2020

[redacted]	Specification [redacted]	Issue III 11.03.2019 r.
	Customer: [redacted]	

Product: Packaging film

Material: High density polyethylene PE-HD

Colour: transparent

Perforation: P1010, P1515, P2025

Cuts: N2333, N3030, N2545

Parameter	Unit	Dimension/tolerance	Test method
Width	mm	[redacted]	PN-ISO 4592:1999
Thickness	mm		PN-ISO 4593:1999
Density	g/cm^3		According to TDS of material
Tensile strength*	MPa		
MD			PN-EN ISO527-1:2012
TD			PN-EN ISO527-3:1998
Elongation*	%		
MD			PN-EN ISO527-1:2012
TD			PN-EN ISO527-3:1998
Corona pretratment double sided	mN/m		I-8.2/DZJ/13

*for thickness ≥0,02 mm without perforation

Packaging

Max diameter od rolls	(b) (4)
Palette	
Packaging	
Coreboard- inside diameter	
Label	

PERFORATION

N2333

(b) (4)

N3030

N2545

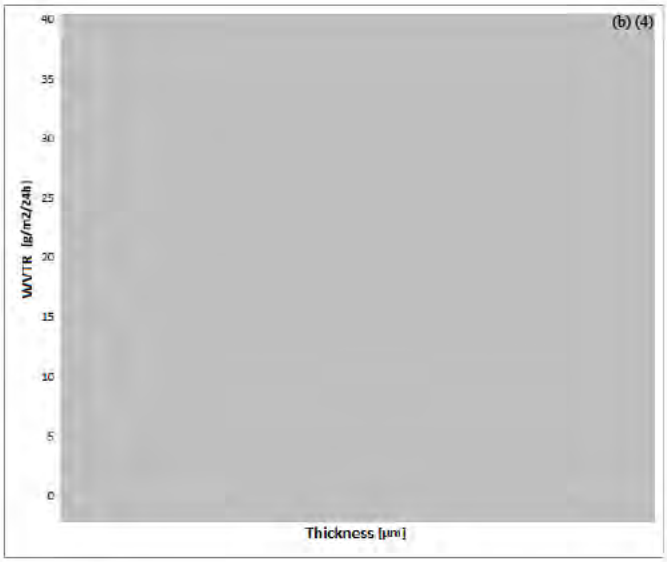
P1010

P1515

P2025

(b) (4)

