Protein Efficiency Ratio (PER) Rat Bioassay Studies to Demonstrate that a New Infant Formula Supports the Quality Factor of Sufficient Biological Quality of Protein: Guidance for Industry

Draft Guidance

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For questions regarding this draft document, contact the Center for Food Safety and Applied Nutrition (CFSAN) at 240-402-1450.

U.S. Department of Health and Human Services
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Protein Efficiency Ratio (PER) Rat Bioassay Studies to Demonstrate that a New Infant Formula Supports the Quality Factor of Sufficient Biological Quality of Protein: Guidance for Industry[1]

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I. Introduction

The Infant Formula Act of 1980 (Pub. L. 96-359) amended the Federal Food, Drug, and Cosmetic Act (FD&C Act) to include section 412 (21 U.S.C. 350a). In 1986, Congress amended section 412 of the FD&C Act to require FDA to establish quality factors for infant formula and stipulated that an infant formula would be considered adulterated if it does not meet the quality factor requirements.[2] On June 10, 2014, as part of the final rule*, Current Good Manufacturing Practices, Quality Control Procedures, Quality Factors, Notification Requirements, and Records and Reports, for Infant Formula* (Infant Formula Final Rule)*,* FDA established requirements for quality factors for infant formulas (79 FR 33057), including the quality factor of sufficient biological quality of protein (21 CFR 106.96(e) and (f)).

An infant formula must meet the quality factor of sufficient biological quality of protein (21 CFR 106.96(e)). Specifically, 21 CFR 106.96(f) describes how an infant formula manufacturer must demonstrate that a formula meets this quality factor:

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[1] This guidance has been prepared by the Office of Nutrition and Labeling, Infant Formula and Medical Foods Staff in the Center for Food Safety and Applied Nutrition in cooperation with the Office of Regulations and Policy at the U.S. Food and Drug Administration.

[2] See Anti-Drug Abuse Act of 1986, Pub. L. 99-570, § 4014.

> A manufacturer of an infant formula that is not an eligible infant formula shall demonstrate that a formula meets the quality factor of sufficient biological quality of protein by establishing the biological quality of the protein in the infant formula when fed as the sole source of nutrition using an appropriate modification of the Protein Efficiency Ratio (PER) rat bioassay described in the "Official Methods of Analysis of AOAC International," 18th ed., sections 45.3.04 and 45.3.05, "AOAC Official Method 960.48 Protein Efficiency Ratio Rat Bioassay," which is incorporated by reference at § 106.160 (see Appendix 1). The PER rat bioassay shall be conducted on a formula and the results evaluated prior to the initiation of a growth monitoring study of the formula that is required under 21 CFR 106.96(b).

Conducting a PER study in an animal model[3] permits a determination of a formula's protein quality before infants are exposed to the formula.[4] This approach ensures that infants will not be fed a formula with inadequate or biologically unavailable protein.[5]

We have developed this guidance to help manufacturers and laboratories in the design, conduct, evaluation, and reporting of PER studies. The guidance is intended to explain how the PER study can be used to provide assurance that a new infant formula meets the quality factor of sufficient biological quality of protein in the infant formula when fed as the sole source of nutrition using appropriate modifications of AOAC Official Method 960.48 (the AOAC Method; Reference 1) (see 21 CFR 106.96(f)).

In general, FDA's guidance documents do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word should in Agency guidances means that something is suggested or recommended, but not required.

II. Background

The purpose of the PER methodology, as defined in the AOAC Method, is to determine the efficiency (i.e., quality) of a test protein ingredient compared with that of the reference casein protein from a rat bioassay. The quality is determined by comparing growth rates from rats consuming a diet containing the test protein ingredient with those of rats consuming a diet containing the reference casein protein. The two diets should be similar in composition to avoid the possibility of creating confounding variables. The PER method defines the dietary

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[3] We support the principles of the "3Rs" to reduce, refine and replace animal use in testing when feasible. We encourage sponsors to consult with us if they wish to use a non-animal testing method they believe is suitable, adequate, and validated to demonstrate that the formula supports the quality factor for the biological quality of the protein as described in 21 CFR 106.96(g)(3). We support alternative methods by exemption in 21 CFR 106.96(f) which allows the manufacturer to request an exemption and provide certain required assurances described in 21 CFR 106.96(g). The applicability of this exemption is not the subject of this guidance.

[4] Current Good Manufacturing Practices, Quality Control Procedures, Quality Factors, Notification Requirements, and Records and Reports, for Infant Formula, Interim Final Rule, 79 FR 7934, at 8023, Feb. 10, 2014.

[5] 79 FR at 8023.

components in the protein evaluation basal diet and adjustments needed to ensure that all comparisons between the test sample and the reference casein are made with diets having a similar content of nitrogen, fat, ash (i.e., inorganic residual remaining after water and organic matter have been removed by heating), moisture, and crude fiber.

Despite recognized limitations, the PER bioassay is unique in its ability to assess relative protein utilization. Chemical measurements of total protein (i.e., measurement of nitrogen) and determination of amino acid patterns are also possible and may be appropriate for certain aspects of protein quality determinations (Reference 2). However, such chemical measurements do not address the bioavailability of a protein. The PER study permits a comparison of the bioavailability of different protein sources. We are not aware of any other method to assess protein bioavailability. In addition, the PER study's standardization as the AOAC Method has led to improved reproducibility (Reference 3).

FDA regulations require that the protein in an infant formula be evaluated as part of the whole formula matrix, rather than as a single protein ingredient, to account for the effects of processing or other components on digestion and protein availability.[6] One challenge with this requirement is how to control for components in the formula matrix that fall outside of the original description of the bioassay in the AOAC Method (References 4 through 7).

It is well-recognized that the composition of certain food products can influence the assessment of the PER (References 4, 8). Modifications to the diets described in the AOAC Method are necessary because infant formulas have relatively high fat and low protein contents and may contain high concentrations of lactose. The aim of specific diet modifications is to ensure that the infant formula containing the test diet and the casein reference diet are as similar as possible so that protein quality can be evaluated without being confounded by other differences between the test and reference diets. Adjustments to diet compositions from those described in the AOAC Method are made to attain similar levels and types of fat, carbohydrate, and ash content. In addition, an infant formula brings its own complement of vitamins and minerals into the PER study test diets. The matching of these additional nutrients in the PER study reference and test diets is challenging. Without proper modifications to the reference diets from what is specified in the AOAC Method, the PER values of infant formulas may be underestimated in comparison to casein (Reference 7).

Modifications to the diets described in the AOAC Method are also necessary to bring the AOAC Method into alignment with current knowledge of rat nutrition. Since the development of the AOAC Method in the 1960s, there have been significant developments in understanding the nutrient requirements of rats. With respect to infant formula, FDA considers modifications to the diet described in the AOAC Method that are based on updated knowledge of rat nutrition to be "appropriate modifications" of the AOAC Method within the meaning of 21 CFR 106.96(f).

[6] See 21 CFR 106.96(f).

Section III provides information about the AOAC Method as it was originally written.

Section IV provides recommendations that address "appropriate modifications" to update the AOAC Method to bring it in line with current knowledge of rat nutrition, as well as recommendations that address "appropriate modifications" of the AOAC Method for infant formula.

III. Overview of AOAC Official Method 960.48 – Protein Efficiency Ratio (PER), Rat Bioassay

The AOAC Method was developed in the early 1960s and provides a procedure by which the quality of a protein can be evaluated and compared with those of other proteins. Protein "quality" can be defined as the ability of a protein to meet the essential amino acid needs of an animal, including humans. The AOAC Method is a standardized bioassay, and its collaborative study data have been published[7] (References 9, 10).

The AOAC Method permits the calculation of a PER as the ratio between the average animal body weight gain per gram of protein consumed of a test protein versus casein after a 28-day feeding period. Typically, to improve the sensitivity of the method, the protein concentration of both the test and reference diets is set at about 10%, a level that is below the estimated level required for growth of 15% (References 3, 11). While growth is slower at 10% protein than at 15% protein, the lower protein levels ensure that available protein is used efficiently.

PER studies need to be carried out under standardized conditions that include age and species of rat, diet composition including protein levels, and duration of feeding and feeding method, so that studies with matrices containing different proteins can be compared (Reference 12). These conditions are defined in the AOAC Method. Certain conditions of the PER study are not defined (e.g., strain of rat, diet fed during acclimation period), while others are defined within specified ranges (e.g., age of rats, length of acclimation period, number of rats per group, frequency of measurements).

While the AOAC Method is flexible in many respects, ensuring consistency between the test and control groups with regard to diet composition is critical to ensure the assay is specific for protein quality and the comparison is not influenced by other uncontrolled differences between the test and reference diets. To ensure the test and reference diets are matched except for protein source, adjustments are needed to the reference diet specified in the AOAC Method. In

[7] Two multi-laboratory collaborative studies provided validation data for the AOAC Method. PER values for the casein control groups were (grams (g) body weight gain/g protein consumed for the 28-day study period): 2.79 ± 0.34 (Reference 9 sucrose as carbohydrate source), 3.12 ± 0.22 (Reference 10 starch as carbohydrate source), and 2.66 ± 0.26 (Reference 10) sucrose as carbohydrate source). FDA considers PER values between 2.4 and 3.3 to be reasonable for casein control groups under the original conditions of the assay.

conducting a PER assay, the performance of the contemporaneous reference group[8] is as critical to the evaluation of the results as the performance of the test group because the results are reported as relative rather than as absolute values (Reference 4).

The PER study defined in the AOAC Method (see Appendix 1) provides a specific nutrient composition and formulas for adjustments that are needed to ensure that the test and reference diets have similar contents of nitrogen, fat, ash, moisture, and crude fiber. General compositional requirements and adjustments are summarized below. An example of the formulation for the control diet for a PER study as originally described in the AOAC Method is shown in Appendix 2.

AOAC Method Diet Formulation and Adjustments

Ingredient	**Dietary level, %**	**Dietary adjustment**
Protein	…...10	Protein from test sample
Cottonseed oil	8	Minus fat content of test sample
Salt mixture	5	Minus ash content of test sample
Vitamin mixture	1	No adjustment described
Cellulose	1	Minus fiber content of test sample
Water	5	Minus moisture content of test sample
Sucrose or corn starch	To 100	

According to the AOAC method, proximate analysis[9] is needed to adjust the diets so that all comparisons between the test protein ingredient and the reference casein protein are made with diets having similar overall contents of nitrogen, fat, ash, moisture, and crude fiber (Reference 1). Thus, the minimum specifications for chemical analyses for PER study diets include the determination of nitrogen, fat, ash, moisture, and crude fiber. Additional analyses can be performed if desired, and many methods are available (e.g., Official Methods of Analysis of AOAC International, AOAC International, Rockville, MD; Official Methods and Recommended Practices of the American Oil Chemists Society, American Oil Chemists Society, Urbana, IL; and Approved Methods of Analysis of the Cereals and Grains Association, Cereals and Grains Association, St. Paul, MN).