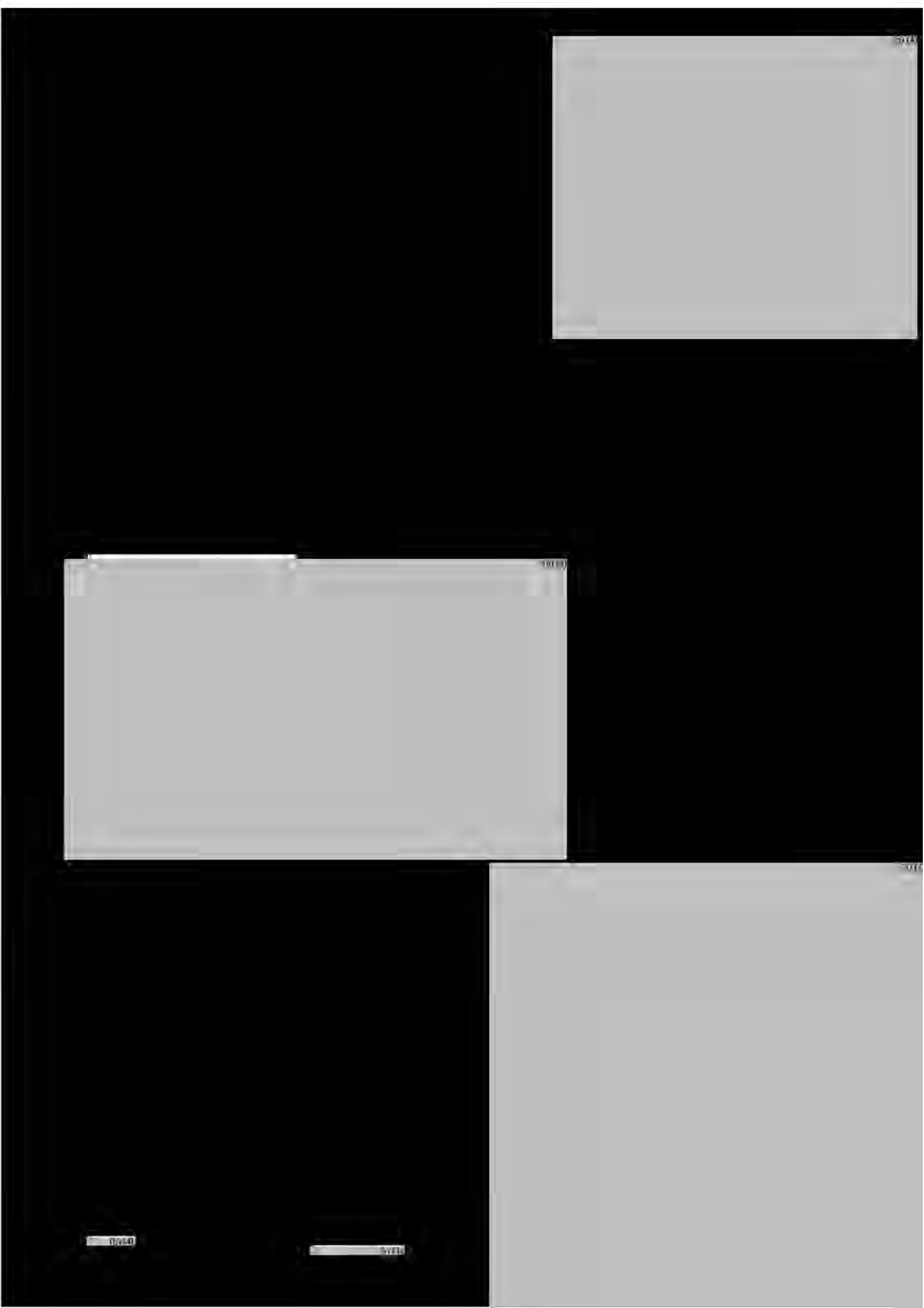
WVTR



MEASUREMENT CONDITION:
38°C, 90% ΔRH

(b) (4)

Customer:



Feed Anti Caking Agents Market

Feed Anti Caking Agents Market Analysis by Source (Natural, Synthetic), By Application (Food, Feed, Fertilizer), By Region (North America, Europe, Asia Pacific, Latin America, and the Middle East and Africa) – Global Industry Size, Share, Growth, Trends, Demand and Forecasts 2021-2026

Pulished: April, 2021 ID: 5046
Pages: 175

Product Description | Table of Contents | Scope of Report
List of table & Figure | Request Sample

Feed Anti Caking Agent Market Overview Analysis:

Feed Anti Caking Agent Market is anticipated to enroll a CAGR of 4.6% during the conjecture time frame. An anticaking agent is an added substance utilized by the feed and manure industry to keep away from the arrangement of irregularities in feeds and composts. It is likewise used to improve the packaging and transportation of these feeds and composts. Anticaking agents give better answers for the issue of dampness adsorption, nitrogen mishap, and building up in granulated manures. Anticaking agents include surface pressure modifiers, ground-breaking surfactants, precious stone propensity modifiers, and separating agents. Anticaking agents are dissolvable in liquor, water, and other natural solvents.

The rising need for an excellent feed and an expansion in the quantity of feed added substances, particularly in the compound feed industry, are the significant development drivers of the market considered.

The anti-caking agent is an additive, utilized in the food industry including seasoning & condiments, dairy bakery, products, meat products, soups & sauces, and sweeteners. It is utilized in the form of powder or granules for preventing the formation of lumps and for easing consumption, packaging, and transport. They are nutritionally void. Few commonly used anti-caking agents in the food industry are calcium silicate, magnesium stearate, powdered cellulose, sodium aluminosilicate, and silicon dioxide, sodium bicarbonate, magnesium trisilicate, calcium Ferro cyanide potassium aluminum silicate, sodium Ferrocyanide, stearic acid, tricalcium phosphate, and polydimethylsiloxane. Sodium aluminosilicate is utilized as an anti-caking agent in table salts.

Impact of Covid-19 on the Global Feed Anti-Caking Agents Market:

Because of the Covid-19 pandemic, there has been a complete shutdown in the manufacturing and distribution network. There is a huge decline in the overall revenue in this industry in 2020, which is supposed to take a few years to revive.

Market Trends:

Extension of the Compound Feed Industry is driving the market growth;

The market considered is basically determined by the extension of the animal feed market. As the domesticated animal's industry enrolled a low development rate, there is an expanding requirement for animal feed. This is required to drive the interest for feed added substances, especially feed anti-caking agents. In addition, silicates are broadly utilized as anti-caking agents, as silicates can assimilate, both, water and oil. Sodium and calcium items, for example, sodium ferrocyanide and tricalcium phosphate are seeing

calcium items, for example, sodium ferrocyanide and tricalcium phosphate are seeing appeal, as they diminish the singular grip of feed.