[8] Historical control group data (e.g., data from groups of rats fed casein diets in earlier studies) are generally unable to replace a contemporaneous control group because diets used in generating such data are unlikely to match the composition of the infant formulation under consideration. It is also unlikely that details of such historical studies including age of rats, duration of study, acclimation period and diet, study diet compositions, etc. would be the same as those in the AOAC Method.

[9] Proximate analysis refers to the quantitative analysis of protein, fat, moisture, ash, and crude fiber. Carbohydrate is calculated by difference.

Formulas are provided in the AOAC Method to allow calculation of reference diet composition based on results of proximate analysis of the test material. Examples of applications of such calculations are shown in Appendix 2.

IV. "Appropriate Modifications" of AOAC Official Method 960.48

A. Need for "Appropriate Modifications" to Update the AOAC Method and for Use of Infant Formulas in PER Bioassays

Since the publication of the AOAC Method in the 1960s, there have been significant developments in our understanding of the nutrient requirements of rats. Thus, for PER studies generally, there is a need for updates to the AOAC Method to ensure such studies are conducted in a manner consistent with the most current understanding of nutrient requirements of rats. With respect to infant formula PER studies, FDA considers changes to the AOAC Method that are based on such current understanding of nutrient requirements of rats to be "appropriate modifications" of the AOAC Method under 21 CFR 106.96(f).

There is also a need for "appropriate modifications" of the AOAC Method when determining the PER of infant formulas. In the PER study as originally described in the AOAC Method, a protein ingredient was assayed at 10%, and other potential variables (e.g., age of rats, diet compositions, length of study) were standardized to minimize confounding variables (Appendix 1). Vitamin composition, moisture, ash, carbohydrates, fat, and fiber were similar for both reference and test diets. Use of a test diet that includes an infant formula in its entirety introduces matrices of high fat content and additional vitamins, minerals, and other ingredients as well as low protein content. A major challenge in analyzing infant formulas by the AOAC Method is matching the reference and test diets to achieve dietary groups with as few confounding variables as possible.

Studies that have evaluated variables that might be encountered when determining the PER of infant formulas have found that modifications of the levels and sources of fat and carbohydrate in casein reference diets to match those in various infant formulas led to significant changes in the resultant PER study values (Reference 7). For example, PER values (g body weight gain/g protein consumed for the 28-day study period; mean ± SEM[10]; 10 rats/group) varied from 3.2 ± 0.13 in an unmatched casein reference diet to 2.3 ± 0.24 and 2.1 ± 0.16 in two matched, milk-based formula test diets (Reference 7). The PER value of 2.1 is 66% of the value measured in the unmatched reference group and shows the magnitude of the difference that may result when reference and test diets are not well-matched. In these data, the PER values of the milk-based infant formulas appear to be significantly underestimated in comparison to the PER value of the casein reference group. The protein quality evaluation of infant formulas using the PER

[10] Standard Error of the Mean (SEM) is an estimate of how far the sample mean is likely to be from the population mean.

bioassay warrants the use of matched casein reference diets for each type of formula (Reference 7).

B. Conduct and Analysis of a PER Study with "Appropriate Modifications" (matching the casein reference (control) and test diets)

1. Preparation of Experimental Diets

Limitations with respect to the assessment of the quality of protein sources for infant formulas using the AOAC Method can be greatly reduced by appropriate modifications of the test and reference diets. Generally, studies with groups of rats fed unmatched diets are very difficult to interpret because the results are confounded by too many variables. For this reason, the compositions of the reference and test diets should be followed throughout the manufacturing process and adjustments to procedures made, as needed, to avoid such confounding variables. The composition of the reference diet in a PER study is within the scope of the investigator's responsibility, and many adjustments can be made in order to match the composition of the casein reference diet to that of the infant formula-based test diet. In the sections below, references to the "casein control" or "control" or "casein reference control" refer to the casein reference diet.

As stated in FDA's interim final rule, *Current Good Manufacturing Practices, Quality Control Procedures, Quality Factors, Notification Requirements, and Records and Reports, for Infant Formula* (Infant Formula Interim Final Rule):

> Prior to study initiation, the test product (finished infant formula) and the casein control are subjected to a compositional assessment (proximate analysis). The diets are then formulated to contain matching amounts of protein, fat, minerals, fiber, and moisture. These diets are analyzed for protein to confirm that they were formulated correctly, which information is used to calculate the PER at completion of the trial.

79 FR 7934, 8023 (Feb. 10, 2014).[11] The Infant Formula Interim Final Rule also stated, "Although the method has limitations with respect to assessment of the quality of protein sources for infant formulas, the limitations are greatly reduced by modification of the test and control diets" (79 FR 7934 at 8023) and provided details on "[t]hree dietary adjustments commonly required for evaluation of the protein quality of infant formulas": (1) matching of the fat content of the reference and test diets; (2) matching of the carbohydrate composition of the reference and test diets; and (3) removal of water from liquid infant formula to achieve the lower limit of nitrogen (1.8% by weight) specified by the PER bioassay (79 FR 7934 at 8023 to 8024). FDA also has considered other areas in which modifications may be appropriate, including the

[11] The preamble to FDA's interim final rule describes the "appropriate modifications" of the AOAC Method. The Infant Formula Final Rule adopted, with some modifications not applicable here, the Infant Formula Interim Final Rule. See 79 FR 33057.

matching of minerals and vitamins in the reference and test diets, acclimation diets and length of acclimation, and record-keeping.

In addition, as noted above, FDA also has considered areas in which modifications may be appropriate to update the AOAC Method to reflect current knowledge of nutrient requirements of rats.

The sub-sections below describe FDA's current thinking with respect to preparation of PER study casein reference diets; protein source, conversion of nitrogen to protein; fats and carbohydrates (including a discussion of vitamin E as it relates to content of polyunsaturated fatty acids (PUFA)); removal of water from liquid infant formulas and determination of moisture in PER study diets; mineral content; vitamin content; fiber; sulfur amino acids (SAA) (methionine, cystine); other constituents (including vitamin C); developing estimates of compositions of PER study reference and test diets (minerals and vitamins); and chemical analysis (demonstrating the appropriateness of modifications).

a. Preparation of PER study casein reference diets

Preparation of PER study casein control diets that are matched as closely as possible to the test diet poses several challenges because of the high fat and high lactose contents of many infant formulas. Mixing of the ingredients may result in a reference diet of gummy or pasty consistency, which may be unacceptable to the young animals. Such consistency also may lead to problems with poor digestibility and reduced nutrient availability, and, as a result, the PER study reference diet may lead to very slow or no growth during the 28-day study period. Manufacturers and laboratories may want to consider blending or combining the fats, protein, carbohydrates, vitamin, and mineral mixtures, and other ingredients using general processes and equipment such as that used in the manufacture of infant formulas, if such are available. Use of such procedures may reduce variables related to differences in diet consistency (texture) between the casein reference and the infant formula test diets. Records of how the PER diets are prepared should be included in the protocol, maintained with the diet preparation records of the study, and included in the final report (see Section V.A., below).

b. Protein Source, Conversion of Nitrogen to Protein

In the AOAC Method, the protein content of a product is calculated from its nitrogen content by applying a factor considered suitable for converting nitrogen to protein in the food. Such factors are based on the nitrogen content of the predominating protein present in various foods. The AOAC Method specifies the use of the factor 6.25 as the nitrogen conversion factor for setting protein levels.

The AOAC Method identifies the Animal Nutrition Research Council (ANRC) reference casein as the protein source for the reference diet. Several high-purity caseins (≥ 85%; ≥ 90%) are available from commercial suppliers. Results of amino acid analyses are often available with the specifications for these products. For PER studies generally as well as infant formula PER studies specifically, FDA considers these high-purity caseins to be appropriate for use as the protein source for the reference diet. Regardless of the casein product used, we recommend that

all specifications for the casein used in PER studies, including the results of the amino acid analyses, be retained because it is helpful in confirming the adequacy of the reference diets with respect to amino acid composition.

c. Fats and Carbohydrates

Fat content: The original protein evaluation basal diet described in the AOAC Method contained 8% cottonseed oil, which was readily available in the past, but is no longer readily available.[12] With respect to infant formula, FDA stated the following in the Infant Formula Interim Final Rule:

> In most cases, when the infant formula is incorporated into the protein evaluation diet based on the nitrogen content, the fat content will be above the limit (8 percent) specified by the AOAC Official Method. The fat content of the reference control (casein) diet must be adjusted to match the fat content of the infant formula test diet.

(79 FR 7934 at 8024.)

Infant formulas contain a variety of fat sources, and FDA considers it appropriate and possible to match the fat content and fatty acid compositions of the PER study reference diet both quantitatively and qualitatively to that of the infant formula-based test diet. This matching of fat and fatty acid compositions is important because, in both humans and animals, the body's need for vitamin E increases with an increase in consumption of PUFAs and with the degree of unsaturation of dietary PUFAs.

The rat requirement for vitamin E (*RRR-α*-tocopherol; formerly called d-α-tocopherol) is 18.0 milligrams per kilogram (mg/kg) diet (equivalent to 27 international units (IU)/kg) when lipids comprise less than 10% of the diet (e.g., a diet containing 50.0 grams (g) fat/kg, 6.0 g linoleic acid (n-6)/kg, and an estimated requirement of 2 g/kg linolenic acid (n-3), which can be substituted with other long-chain n-3 PUFA)[13] (Reference 11). Higher concentrations of vitamin E may be required if high-fat diets are fed (Reference 11). Harris and Embree reviewed studies in which rats were fed diets containing varying ratios of vitamin E:PUFA (expressed as mg of *d-α*-tocopherol to grams of PUFA), which were sufficient either to induce vitamin E deficiency or to relieve it (Reference 13).

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[12] Previously, many laboratories used corn oil (Reference 5) or blends of coconut and corn oils or coconut and soy oils (References 7, 8) in place of cottonseed oil. While corn oil is still readily available, many oils are now blends of the most economically available vegetable oils (Reference 6). No consensus seems to have developed in favor of the inclusion of a specific oil or oil blend for the 8% fat PER study diets. However, in the studies cited, the same oil or oil blend was used at the same concentration in both PER study reference and test diets.

[13] The terms "n-3" (or omega-3) and "n-6" (or omega-6) identify fatty acids belonging to two series of polyunsaturated fatty acids. The "n-3" and "n-6" refer to where the first double bond occurs in the molecule. In the omega-3 fatty acids, the first double bond occurs on the 3rd carbon atom - counting from the methyl (or omega) end. In the omega-6 fatty acids, the first double bond occurs on the 6th carbon atom, again counting from the methyl (or omega) end.