North America and Europe are the major regions in the market;

The North American locale is the biggest land portion in the worldwide feed anti-caking agents market, firmly followed by Europe. Poultry and pig represented 40% and 25% of the piece of the pie, individually. The United States represented practically 70% of the local piece of the pie, chiefly because of its settled domesticated animal's production industry. Moreover, the requirement for top-notch feed is driving the development of the market contemplated.

Recent Developments:

1. In September 2015, Solvay initiated South-East Asia's biggest sodium bicarbonate plant situated in Thailand to satisfy the developing need for high-quality items in the medicinal services, food, and other purchaser merchandise markets all through Asia.
2. In March 2014, Evonik opened another plant in Thailand to expand the production limit of hastened silica in Southeast Asia. The encouraged silica is utilized in the car, food, and animal feed ventures.
3. In May 2015, Brenntag obtained Quimicas Meroño, S.L (Spain), mechanical synthetics wholesaler, to extend its business in Spain. This securing helped Brenntag's neighbourhood conveyance organize in Eastern Spain and furthermore empowers the organization to get to a few key European markets, which makes development openings, essentially in key divisions, for example, food and oil and gas.
4. In July 2015, Univar procured the advantages of Chemical Associates Inc. (US). The vital securing helped the organization upgrade its market nearness by going into new markets and sections, for example, the food and personal care businesses. This procurement likewise helped in extending its production limit and its current items portfolio in the US office.
5. In February 2017, Brenntag has acquired Lionheart Chemical Enterprises (Pty) Ltd (South Africa). The acquisition has fuelled Brenntag to reinforce its situation in the South African compound distribution market. Lionheart's item portfolio likewise bolstered different exercises of Brenntag in the South African area for Multisol and Crest Industrial Chemical organizations. It further helped the organization's capability to build its deals.

Market Dynamics:

Drivers and Restraints:

The global feed anti-caking agent market is driven by the quick extension of the animal feed industry which quickens the interest for these agents. The feed makers are more concerned with forestalling hardening, lumping, connecting, of their feed items. This has driven their methodology towards the feed anti-caking agents. Also, an issue in regards to the simplicity of packaging and transport is another prime factor for the feed items. The feed anti-caking agent is additionally liable to improve things and packaging of the feed items which likewise expands its interest in the market. Moreover, silicates are among the broadly utilized feed anti-caking agents inferable from its retention capacity of both water and soil. Besides, the fast development of the domesticated animal's division is likewise advancing the utilization of feed anti-caking agents as the developing nations are progressively moving their eating regimens to animal items. The utilization of meat items is developing quickly in the rising locale as it speaks to a huge populace on the planet. It has expanded the development of the animal segment over the world just as it builds interest in feeding anti-caking agents.

But, analysis of anti-caking agents has been indicating that anti-caking agents may adversely affect food nourishment. For example, a particular report result has referenced that most enemies of building up agents brought about the extra debasement of Vitamin C added to food. Such downsides of anti-caking agents have been hosting the development pace of the anti-caking agents market.

Market Segmentation:

The worldwide feed anti-caking agents market can be divided depending on the source, application and region.

As far as source, the anticaking agent for the feed market is sectioned into natural and synthetic.

By application, the anticaking agent for the feed and manure market can be isolated into fertilizer, poultry and dairy cattle, and others. Fertilizer is the significant application portion of the anti-caking agent for the feed and manure market. Anti-caking agents are basically utilized in nitrogen, potassium, ammonium, and phosphorus-based manures to keep them from retaining dampness from the air and framing a cake. In poultry and dairy cattle, anticaking agents are utilized in feed to quit hardening and agglomeration and increment the stream properties of feed ingredients.

Regional Analysis:

In view of the region, the global feed anti-caking agent market can be sectioned into North America, Europe, Asia Pacific, the Middle East and Africa, and Latin America. North America is foreseen to rule the worldwide market, trailed by Europe and Asia pacific. Extension in the domesticated animals' industry in North America is evaluated to help the local market. Interest in animal feed is rising continually, which is anticipated to drive the feed added substances market, especially in feed anti-caking agents. Ascend in population for the anticaking agent for feed and manure in the farming business in the Asia Pacific is foreseen to help the general market during the gauge time frame. Government activities to expand crop yield and great strategies empowering economical and effective rural practices are likewise assessed to assume basic jobs in boosting the market in the district during the conjecture time frame. The feed anti-caking agent market in the Middle East and Africa and Latin America is additionally anticipated to grow generously during the following few years.