Harris and Embree (Reference 13) found that across diets varying from 5 to 22% in total fat content and including fat sources such as lard, cod liver, linseed oil, corn oil, and menhaden oil, a minimum ratio of vitamin E:PUFA of 0.48 ± 0.28 mg of *d*-*α*-tocopherol to grams of PUFA (mean ± standard deviation; n=9 studies) was needed to protect against vitamin E deficiency.

While it is not possible to provide a rule for predicting the requirement for a nutrient without taking into account other components of the diet, the vitamin E:PUFA ratio may serve as a useful guideline. The following examples illustrate the use of the minimum ratio value for vitamin E:PUFA: a diet containing 10% soybean oil with 58% PUFA and a vitamin E content of 1.8 mg/100 g has a vitamin E:PUFA ratio of 0.31 (1.8/5.8=0.31), which may have an adverse effect on vitamin E nutrition. The ratio can be increased by adding more vitamin E to the diet as follows: the same diet containing 10% soybean oil with 58% PUFA and a vitamin E content of 5.0 mg/100 g has a vitamin E:PUFA ratio of 0.86 (5.0/5.8=0.86).

We suggest that the total PUFA contents of the PER study test and reference diets be estimated from the Certificates of Analysis or other information and used with dietary concentrations of vitamin E to calculate the ratio of vitamin E:PUFA as mg vitamin E/g PUFA for each diet. Modifications in the concentration of vitamin E in the reference diet may be made, if needed.

Carbohydrates: As originally developed, PER study diets were made up to 100% with sucrose or corn starch. Rats can utilize several carbohydrates, including glucose, sucrose, maltose, fructose, and a variety of starches (e.g., corn, wheat, rice) (Reference 14). No consensus seems to have developed for use of a specific carbohydrate composition. As noted in References 6 through 8, the same type and level of carbohydrates were used in both the PER study reference and test diets.

With respect to infant formula, as FDA stated in the Infant Formula Interim Final Rule:

> Lactose is the carbohydrate component of most milk-based infant formulas. Rats do not tolerate lactose well and often develop diarrhea, which may lead to an underestimate of protein quality of the formulas. The casein reference control diet(s) must contain levels of lactose comparable to the amount in the infant formula test diet to adjust for possible confounding of the estimation of protein quality. If an infant formula contains a carbohydrate source other than lactose (e.g., sucrose, corn syrup solids), the source of carbohydrate in the formula should be used in the control diet as well.

(79 FR 7934 at 8024.)

Products high in lactose represent a special category of foods because of the adverse responses of rats to diets with lactose (Reference 15). FDA's current thinking is that it is critical to the conduct of a well-designed PER study that the casein reference diet contain a level of lactose comparable to amounts present in the infant formula test diet. The need for inclusion of lactose in acclimation diets fed prior to a PER study with a high-lactose test diet is considered below.

d. Removal of Water from Liquid Infant Formulas and Determination of Moisture in PER Study Diets

Removal of Water from Liquid Infant Formulas: As stated in the Infant Formula Interim Final Rule:

> Infant formula is incorporated into the protein evaluation diet based on its nitrogen content. Because of the high water content of infant formulas in liquid form, these products are below the limit of total nitrogen (1.8% by weight) required for the PER bioassay. Liquid infant formulas must be freeze dried so that the test sample contains more than 1.8% nitrogen before the infant formula test diet is formulated.

(79 FR 7934 at 8024.)

Either powdered or liquid forms of an infant formula may be used in a PER study. Freeze-drying (lyophilization) is a reasonable way to accomplish the removal of water from a liquid infant formula. If a powdered form of an infant formula is used in a PER study, the amount used should be adjusted so that the test sample contains more than 1.8% nitrogen before the infant formula test diet is formulated.

Determination of Moisture in PER Study Diets: The appropriate modifications that FDA discussed in the Infant Formula Interim Final Rule did not address the issue of water in the PER diets (a separate issue from the removal of water from liquid infant formulas with the goal of bringing the nitrogen level to the concentration specified in the AOAC Method). Furthermore, the issue of the water content of the PER study diets is distinct from the issue of providing drinking water to the animals during the study,[14] and one cannot substitute for the other. The water content of the diets needs to be considered in formulating the PER study diets because adjustment for water (expressed as "moisture" in the formula shown in the AOAC Method; Appendix 1) is one of the primary specifications in the development of the PER study diets. PER study reference and test diets should both contain equal moisture contents because the level of hydration may influence PER study results (Reference 4). The AOAC Method provides a formula for adjusting the water content of PER diets to about 5% based on the moisture content of the test material. Earlier studies have shown that differences in water content may influence PER study results, with PER values for casein increasing with the percent of water added to the diets (Reference 4 and references therein). A second effect of water that should be considered is the point at which water is added to the diets. In earlier studies, higher PER values were obtained when water was added to casein at the beginning of the diet mixing process than when water was added to the final diet after mixing other ingredients (Reference 15). FDA recommends that the issue of water in PER study diets be discussed with diet formulators with experience in blending diet ingredients such as oils and carbohydrates to avoid problems with diet consistency or palatability.

[14] See Section IV.B.3.a for further information about providing drinking water to animals during the study.

e. Mineral Content

The AOAC Method is flexible with respect to the mineral mixture used to meet the mineral levels defined in the reference and test diet preparations. The AOAC Method provides the composition of a specific United States Pharmacopeia (USP) salt mixture (See Appendix 1) and also states that other salt mixtures having essentially the same proportions of all essential elements, including sulfur, may be used. The mineral mixture described in the AOAC Method is USP XIX (1975) (p. 612). The corresponding item in USP XX (1980) is found in USP XX (141) at page 902. Both citations refer to the same salt mixture, which is part of USP's Protein-Biological Quality Test. When used at 5% of the diet, this mineral mixture provides the minerals shown in Appendix 3.

In the formulation of the mineral mixture used in the AOAC Method diets, magnesium (Mg), iron (Fe), manganese (Mn), Zn, and copper (Cu) are provided as their sulfate salts. These salts provide about 96.4 mg sulfur/100 g to the final diet (Appendix 3).

The following paragraphs describe FDA's recommendations regarding mineral content, specifically: (1) FDA's recommendations regarding the mineral mixture to be used, taking into consideration the sulfur/sulfate content of the final diets, with recommended changes in the concentrations of specific minerals in the mineral mixture to update the AOAC Method to bring it into alignment with current knowledge; and (2) FDA's recommendations regarding ash content and evaluating the mineral composition of the final PER study test diet relative to that of the reference diet.

Mineral mixtures: Except for zinc (Zn), selenium (Se), and molybdenum (Mo), the concentrations of minerals provided in the USP salt mixture are within the acceptable range in the estimated nutrient requirements for growth of the laboratory rat provided in the National Academy of Sciences, National Research Council (NRC) Nutrient Requirements of Laboratory Animals (Reference 11). These estimated nutrient requirements for growth are minimal requirements and do not include a margin of safety (Reference 11). The concentration of iodine (I), while not at a potentially toxic level, is about 200-times higher than its concentration in currently available mineral mixtures.

FDA considers the mineral mixture described in the AOAC Method with the following changes to be appropriate for use at 5% with the 10% protein PER study diets that are low in methionine (Reference 16): (1) the concentration of potassium iodide (KI) should be reduced from 0.79 g/kg of mixture to 0.0079 g/kg of mixture; (2) the concentration of zinc sulfate ($ZnSO_4$ $7H_2O$) should be increased from 0.548 g/kg of mixture to 1.1 g/kg of mixture; (3) sodium selenate (Na_2SeO_4) should be added at 0.0080 g/kg of mixture; (4) ammonium paramolybdate ($(NH_4)_6Mo_7O_{24}$ $4H_2O$) should be added at 0.0056 g/kg of mixture; and (5) cobalt (Co), which is not required by rats (Reference 11), should be omitted. When the resultant mineral mixture is used at 5% of the diet, the concentrations of I, Zn, Se, and Mo will be (g/kg diet) 0.00030, 0.0125, 0.00017, and 0.00016, respectively, and will meet the specifications of the NRC Nutrient Requirements of Laboratory Animals (Reference 11).

We also consider the use of the Bernhart-Tomarelli mineral mixture (Reference 17) to be appropriate when 0.010 g Na_2SeO_4 and 0.0070 g $(NH_4)_6Mo_7O_{24}\ 4H_2O$ are added per kg of the mixture. When used at 4% of diets, this Se- and Mo-supplemented mixture will provide 0.00017 g Se/kg diet and 0.00016 g Mo/kg diet and will meet the specifications in the NRC's Nutrient Requirements of Laboratory Animals (Reference 11).

Other mineral mixtures are commercially available from manufacturers of animal feeds. The mixtures include "AIN-76A" and "AIN-93G," which are constituents of, respectively, rodent diets AIN-76A and AIN-93G. We do not recommend the use of the AIN-76A or AIN-93G mineral mixtures in the 10% casein PER study diets because of their sulfur/sulfate content. These mineral mixtures provide sulfate, as potassium sulfate, at 0.301-0.337 g/kg diet (Appendix 3) compared with 0.501 g sulfate/kg from the Bernhart-Tomarelli mineral mixture (used at 4%) and 0.964 g sulfate/kg from the USP XIX mineral mixture (used at 5%) in the AOAC Method control diet. Use of the AIN-76A or AIN-93G mineral mixtures alone leads to situations in which the inorganic sulfur/sulfate contents of the final diets are lower than that of the finished diet described in the AOAC Method. Our current thinking is if a decision is made to use one of the AIN mineral mixtures, then the inorganic sulfate concentration of the diets should be adjusted to provide the inorganic sulfate content set forth in the AOAC Method (i.e., 0.964 g/kg diet).[15]

The composition of each of the above mineral mixtures is listed in Appendix 3.

Ash content and evaluation of the mineral composition of the diets: Regardless of which mineral mixture is used, FDA recommends a thorough evaluation of the mineral composition of the final PER study test diet relative to that of the reference diet. The AOAC Method as originally written specifies the use of a mineral mixture at 5% of the diets, less an adjustment for the ash content of the test material. Our current thinking is that the ash content of diets is not an appropriate surrogate for mineral composition when PER studies are conducted with infant formulas.

Infant formulas contain significant quantities of minerals, and the usual adjustment, as specified in the AOAC Method, is to add less of a mineral mixture to the test formula diet than is added to

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[15] Sulfur has not been classified as a required nutrient in experimental rat diets but is an integral part of the sulfur-amino acids and vitamins (Reference 14). However, Michels and Smith showed that dietary sulfate is readily incorporated into cartilage of adult rats and will spare methionine for this purpose (Reference 18). They suggested that 0.1% dietary sulfur be included when methionine is minimal. These findings were supported by Bernhart and Tomarelli, who reported that a mineral mixture that met the NRC (1963) mineral specifications for rats (which did not include sulfur) was improved by the inclusion of 0.1% sulfate when fed in a low-protein diet (8.8% lactalbumin) (References 17, 19). The authors concluded, based on their results and other studies cited in Reference 17, that with diets containing suboptimal levels of protein and low levels of sulfate, the addition of sulfate results in increased growth and PER. Therefore, a salt mixture for comparing protein quality should contain enough sulfate to allow for variations in the sulfate content of the proteins tested (References 16, 17). Based on these and several other reports, the NRC's Nutrient Requirements of Laboratory Animals (1978) (Reference 14) for rats included a specification for sulfur (i.e., 0.03 g/100 g diet; 0.03%; 0.3 g/kg diet). The NRC's Nutrient Requirements of Laboratory Animals (Reference 11) also cited studies reported in Reference 13 and concluded that 300 mg sulfur/kg diet (0.3 g/kg diet) as inorganic sulfate may be beneficial in diets containing 15% protein.

the casein reference diet based on ash content (Appendix 1, Appendix 2). The magnitude of this adjustment is much greater when infant formula is used as the test material than when an isolated protein source is used as the test material. For example, a PER study reference diet formulated to contain 5% of a mineral mixture and 10% casein with an ash content of 1.51% would contain 4.85% of the mineral mixture (5.0% – (10%)(1.51)/100 or 5.0% - 0.15% = 4.85%). The corresponding PER study test diet formulated with 84.25% of an infant formula with an ash content of 3.83% would contain only 1.77% of the same mineral mixture (5.0% – (84.25%)(3.83)/100 or 5.0% – 3.23% = 1.77%). The resultant ash contents of both finished diets will be the same (i.e., 5.0 % in the example above). However, calculations of amounts of individual minerals added by the infant formula and those added by the mineral mixture will likely show that the overall mineral compositions of the PER study test and reference diets are not comparable because the relative proportions of minerals added to the test diet by the infant formula plus the mineral mixture differ significantly from those added to the reference diet by the mineral mixture alone.

Thus, while our current thinking is that the ash content alone is not an appropriate surrogate when matching minerals in test and reference diets in a PER study with infant formulas, we recommend that this issue be resolved by developing estimates of compositions of projected PER study test and reference diets prior to initiation of the PER study. In Section IV.B.1.j. and Appendix 6, FDA provides a discussion and a table that shows how the compositions of PER study test and reference diets can be developed from the composition of the infant formula.

f. Vitamin Content

The AOAC Method provides the composition of a vitamin mixture and specifies its use at 1% in both the PER study reference and test diets. This specification indicates that the expectation of the AOAC Method is that both reference and test diets will have the same vitamin compositions, thus reducing the confounding effects of having different vitamin compositions between the reference and test groups.

The vitamin mixture as listed in the AOAC Method is not commercially available. Three commercially available vitamin mixtures are pertinent to the PER study: (1) the AOAC vitamin mixture, which differs from the originally described AOAC Method vitamin mixture only in providing vitamins A and D together as a powder; (2) vitamin mixture AIN-76A; and (3) vitamin mixture AIN-93G (Appendix 4). These vitamin mixtures are specified for use at 1% of diets. The three commercially available mixtures specify the forms of many of the vitamins, which was not done with the original AOAC Method formulation (see Appendix 1).

The AOAC Method does not provide a formula to describe how to make adjustments in vitamin composition. This may be because the use of the PER method with infant formulas or other foods that contained significant quantities of vitamins may not have been anticipated at the time the method was developed. Furthermore, because there is not a single surrogate measure for all vitamins as there is for all minerals (i.e., ash), we are not aware of formulas that would encompass adjustments for all potential vitamin compositions of various formulas that might be tested. Adjustments should be made on a formula-by-formula and vitamin-by-vitamin basis.

We recognize that the issue of comparability of vitamin compositions between the casein reference and infant formula test diets may be more complicated than that with mineral compositions, but it is still amenable to adjustment. Our current thinking is that use of different amounts of a defined vitamin mixture in the reference and test diets may be one step toward ensuring matching levels of vitamins in both diets.

Because most infant formulas provide significant quantities of vitamins as well as minerals, careful calculations should be made before diet formulation begins to determine the contributions of vitamins anticipated from the test formula and from the vitamin mixture(s). In some cases, the infant formula itself may provide most or perhaps all vitamins, as per the NRC's Nutrient Requirements of Laboratory Animals (Reference 11). Adjustments in amounts of vitamin mixture added to the infant formula-based test diet or preparation and use of a specific vitamin mixture to adjust for such a finding may be needed.

Specific recommendations for several vitamins follow:

- **Vitamin E**: Comments with respect to vitamin E as it relates to fat composition in the diets are found in Section IV.B.1.c., above.

- **p-aminobenzoic acid (*p*-ABA)**: The vitamin *p*-ABA is included in the AOAC vitamin mixture, but not in the AIN-76A or AIN-93G vitamin mixtures (Appendix 4). *p*-ABA, a vitamin that is a precursor of folic acid, is not needed by the rat and can be omitted from the vitamin mixture. It may have been added to the original AOAC Method vitamin mixture to facilitate the synthesis of folate by intestinal bacteria.

- **Inositol**: Inositol is included in the AOAC vitamin mixture, but not in the AIN-76A or AIN-93G vitamin mixtures (Appendix 4). Inositol is a type of sugar that was once considered to be part of the B vitamin family and known as vitamin B8. It can be synthesized in the animal body from glucose and is no longer considered to be a vitamin. Inositol is not required by rats under conventional conditions, but a requirement has been reported in lactating rats fed antibacterial drugs (Reference 11). If inositol is added to an infant formula, we recommend that it be included in vitamin mixtures used in PER studies of such formula to facilitate matching of inositol levels in the test diet.

- **Vitamin A**: The estimates of vitamin requirements for rats have changed over the years. This is most obvious in the concentrations of vitamin A included in vitamin mixtures and diets. The AOAC Method vitamin mixture as listed in the AOAC Method and the AOAC vitamin mixture each provide 20,000 IU vitamin A per kilogram diet versus the current NRC (1995) (Reference 11) specification of 2,300 IU per kilogram diet. Infant formula typically has a high concentration of vitamin A. Because of the importance of matching the reference and test diets, for infant formula, the concentration of vitamin A in the reference diet should be matched to

that of the test diet even if such matching results in reference diets of high vitamin A content.[16]

- **Vitamin K**: Menadione (vitamin K3) is specified as the form of vitamin K in the AOAC Method vitamin mixture, and its use was continued in the AIN-76A vitamin mixture. More recent vitamin formulations (e.g., AIN-93G vitamin mixture) include vitamin K as phylloquinone (vitamin K1). Menadione is approximately one-tenth as active as phylloquinone. We recommend that phylloquinone, rather than menadione, be used as the source of vitamin K. The amounts and forms of vitamin K included in various vitamin mixtures are listed in Appendix 4.

- **Choline**: Choline is an essential nutrient and serves as a methyl-donor in many physiological reactions. While choline is not a vitamin, it is frequently listed with the vitamin component of diets (References 11, 14). Choline (form unspecified) is included as a part of the AOAC Method vitamin mixture listed in the AOAC Method (Appendix 1). Its dihydrogen citrate salt is used in the commercially available AOAC vitamin mixture (Appendix 4). Choline is not included in the AIN-76A and AIN-93G vitamin mixtures (Appendix 4). Rather, the AIN-76A and AIN-93G diet formulations include choline bitartrate as separate additions to the final diets. The consequence of this change is that if the AIN-76A or AIN-93G vitamin mixtures are used to replace the AOAC Method vitamin mixture, and the need for the separate addition of choline is overlooked, the resulting finished casein reference diet will be deficient in choline. The situation with respect to choline in the PER study test diet will need to be determined by evaluation of the choline content of the infant formula and its rate of addition in the test diet. The levels of choline in the reference diet and in the infant formula test diet should be matched. FDA's current thinking is that choline may need to be included as a separate addition to both diets.

- **Vitamin C**: Vitamin C is not required by rats. It is included in infant formulas and will be present in PER study test diets. The inclusion of vitamin C in PER study test diets is discussed in Section IV.B.1.i., below.

The development of estimates of the compositions of the reference and test diets before beginning diet preparation, described in section IV.B.1.j. and Appendix 6, is applicable to matching vitamin compositions as well as mineral compositions.

g. Fiber

The PER study diets described in the AOAC Method (Section III, above) include 1% fiber as cellulose. For the test diet, this ingredient is calculated as 1% cellulose minus the fiber content

[16] If the AOAC Method is used for an optional, additional control group (i.e., a standard reference control group), it is possible to reduce the vitamin A in the vitamin mixture to a concentration closer to the current estimated requirement (i.e., 2,300 IU per kilogram diet).

of the test sample and is measured as crude fiber in the proximate analysis. A review of Certificates of Analysis submitted with recent PER studies showed that AOAC Official Method 962.09, a method for crude fiber in animal feed and pet food, is often used for determination of crude fiber.

Rats do not require fiber in their diets. The NRC's Nutrient Requirements of Laboratory Animals does not provide a value or a recommendation for fiber in rat diets and notes only that inclusion of fiber may be potentially beneficial (Reference 11). However, although infant formulas do not contain cellulose, but do contain other fiber-like carbohydrates, we have considered whether to add cellulose to PER study diets conducted with infant formulas (i.e., to both the reference and test diets).[17]

The NRC's Nutrient Requirements of Laboratory Animals (Reference 11) states:

> Some carbohydrates that cannot be properly called fiber also elicit some responses similar to those observed with true fibers. Lactulose (disaccharide), raffinose (trisaccharide), and fructo-oligosaccharides are not absorbed in the small intestine but are rapidly fermented in the hindgut.

Infant formulas do not contain cellulose or other sources of crude fiber. They do, however, frequently contain non-digestible carbohydrates such as fructo-oligosaccharides (FOS) or galacto-oligosaccharides (GOS). Our current thinking is that it is not necessary to add cellulose to the infant formula-based test diet or to its matched casein reference diet when the infant formula includes non-digestible carbohydrates such as FOS and/or GOS. When FOS or GOS are present in the infant formula, they should be included in the matched casein reference diet and analyzed by appropriate specific methods.

Due to our uncertainty as to the outcome of PER studies conducted in completely fiber-free diets,[18] we recommend that 1% cellulose be added to the test and matched casein reference diets

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[17] In Section IV.B.1.f. and j., above, and in Appendix 6, we suggest additions to the infant formula-based test diet that are limited to those necessary to ensure that the test diet will meet the nutritional requirements of the rat (with the exception of protein). In practice, the infant formula may provide the concentrations of certain minerals and vitamins in the test diet, which exceed the NRC's Nutrient Requirements of Laboratory Animals (Reference 11) and are much higher than those of casein reference diet in the AOAC Method. In such situations, the reference diet should then be formulated to be comparable in all of its ingredients to the final test diet. In general (see Section IV.B.1.j, below), we do not recommend the addition to the infant formula-based test diet of ingredients that are not initially present in the infant formula or those for which there is not a recommendation by the NRC's Nutrient Requirements of Laboratory Animals (Reference 11). However, the question of whether fiber should be added to the test diet and matched casein reference diet under certain conditions raises this as a separate issue.