Key Players

- Novus International
- Evonik Industries
- Bentonite Performance Minerals LLC
- PPG Industries
- Kao Chemicals
- Brenntag AG
- Univar Inc
- Solvay S.A
- PQ Corporation
- Agropur Ingredients

Related Reports

North America Feed Anti Caking Agents Market	Apr 2021	Download Sample
Europe Feed Anti Caking Agents Market	Apr 2021	Download Sample

(b) (4)

(b) (4)

(b) (4) brand

Dry Anti-Caking Aid

ITEM CODE
016508

DESCRIPTION
An anti-caking aid and non-nutritive carrier to be used for further manufacturing of animal feeds.

INGREDIENTS
Hydrated Calcium Aluminosilicate, Hydrated Sodium Aluminosilicate and Mineral Oil.

QUALITY SPECIFICATIONS

Parameter	
Residue	0 – 5% (20 mesh screen)
Moisture	0 – 18%
Color	Off white to pale green

TYPICAL CHARACTERISTICS

Parameter	
Bulk Density	45 – 60 lb/ft^3

TYPICAL COMPOSITION

NUTRITIONAL VALUES[1]	
Moisture	12.00% (max)
Crude Fat	3.00% (min)
Ash	87.00% (max)
Sodium	0.10% (min) and 0.60% (max)
Potassium	2.00% (min)
Calcium	1.50% (min) and 2.00% (max)
Iron	6,000 ppm (min)
Copper	5 ppm (max)
Zinc	35 ppm (min)
Barium	1,000 ppm (max)
Lead	30 ppm (max)

[1] All non-listed values are zero (energy, CP, other minerals, etc.)

(b) (4)

INSTRUCTIONS FOR USE

Use at the rate of 2-12 lbs per ton in total mixed rations or in the finished feed. Not to exceed 2.0% in finished feed.

STORAGE

Store in a cool, dry place. Keep bag closed when not in use.

PACKAGING

25 KG bag, 853 KG and 1,000 KG bulk sack

[redacted]

Section 1 Company Information

Company Name [redacted]
Telephone
Address

Product Name

Section 2 Product Information

DESCRIPTION
SODIUM CALCIUM ALUMINOSILICATE ANIMAL FEED INGREDIENT

[redacted]onsists of an extremely pure form of clinoptilolite zeolite, or sodium aluminosilicate, that acts as a buffer, an anticaking agent and much more when included in feed as a dietary supplement.

[redacted]offers many benefits when used as a feed additive for beef cattle & dairy cows, swine, poultry, and horses.

Since zeolite is the world's only naturally occurring negatively charged mineral a great number of benefits in the feed process can result from adding it to animal feed. FreeFlow feed supplement is inert, stable, non-toxic and approved for animal feed (CFR 582-2727).

[redacted] a naturally occurring mineral formed when prehistoric volcanic ash fell over fresh spring water. The result is a unique aluminosilicate molecule with a highly porous, and negatively charged hexagonal structure. This negative charge, combined with a voluminous microbial surface area creates an environment for high cation exchange rates.

Section 3 Certifications

OMRI Listed
Certified Organic

Section 4 Properties

Chemical Formula	Na6[Al6Si30O72]24H2O
Clinoptilolite Content	[redacted]
Form	
Shape	
Color	
Pore Diameter	
Specific Gravity	
Specific Surface Area	
Bulk Density	
pH stability	
Hardness	
Swelling Index	
Cation Exchange Capacity (CEC)	

Section 5 Chemical Analysis

SiO2	Al2O3	Fe2O3	CaO	MgO	Na2O	K2O	MnO	TiO2
[redacted]								

Section 6 Major Exchangeable Cations

[redacted]

Section 7 Hazards

Potential Acute Health Effects
Slightly hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation.

Potential Chronic Health Effects
Hazardous in case of skin contact (corrosive).
Slightly hazardous in case of skin contact (irritant).

Carcinogenic Effects
3 (Not classifiable for human.) by IARC.

Mutagenic Effects
Not available.

Teratogenic Effects
Not available.

Developmental Toxicity
Not available.

Section 8 First Aid

Eye Contact
Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention if irritation occurs.

Skin Contact
Wash with soap and water. Cover the irritated skin with an emollient. Get medical attention if irritation develops.

Inhalation
If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. *Get medical attention.*

Ingestion
Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately.

Section 9 Warnings

CONTACT WITH TURPENTINE, VEGETABLE OIL, UNSATURATED ORGANIC COMPOUNDS AND HYDROFLUORIC ACID MAY GENERATE HEAT. DO NOT USE WITH THESE COMPOUNDS.

Section 10 Recommended Use

Include between 2% - 5% FreeFlow additive to animal feed. Sprinkle over bedding and flooring as needed for odor and moisture control.