[18] While we have reviewed successful PER studies (i.e., those with PER values for the casein control group of 2.20 ± 0.14 g body weight gain per g protein consumed (see Reference 7)) in which no cellulose was added to the test and matched casein reference diet, the infant formulas that were the subjects of these submissions all contained either FOS or GOS. Thus, both the test and matched casein reference group diets contained non-digestible carbohydrates. To date, we have not reviewed a PER study submission in which no source of non-digestible carbohydrate was included in the infant formula or no cellulose was added to the test and matched casein reference group diet (e.g., a PER conducted with fiber-free test and matched casein control diets).

when the infant formula does not contain a source of non-digestible carbohydrate. We note that when Mitchell *et al.* (1985) (Reference 7) and Harris *et al.* (1988) (Reference 8) performed PER analysis on a variety of soy- and casein-based infant formulas, they added 1% cellulose to all test diets. The infant formulas themselves did not contain complex carbohydrates.

Finally, if a manufacturer and laboratories chooses to add an additional experimental group following the original AOAC Method (i.e., 10% protein, 8% fat; often identified as a "standard casein control group"), then 1% cellulose should be added to the diet for this group (See Appendix 1).

h. Sulfur Amino Acids (SAA) (methionine, cystine)

We recommend that the PER casein reference control diets contain 0.964 g/kg inorganic sulfur as specified in the AOAC Method. If there is concern about a potential failure of the casein reference group because of low concentrations of cystine in casein, manufacturers and laboratories may want to consider matching the sum of methionine plus cystine (i.e., methionine + cystine) concentrations in the casein reference control and test diets. If time and resources permit, manufacturers and laboratories may want to prepare the (methionine + cystine)-matched diet and include it as a second casein reference control group in their PER study.

The following section provides background information to support these recommendations on the SAA methionine and cystine, then describes our current thinking with respect to an option for matching the concentrations of (methionine + cystine) in the control and test diets. Additional information regarding the concentrations of sulfur/sulfate salts in PER study diets is found in Section IV.B.1.e.

Methionine and cystine — limiting amino acids in casein: When an essential amino acid such as methionine is not provided in adequate quantities in a diet (e.g., in a protein-deficient diet), protein synthesis and growth are limited to the rate at which the essential amino acid is available. In such circumstances, the essential amino acid is referred to as the "limiting" amino acid. In simpler terms, the "first limiting" amino acid is the essential amino acid that first becomes deficient in a diet. The SAA methionine and cystine are first limiting in diets containing 8-10% protein from casein (Reference 20). In addition, in casein, the concentration of cystine relative to that of methionine is quite low. The concentration of cystine in casein (i.e., 0.42 g/16 g N) is only 14.3% of the concentration of methionine (i.e., 2.94 g/16 g N; Cys/Met x 100 = 14.3%) (Reference 21). In comparison, the concentration of cystine in skim milk (i.e., 0.85 g/16 g N) is much higher relative to that of methionine (i.e., 2.32 g/16 g N; Cys/Met x 100 = 36.6%) (Reference 21).

The rat requirement for SAA is expressed as the sum of methionine plus cystine (i.e., methionine + cystine). This value is 9.8 g/kg (0.98 g/100 g diet; 0.98%) for rats fed diets containing 15% protein (Reference 11). While the corresponding value for rats fed 10% casein protein diets has not been determined, Peace *et al.* found that optimal feed-to-weight-gain ratio, relative net protein ratio, and plasma amino acid parameters were obtained when weanling rats were fed 8% casein plus amino acid diets containing 0.44% (0.44 g/100 g diet; 4.4 g/kg diet) total SAA with cystine replacing 33 to 60% by weight of dietary methionine (Reference 20). The inclusion of

cystine at the expense of methionine in 8% protein diets improved overall rat performance and utilization of dietary methionine (Reference 20). Provision of cystine may decrease the requirement for enzymatic conversion of methionine to cystine and its derivatives, permitting more efficient utilization of methionine for protein synthesis (Reference 20). The ability of cystine to substitute for a portion of the methionine requirement is important because cystine is present in limited quantities in 10% casein diets. Thus, the contribution that cystine can make to overall methionine metabolism is reduced in rats fed 10% casein protein diets.

SAA and sulfur/sulfate content: The AOAC Method does not include the addition of cystine. To better understand why, we evaluated the composition of the mineral mixtures used in the diet. The AOAC Method mineral mixture includes several sulfate salts, and the diet, when prepared with 5% of the mineral mixture, provides 0.964 g sulfur/kg. Use of the Bernhart and Tomarelli mineral mixture gives PER results comparable to those obtained with use of the mineral mixture described in the AOAC Method. The Bernhart and Tomarelli mineral mixture provides 0.501 g sulfur/kg when used at 4% (Appendix 3). The original AOAC Method mineral mixture and the Bernhart and Tomarelli mineral mixture both provide considerably more sulfur than provided by either the AIN-76A or AIN-93G mineral mixtures (e.g., 0.337 and 0.301 g sulfur/kg, respectively, when used at 3.5%) (Appendix 3).

The diets of PER study control groups for which we have data (see below Section IV.B.2) used either the AOAC Method mineral mixture or the Bernhart and Tomarelli mineral mixture. Both mixtures provided sulfur at concentrations considerably higher than the value of 0.3 g/kg diet identified in the current NRC (1995) Nutrient Requirements of Laboratory Animals (Reference 11) as potentially beneficial in diets of 15% protein. The increased inorganic sulfur from the AOAC Method mineral mixture or the Bernhart and Tomarelli mineral mixture may have contributed significantly to sulfur metabolism and compensated for the low SAA content of the 10% casein diets. Under such circumstances, a separate addition of cystine was not needed in the original AOAC Method casein reference control diet.

While we do not have data that would assist in making a precise evaluation regarding addition of inorganic sulfate in the PER study casein reference control diets in studies with infant formulas, our current thinking is that inorganic sulfur should be included in diets at levels like those found in PER study control diets described above (i.e., between 0.50 and 0.96 g/kg diet). Our current thinking is that a concentration at the high end of the 0.50 and 0.96 g/kg range should be used as a target concentration because it will provide a margin of safety. Therefore, we recommend that the concentration of inorganic sulfur in the PER study casein reference control diets when used with infant formulas be adjusted to provide the inorganic sulfate content set forth in the AOAC Method (i.e., 0.964 g/kg diet).

Possible addition of cystine: The information above suggests that in PER study diets with significant inorganic sulfur content (e.g., 0.50-0.96 g/kg, or 0.05-0.096%), the addition of cystine may not be needed. However, some losses of nutrients (e.g., due to diarrhea) may occur based on the composition of the diet (e.g., high lactose), and under such conditions, a low cystine concentration in the casein reference control diet (e.g., perhaps 20-30% of the value in the test diet) might cause a growth failure in the group. This suggests that the addition of cystine to the

casein control diet sufficient to match the amount of (methionine + cystine) in the test diet may be considered.

Matching the concentrations of (methionine + cystine) in PER study test and casein reference diets and inclusion of the group as a second reference control group in PER studies: Manufacturers and laboratories may want to consider matching the concentrations of (methionine + cystine) in the PER study test and reference casein diets if there is concern about a potential failure of the casein reference group because of low concentrations of cystine in casein. The following paragraphs describe a process by which this matching may be accomplished.

The infant formula and the casein for the control diet can be analyzed for their methionine and cystine content prior to diet preparation. The concentrations of (methionine + cystine) can be calculated from the results of these analyses. The calculation can be corrected for the rate of use of the infant formula in the PER study test diet. A similar calculation can be made from the amino acid composition information for the casein that will be used in the control diet. If the analyzed concentration of cystine in the control diet is < 80% of the value for cystine in the test diet (calculated from the analyzed value in the infant formula and corrected for its rate of use), then cystine can be added to the control diet so that the concentrations of (methionine + cystine) are similar in both diets. In practice, this will generally involve only calculation of the amount of cystine to be added to the control diet. The final concentration of (methionine +cystine) in the control diet will be within ± 20% of the value for (methionine + cystine) in the test diet (see Appendix 6).

If time and resources permit, manufacturers and laboratories may want to prepare the (methionine + cystine)-matched diet described above and include it as a second casein reference control group in their PER study. The first control group would meet FDA recommendations regarding modifications with respect to matching fat types, lactose, vitamins, minerals, and other nutrients, and would contain the amount of inorganic sulfur specified in the AOAC Method. This primary casein reference control group is usually referred to as a Modified Casein Control Group. The second control group would have the same composition as the first and, additionally, would be matched for concentrations of (methionine + cystine) in the control and test diets. Comparison of PER results with these two groups will assist in determining the relative importance of the need for increased inorganic sulfur versus increased SAA in the PER study casein reference diets.

Lack of justification for addition of methionine: We are unaware of a justification for adding methionine alone to raise the (methionine + cystine) concentration in the control diet above the level of (methionine + cystine) in the test diet because there is insufficient information from which to derive an estimate of the concentrations of total SAA or of methionine alone that are needed for 10% casein diets. However, the steps outlined above will ensure that the test and control diets are matched within ± 20% in their contents of (methionine + cystine). The purpose of the addition of cystine is to increase the cystine content of the casein control diet so that the (methionine + cystine) contents of both diets are similar. We are uncertain as to whether the addition of methionine alone will suffice to remedy the limited concentration of cystine in the casein control diet. In general, concentrations of methionine among proteins are more similar

than are concentrations of cystine. In some instances, the concentration of methionine in the casein control diet may be higher than that in the infant formula-based test diet. In such a case, if addition of methionine only were under consideration, then addition of methionine would appear unnecessary. However, the concentrations of cystine in the test and control diets (and the values for (methionine + cystine)) would remain unmatched.

We note that *DL*-methionine was the recommended supplement to the AIN-76A diet. During the formulation of the AIN-93 diets, the AIN noted that compared with other milk proteins, *L*-cysteine or *L*-cystine and not *L*-methionine were the amino acids found in small amounts in casein. For this reason, the AIN recommended *L*-cystine instead of *L*-methionine as the supplement to the AIN-93 diets (Reference 22).

In cases in which the concentration of (methionine + cystine) in the test diet is less than the corresponding sum in the control diet, and the concentration of cystine in the control diet is < 80% of the concentration of cystine in the test diet, then sufficient cystine can be added to the control diet to match its concentration in the test diet.

Our current thinking is that the approaches described above will reduce the risk of a failure of the PER study casein reference control group.

i. Other Constituents (including vitamin C)

Other constituents: Infant formulas may contain other components (e.g., nucleotides, taurine, oligosaccharides) that would not normally be included in rat diets. Our current thinking is that the composition of the PER study reference diet should be matched as closely as possible to that of the infant formula-based test diet. Concentrations of constituents such as inositol, carnitine, taurine, nucleotides, docosahexaenoic acid (DHA), arachidonic acid (ARA), GOS, or FOS, or others in the infant-formula-based test diets should be evaluated, and such constituents should be included in the PER study reference diet at the same concentrations found in the test diet. Results of the analyses by appropriate and specific methods of components that are added to the PER study casein control diet to match their concentration in the infant formula test diet should be included in the final report of the PER study.

Vitamin C: Infant formulas must contain vitamin C, and it will be present in the PER study test diets.[19] Vitamin C is not needed by the rat. Vitamin C should be included in the PER study reference diet at a concentration equivalent to its concentration in the PER study test diet.

j. Developing Estimates of Compositions of PER Study Reference and Test Diets (minerals and vitamins)

In Appendix 6, we recommend an approach that can be used to develop the compositions of PER study test and reference diets from the composition of the infant formula that is the subject of the

[19] See 21 CFR 107.100

submission. The first step using this approach is to list all components of the infant formula in units/100 g or units/kg. The rate of addition of the formula to the test diet will provide the starting point for formulation of mineral and vitamin premixes. The sum of nutrients provided by the infant formula at its rate of addition and contributions from mineral and vitamin mixtures will provide information to formulate the reference diet. We recommend matching the mineral and vitamin compositions of the PER study reference diet within ± 20% of the mineral and vitamin compositions of the PER study test diet.

In Appendix 6, we recommend a format that may be useful for developing estimates of the mineral and vitamin compositions of the test and reference diets. In addition, we provide worked examples, which includes values for parameters, for several minerals and vitamins and show how to view the information to provide a comparison of nutrients in both the test and reference diets. Review of the NRC's Nutrient Requirements of Laboratory Animals for rats at various steps of diet development (e.g., during formulation of premixes, during diet preparation) will be helpful in assessing the adequacy of the test and reference diets (Reference 11).

k. Chemical Analyses (demonstrating the appropriateness of modifications)

The AOAC Method stipulates proximate analyses (e.g., for nitrogen, fat, ash, moisture, and crude fiber) be performed to match major components of the PER study test and reference diets. Calculations of anticipated diet compositions should be performed to determine what additional analyses might be appropriate. Following such comparisons, FDA recommends that manufacturers and laboratories analyze several vitamins and minerals to ensure that the diet compositions are as anticipated. Analysis of samples from different locations in the mixing bowl or other mixing apparatus (e.g., top, middle, and bottom of a mixing container) is useful for assessing homogeneity of diet preparation. Consideration should also be given to analysis of vitamins that may be present in different forms in the test and control diets (e.g., vitamin K).

A demonstration that appropriate modifications have been made includes documentation that proximate analyses have been performed and that "appropriate modifications" were addressed in the diets. In addition, when full compositions of the PER study test and reference diets are provided to FDA (e.g., in a protocol or in the PER study final report), it is possible for us to confirm by calculation that appropriate modifications were made. Our current thinking is that the inclusion of a full specification (e.g., a Certificate of Analysis (CoA)) for the lot/production run of infant formula used in the PER study with nutrients expressed as units/100 g or units/kg and minerals expressed as their elemental concentrations (i.e., calcium, not calcium carbonate) is essential for use in verifying the accuracy of the composition of the test diet. The addition rate of the formula to the PER study test diet should be stated in the diet preparation records. Complete diet preparation records should be summitted with the protocol and final report. A manufacturer may wish to include examples of calculations used in preparing the PER study diets, and we encourage the inclusion of this information. Inclusion of records of all chemical analyses and their methods and results also are also helpful in determining the appropriateness of the compositions of the PER study test and reference diets.

2. PER Values for Casein Reference Control Diets

The following paragraphs describe our current thinking on attainable values for casein reference control groups when the AOAC Method is performed as originally described, when the AOAC Method is performed with appropriate modifications with lactose-free infant formulas, and when the AOAC Method is performed with appropriate modifications with high-lactose infant formulas.

Casein reference control data — Original conditions of the AOAC Method: The available data show that when the AOAC Method is performed as described (including, among other components, 10% protein, 0% lactose, 8% fat, and 5% AOAC-specified mineral mixture), additional cystine is not needed, and sustained growth can be achieved in the control group over the 28-day bioassay period. FDA considers that PER values for the casein control group between 2.62 and 3.09 g body weight gain per g protein consumed over the 28-days are attainable under the original conditions of the AOAC Method, which includes use of a mineral mixture providing 0.50-0.96 g sulfur/kg diet (see Footnote 6 for data and Appendix 3 for compositional information).

Casein reference control data — AOAC Method modified for infant formulas — Lactose-free formulas: When the AOAC Method is appropriately modified for use with infant formulas and lactose-free infant formulas are studied, with PER diets containing, among other components, 10% protein, 0% lactose, and 21-28% fat, PER values for the casein control group between 2.62 and 3.09 g body weight gain per g protein consumed over the 28-days are attainable.

Casein reference control data — AOAC Method modified for infant formulas — High-lactose formulas: We have also identified attainable PER values when the AOAC Method is appropriately modified for use with infant formulas and high-lactose infant formulas are studied. The limited data available were obtained from studies in which the AOAC Method was performed with infant formulas with diets including, among other components, 10% protein, 43% lactose, 24% fat, and 4% of the Bernhart and Tomarelli mineral mixture (Appendix 3; Reference 7) for a 28-day bioassay period. Cystine was not added to the control group diet in these studies. FDA considers that PER values for the casein control group of (mean ± SD; n=2) 2.2 ± 0.14 g body weight gain per g protein consumed over the 28-day bioassay period (range 2.06-2.36) are attainable under dietary conditions of high lactose and high fat when mineral mixtures are used that provide 0.50-0.96 g sulfur/kg diet (Appendix 3).

Summary: FDA considers the following ranges of PER values (g weight gain/g protein consumed for 28 days) for casein reference control groups to be attainable:

- Under original conditions of the AOAC Method: 2.62 – 3.09
- Under modified conditions for infant formulas: Lactose-free formulas: 2.62 - 3.09
- Under modified conditions for infant formulas: High-lactose formulas: 2.06 – 2.34

3. Experimental Animals

a. Age, Weight, Number/Group, Acclimation Period, Housing, Acclimation Diet

Age and Weight. The AOAC Method specifies that experimental animals be weanlings ≥ 21 days of age but ≤ 28 days of age. The range of individual rat weights among animals used should be ≤ 10 grams. FDA recommends that these ranges be maintained to limit the variability associated with the bioassay.

Number/Group. Groups of ≥ 10 rats are needed for a PER study. A reference group that will receive the casein reference diet is needed for each test formula group. If the test formulas are sufficiently similar, one reference casein group can be used for a concurrent assay of more than one test material. As stated in the AOAC Method, when assembling all groups is complete, the total number of rats in each group should be the same, and the average weight of rats in any one group at the beginning of the assay period should not exceed by > 5 grams the average weight of rats in any other group (see also Appendix 1).

A randomization method is not specified in the AOAC Method, but several procedures are available (e.g., stratification by weight followed by randomization; or use of randomization statistical functions in commercially available software).

Acclimation Period. The AOAC Method specifies acclimation periods of ≥ 3 days, but < 7 days. Most experimental animals are transported from breeding facilities to the sites where the studies will be carried out. FDA's current thinking is that acclimation periods of 1 to 2 days are usually needed to acclimate the young animals to their new surroundings. Several additional days, running concurrently, may be needed to acclimate the young animals from nursing to individual feeders and from milk to solid diets.

Housing. Housing animals individually during acclimation, while not specified in the AOAC Method, will facilitate the transfer to individual housing and individual feeding that is required during the 28-day PER study experimental period (Reference 1). Housing in wire-bottom cages throughout will reduce coprophagy (i.e., eating of feces).[20]

Acclimation Diets. Acclimation diets are not specified in the AOAC Method, and specific literature on acclimation diets is limited. We recommend feeding diets of 10% casein (e.g., such as a PER study reference diet) to both the test and control groups during the acclimation period (Reference 23). The alternative of feeding diets with high protein levels (e.g., chow-type diets, generally ≥ 16% protein) will likely bring in high levels of minerals and vitamins, and this exposure may make it more difficult for the rats to adjust to their PER study diets. Rats should

[20] Feces may contain several vitamins synthesized by intestinal bacteria. Consumption of feces is a practice which can provide such vitamins to the animal.

be weighed at the beginning and the end of the acclimation period, and the records should be maintained with the study records.

The use of infant formulas in PER studies warrants further consideration regarding a suitable acclimation diet. Our current thinking and recommendations are described in the following paragraphs.

Certain carbohydrates may have detrimental effects on growth and PER values when they are present in high concentrations and may result in test samples appearing to be lower in protein value than their true value. Lactose is present in milk-based formulas and is added as a separate ingredient to other formulas. As discussed in Section IV.B.1.a, FDA recognizes that appropriate modifications to the AOAC Method are needed to address the adverse effect of lactose in rats, and the need to have PER reference and test diets matched in lactose content (that is, the reference diet must contain a level of lactose comparable to that of the test diet).

While the need for matching the levels of lactose in PER reference and test diets is well recognized, there is little information regarding the need (or not) for lactose in diets fed during the acclimation period (References 7, 24). Mitchell and Jenkins, in their studies with infant formulas, did not provide lactose-containing diets during their short (2-day) acclimation periods (Reference 7). While Burnette and Rusoff recommended that the feeding of acclimation diets containing 20% lactose should precede the feeding of test diets containing more than 20% lactose, they did not provide data showing the results of such acclimation on results of a subsequent PER study (Reference 24).

DeAngelis *et al.* conducted studies in which weanling rats (21 days old) were fed diets containing 10% protein and varying levels of lactose (0, 1, 2, 5, 10 , 20 and 50%) for 15 days (Reference 25). They observed that PER values (at 15 days) at 10% and 20% lactose were not different from those obtained under control conditions. They noted that growth of weanling rats was normal when lactose in diets was present at levels < 20%. When dietary lactose was 50%, PER values (at 15 days) decreased to 0.92 ± 0.45 (mean, standard deviation) (g weight gain/protein ingested) from PER values of 3.51 ± 0.15 for 0% lactose and 3.04 ± 0.19 for 20% lactose. No acclimation period was reported in this 15-day study. While these data suggest that rats tolerate lactose at levels ≤ 20% , they do not address the need for lactose in acclimation diets.

The high intestinal lactase activity in neonatal rats declines rapidly around the time of weaning (Reference 26). Van de Heijning *et al.* reported that in neonatal Wistar rats, intestinal lactase activity decreased promptly upon weaning (post-natal day 21) and, when the weaned animals were placed on diets containing 30% lactose, remained at a low residual level (about 25%) into adulthood (Reference 26). Their study did not corroborate the theory that keeping the substrate available can lead to maintenance of newborn lactase levels. Their work may also suggest that "adaptation" to high lactose diets does not occur.

On the basis of the limited literature available, we recommend that one of the two options be used for acclimation diets:

(1) Use an acclimation diet containing 20% lactose for an infant formula containing ≥ 20% lactose. The 20% lactose diet can be used to acclimate both the test and reference groups; or

(2) Use an acclimation diet that matches the lactose level in the test diet. As a practical issue, in this case, the casein reference diet can be used as the acclimation diet for both the test and reference groups.

We do not have enough information to recommend whether the acclimation diet should contain 20% lactose or a higher level that matches that of the test group diet. The Van de Heijning *et al.* data suggest that there is little adaptation to high lactose diets, and for this reason, we recommend that acclimation periods be as short as possible (perhaps only as long as is required for the weaning rats to adjust to individual housing and a non-milk diet) (Reference 26). We recommend that manufacturers review their PER studies and, based on their own experience, decide which of these two options is more appropriate for them. We currently consider either approach sufficient to facilitate the adaptation of the rats to their high-lactose PER study diets.

b. Assay Period, Type, and Frequency of Measurements

The AOAC Method specifies an assay period of 28 days with food and water available *ad libitum* throughout. Environmental conditions for test and reference casein groups should be monitored and maintained as uniformly as possible. Body weights of each rat should be recorded on the first day of the assay period. Body weights and food intakes of each rat should be measured at regular intervals, ideally daily but at intervals not greater than 7 days, and on the 28^{th} day after the beginning of the assay period. Measurement of body weights and food intakes at more frequent intervals (e.g., twice per week) are useful with new formulas so that decreases in weight gain or food intake may be identified promptly. We recommend further that during each collection of live animal data, the new records be compared with data collected previously to confirm that patterns of food intake and body weight are as expected. The potential utility of measuring water consumption as well as food consumption should be considered.

c. Monitoring Attrition and Adverse Effects of Diets

Dietary components such as lactose, unusual oils, or proteins of low quality may cause changes in the growth of rats during the acclimation phase and experimental phase of a PER study. For these reasons, FDA recommends that attrition and adverse effects (e.g., oily coats, staining on hair, loose stools, diarrhea, cataracts) of the PER study diets on the reference casein and test group rats be monitored and recorded at regular intervals, ideally daily, but not greater than every 7 days. All such results should be included in the final report of the PER study. Such adverse effects may serve as an early warning of potential problems with components of the infant formula.

4. Tabulation and Calculation of Results (e.g., statistical analyses; reference data; record-keeping)

The AOAC Method specifies that average 28-day weight gains, protein intake, PER (g body weight gain/g protein consumed during the 28-day test period), and ratio x 100 of sample PER to reference casein PER should be tabulated for each group. In addition, FDA recommends that the 28-day weight gain, the food intake and protein (N x 6.25) intake, and the PER (g weight gain/g protein intake) be recorded and calculated for each rat.

The AOAC Method does not specify statistical analyses to be conducted. Thus, statistical analysis of PER data can be performed at the discretion of the laboratory performing the PER study or the manufacturer that reviews the results. Commonly used statistical analyses include the calculation of mean and standard deviations for all continuous data, including overall and weekly body weights and body weight gains, overall and weekly food and protein consumptions, and the PER values. Analysis of variance is frequently used to compare the PER of the test group to that of the casein reference group. A variety of statistical programs are available for this purpose. FDA encourages performing statistical analysis.

Unlike growth monitoring studies for infants, there are no growth reference data for the rat PER studies. However, FDA recommends a review of the PER literature (e.g., PER values, diet compositions) found in Section VI (References) to determine whether unexpected results have been obtained.

Recordkeeping is essential to understanding the details of the conduct of a PER study. This is particularly important in the areas of diet preparation and in details of parameters presented in the AOAC Method as a range. For example, the ages and weights of rats on arrival should be recorded as individual values (rather than as broad ranges) and the individual data should be retained as part of the study records.

V. Miscellaneous

A. Protocols and Reports

The AOAC Method does not mention development of a protocol or final report. We recommend that both be prepared. Manufacturers with in-house facilities and contract laboratories with experience performing PER studies may have a protocol form available that can be used to develop a draft for the conduct of the desired PER study. Such a draft protocol should be developed to ensure that the specifications of the AOAC Method and FDA's "appropriate modifications" are met. The benefit of chemical analyses in addition to proximate analyses to confirm critical aspects of PER study diet preparations may become apparent during protocol development.

While not required, we recommend that the protocol be reviewed by us before initiation of the PER study. Manufacturers that are interested in sending a protocol for review may contact the Infant Formula and Medical Foods Staff (IFMFS) at (240) 402-1450. After protocols are

provided to the IFMFS, reviewers may analyze aspects of the protocol, including the plans for diet preparation, plans for matching test and control diets, proposals for acclimation diets, proposals for data collection, and records to be maintained. In the review, IFMFS may identify areas that the manufacturer or laboratory may want to reconsider such as lack of appropriate matching between control and test diets, proposed use of acclimation diets that do not seem to match either the test or control diets, or situations in which we cannot identify the origin of specific nutrients in the diets. Instances may be identified in which specific pieces of information are missing, or where calculations appear to be inaccurate. IFMFS will then provide feedback to the requestor.

We recommend that complete records for all aspects of a PER study be maintained and that a final report be prepared. The report should include the following:

1. The full specification for the infant formula under consideration, with nutrient composition expressed as units/100g or units/kg (i.e., quantitative formulation and nutrient content);
2. Information related to calculation of compositions of PER study acclimation, reference, and test diets; and
3. Information on the conduct of the live animal phase of the study, including selection criteria for rats upon arrival (e.g., by random numbers), selection criteria for rats when assigned to study groups (e.g., stratified randomization procedures), and descriptions of animal husbandry practices (e.g., type of caging, feeders and water bottles or watering system, animal room temperature and humidity).

The usefulness of weekly measurements of water consumption should be considered. Our current thinking is that laboratories performing PER studies should be accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and should adhere to the Guide for the Care and Use of Laboratory Animals (Reference 27). Documentation of the nature and frequency of live animal observations (including those made during the acclimation phase) should be included in the final report. Other important records that should be included in the final report include all individual live animal data (body weights and food consumption, including food consumption during the acclimation phase), individual protein consumption values for each animal, results of all chemical analyses related to diet compositions (proximate and other analyses), and calculations of individual and group PER values.

The full specification for the infant formula (i.e., quantitative formulation and nutrient content) for the lot/production run of the infant formula that will be used in the PER study and anticipated compositions for the PER study control and test diets should be included in the PER study protocol as well as in the final report. The availability of the complete specification (e.g., CoA) for the batch/lot of infant formula that will be used in the PER study is critical because numerous calculations are developed from it.

B. Reference Guidelines

Nutrient compositions of rat diets vary according to experimental objectives, and many practical diets may include nutrients at levels that exceed specifications as a margin of safety. However, the purpose of the PER study is to compare protein quality with as few confounding variables as possible and to provide a short-term diet that meets or exceeds all rat estimated nutrient requirements (Reference 11), rather than to provide an optimized diet.

We recognize that there is limited information on the specifications for all nutrients in 10% protein diets. As an example of how NRC's Nutrient Requirements of Laboratory Animals specifications may differ in diets of low protein content, Peace *et al.* reported that the sulfur-amino acid requirements for rats fed 8% protein were 0.33% of the diet or 4.1% of dietary protein, rather than the value of 0.98% (*DL*-methionine + cystine) of the NRC diet (References 11, 20). We consider that most nutrient levels would be significantly lower for the 10% protein PER study diets than NRC values for 15% protein diets, but we are not able to determine the reductions that would be appropriate for each nutrient. Thus, we are unable to provide guidance on how best to use the levels provided in NRC's Nutrient Requirements of Laboratory Animals

in such circumstances (Reference 11). These levels should be used with caution and should not be used to adjust the overall compositions of the PER study diets (Reference 11). Rather, they should be used to identify and correct a PER study diet that may be deficient in a specific vitamin or mineral and to identify and correct a PER study diet that may be formulated to contain a potentially toxic level of a specific mineral or vitamin (see, e.g., section IV.B.1.c).

VI. References

The following references marked with an asterisk (*) are on display at the Dockets Management Staff (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852, 240-402-7500, and are available for viewing by interested persons between 9 a.m. and 4 p.m., Monday through Friday; they also are available electronically at https://www.regulations.gov. References without asterisks are not on public display at https://www.regulations.gov because they have copyright restriction. Some may be available at the website address, if listed. References without asterisks are available for viewing only at the Dockets Management Staff. FDA has verified the website addresses, as of the date this document publishes in the *Federal Register*, but websites are subject to change over time.

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VII. Appendices

Appendix #1. AOAC Official Method 960.48
Appendix #2. Adjustments required by the Association of Official Analytical Chemists AOAC Official Method 960.48.
Appendix #3. Contributions to diets (g/kg) of specific mineral mixes used at listed levels.
Appendix #4. Contributions to diets (units/kg) of specific vitamin mixes used at 1%.
Appendix #5. Compositions of the Association of Official Analytical Chemists (AOAC) Official Method 960.48 diet, diets AIN-76A and AIN-93G and National Research Council (NRC) Nutrient Requirements for Rats (1978 and 1995).
Appendix #6. Matching the mineral, vitamin, and amino acid compositions of PER study test and reference diets.

Appendix #1. AOAC Official Method 960.48

45.3.04

AOAC Official Method 960.48
Protein Efficiency Ratio

Rat Bioassay
First Action 1960
Final Action 1962

(Applicable to materials containing >1.80% N.)

A. Reagents

(**a**) *ANRC reference casein.*—Available from New Zealand Milk Products (1269 N. McDowell, PO Box 80816, Petaluma, CA 94975-8016, USA).

(**b**) *Salt mixture USP.*—Either USP salt mixture or salt mixture having essentially same proportions of the elements. Prepare USP XIX (p. 612) salt mixture (or corresponding USP XX item) as follows: Grind in mortar portion of 139.3 g NaCl with 0.79 g KI. Similarly grind together remainder of the NaCl with 389.0 g KH_2PO_4, 57.3 g $MgSO_4$ anhydrous, 381.4 g $CaCO_3$, 27.0 g $FeSO_4·7H_2O$, 4.01 g $MnSO_4·H_2O$, 0.548 g $ZnSO_4·7H_2O$, 0.477 g $CuSO_4·5H_2O$, and 0.023 g $CoCl_2·6H_2O$, finally adding the NaCl–KI mixture. Reduce entire mixture to fine powder.

(**c**) *Vitamin mixture.—See* Table **960.48**.

(**d**) *Cottonseed oil.*

(**e**) *Cellulose.*—Cellu Flour, Solka Floc, or equivalent.

(**f**) *Protein evaluation basal diet.—*

Test sample	X*
Cotton seed oil	$8 - \frac{X \times \%\ \text{ether extract}}{100}$
Salt mixture USP	$5 - \frac{X \times \%\ \text{ash}}{100}$
Vitamin mixture	1
Cellulose	$1 - \frac{X \times \%\ \text{crude fiber}}{100}$
Water	$5 - \frac{X \times \%\ \text{moisture}}{100}$
Sucrose or corn starch, to make 100	$*X = \frac{1.60 \times 100}{\%\ \text{N of test sample}}$

All % figures refer to test sample. Proximate analysis is needed to adjust diet so that all comparisons between test samples and reference material shall be made with diets having same content of N, fat, ash, moisture, and crude fiber. These suggested levels of fat, ash, moisture, and crude fiber are desirable whenever proximate analysis of product permits.

B. Experimental Animals

Laboratory rats, males, shall be from same colony, and maintained during period before weaning upon diet and under environmental conditions that will provide for normal development in all respects; weaned; ≥21 days of age but ≤28 days of age; range of individual rat weights among animals used shall be ≤10 g. When animals are transported from breeding colony to test laboratory, acclimation period of ≥3 days but <7 should precede test.

Table 960.48. Vitamin mixture

Ingredient	mg/100 g ration
Vitamin A (dry, stabilized)	2000 (IU)
Vitamin D (dry, stabilized)	200 (IU)
Vitamin E (dry, stabilized)	10 (IU)
Menadione	0.5
Choline	200
p-Aminobenzoic acid	10
Inositol	10
Niacin	4
Ca-D-pantothenate	4
Riboflavin	0.8
Thiamine·HCl	0.5
Pyridoxine·HCl	0.5
Folic acid	0.2
Biotin	0.04
Vitamin B_{12}	0.003
Glucose to make	1000

C. Assay Groups

Assemble groups of ≥10 rats. In assay of each material provide 1 group that will receive ANRC reference casein. One reference casein group may be used for concurrent assay of >1 assay material. When assembling of all groups is complete, total number of rats in each group must be the same, and average weight of rats in any 1 group on day beginning assay period must not exceed by >5 g average weight of rats in any other group.

D. Assay Period

Throughout assay period keep each rat in individual cage and provide with appropriate assay diet and H_2O ad libitum. During assay period maintain all conditions of environment as uniform as possible with respect to each of groups being compared to ANRC reference casein. Record body weight of each rat on beginning day of assay period and body weight and food intake of each rat at regular intervals, not >7 days, and on 28th day after beginning of assay period.

E. Calculations and Tabulation of Results

Calculate average 28 day weight gain and protein (N × 6.25) intake per rat for each group. Calculate Protein Efficiency Ratio (PER) (weight gain/protein intake) for each group. Determine ratio × 100 of PER for each assay group to PER for ANRC casein reference group. Tabulate 28 day weight gains, protein intake, PER, and ratio × 100 of sample PER to ANRC Reference Casein PER for each assay group. Report protein quality of sample as ratio ×100 of sample PER to ANRC Reference Casein PER.

References: *JAOAC* **43,** 38(1960); **48,** 847(1965).

Appendix #2. Adjustments required by Association of Official Analytical Chemists (AOAC) Official Method 960.48.

The PER study diets described in AOAC Official Method 960.48 are made up to contain 10% protein (N x 6.25), 8% fat, 5% ash, 1% fiber, and 5% moisture. This is accomplished by use of cottonseed oil, a USP salt mixture, cellulose, and water. Amounts of fat, ash, fiber, and moisture supplied by the test sample are accounted for through use of the formulas shown below. Control and test diets are also supplied with 1% each of the same vitamin mixture. Proximate compositions of the finished diets should be the same for each series.

A worked example of the formulation of the basal diet for a PER study is given below. The following analytical values, obtained by proximate analyses, are used for the calculations: Nitrogen, 13.89%; protein (N x 6.25), 86.8%; fat (ether extract), 0.7%; moisture, 7.6%; ash, 1.58%; fiber, 0.47%. The test sample is variously defined as "A" or "X."

Worked Example of the Formulation of the Basal Diet for a PER Study

Ingredient	Formula for calculation	Result	Percent in diet
Test sample (infant formula)	A = (10.0) x 100)/ (% protein (N x 6.25) OR X = (1.6 x 100)/(% N in sample)	(1000)/(13.89)(6.25) = 11.52 OR (160)/(13.89)= 11.52	11.52
Fat (cottonseed oil)	8- (A x % ether extract)/100 OR 8- (X x % ether extract)/100	8- (11.52 x 0.7)/100) = 8 – 0.0806 = 7.919	7.92
Salt mixture (USP)	5-(A x % ash)/100 OR 5- (X x % ash)/100	5 – (11.52 x 1.58)/100 = 5-0.182 = 4.818	4.82
Cellulose	1-(A x % fiber)/100 OR 1-(X x % fiber)/100	1-(11.52 x 1.58)/100 = 1-0.182 = 0.818	0.82
Vitamin mix	None provided	N/A	1
Water	5-(A x % moisture)/100 OR 5-(X x % moisture)/100	5- (11.52 x 7.6)/100 = 5-0.876 = 4.12	4.12
Sucrose or cornstarch, to 100 %			Ingredients: 30.2 %; Sucrose or cornstarch: 100 - 30.2 = 69.8 %

Appendix #3. Contributions to diets (g/kg) of specific mineral mixtures used at listed levels.

Minerals	USP XIX p. 612 (AOAC)[1,2]	USP XIX p. 612 (AOAC)[1]	Bernhart & Tomarelli[3]	AIN-76A[3]	AIN-93G MX[3]	NRC (1995) requirements[4]
Level of addition	4%	5%	4%	3.5%	3.5%	-
	g/kg	g/kg	g/kg	g/kg	g/kg	g/kg
Ca	6.109	7.636	8.997	5.155	5.0	5.0
P	3.542	4.428	7.456	3.984	1.992	3.0
K	4.479	5.5988	2.675	3.602	3.60	3.6
Na	2.192	2.74	0.763	1.019	1.019	0.5
Cl	3.381	4.226	0.743	1.571	1.571	0.5
S	0.771	0.9638	0.501	0.337	0.301	-
Mg	0.463	0.5788	0.603	0.507	0.502	0.5
I	0.0242	0.03025	0.0002	0.0002	0.0002	0.00015 (150 µg/kg)
Fe	0.217	0.2713	0.0373	0.0351	0.036	0.035 (35 mg/kg)
Cu	0.0049	0.00613	0.0065	0.0056	00058	0.005 (5 mg/kg)
Mn	0.0521	0.06513	0.0464	0.0058	0.0105	0.010 (10 mg/kg)
Zn	0.005	0.00625	0.0171	0.0314	0.032	0.012 (12 mg/kg)
F	-	-	-	-	0.001	-
Se	-	-	-	0.00015	0.00015	0.00015 (150 µg/kg)
Mo	-	-	-	-	-	0.00015 (150 µg/kg)
Co	0.000228	0.000285	-	-	-	-

Abbreviations: USP, United States Pharmacopeia; AIN, American Institute of Nutrition; NRC, National Research Council.

[1] USP salt mixture XIX (p. 612)((1975). Corresponding USP XX item (1980) is found in <141> p. 902.
[2] Calculated from 5% level of addition.
[3] Harlan Research Diets_Custom Research Diets.pdf. Harlan Laboratories, Inc., Indianapolis, IN, 46250; http://www.harlan.com, tekladinfo@harlan.com
[4] National Research Council, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board of Agriculture, 1995, Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, National Academy Press, Washington, DC. http://www.nap.edu/catalog/4758.html. (free download).

Appendix #4. Contributions to diets (units/kg) of specific vitamin mixtures used at 1%.

Vitamin	AOAC Official method[1]	AOAC vitamin mix[2]	AIN-76A vitamin mix[2,3]	AIN-93G vitamin mix[2,3]
Vit. A (dry, stabilized)	20,000 IU	-	-	-
Dry vit. A palmitate (500,000 IU/g)	-	-	4000 IU	4000 IU
Vitamin D (dry stabilized)	2000 IU	-	-	-
Vit. D3 trituration (400,000 IU/g)	-	-	1000 IU	-
Vit. D3 (cholecalciferol) 500,000 IU/g	-	-	-	1000 IU
Vits. A and D powder containing vit. A acetate (500,000 IU/g) and vit. D3 (50,000 IU/g)	-	20,000 IU vit A 2000 IU vit D3	-	-
Vit. E (dry stabilized)	100 IU	-	-	-
Dry vit. E acetate (500 IU/g)	-	100 IU	50 IU	-
DL-α- tocopheryl acetate (500 IU/g)	-	-	-	75 IU
Menadione	5 mg	5 mg	-	-
Menadione sodium bisulfite complex (62.5% menadione)	-	-	1.5 mg	-
Vit. K (phylloquinone)	-	-	-	0.75 mg
Choline[3]	2000 mg (2 g)	-	-	-
Choline dihydrogen citrate	-	4.8781 g	-	-
p-aminobenzoic acid	100 mg	100 mg	-	-
Inositol	100 mg	100 mg	-	-
Niacin	40 mg	40 mg	30 mg	-
Nicotinic acid	-	-	-	30 mg
Ca-*D*-pantothenate	40 mg	40 mg	16 mg	16 mg
Riboflavin	8 mg	8 mg	6 mg	6 mg
Thiamine HCl	5 mg	5 mg	6 mg	6 mg
Pyridoxine HCl	5 mg	5 mg	7 mg	7 mg
Folic acid	2 mg	2 mg	2 mg	2 mg
Biotin	0.4 mg	0.4 mg	0.2 mg	0.2 mg
Vit. B12	0.03 mg	-	-	-
Vit. B12 (0.1% trituration in mannitol)	-	0.03 mg	0.01 mg	0.025 mg
Sugar component of vitamin mix	Glucose	Dextrose	Sucrose	Sucrose

NRC (1995) requirements/kg diet for growth: A (retinol), 0.7 mg (2333 IU); D (cholecalciferol), 0.025 mg; E (*RRR-α*-tocopherol, 18.0 mg; K (phylloquinone), 1.0 mg; biotin (*d*-biotin), 0.2 mg; choline (free base), 750 mg; folic acid, 1.0 mg; niacin (nicotinic acid), 15.0 mg; pantothenate (Ca-*d*-pantothenate), 10.0 mg; riboflavin, 3.0 mg; thiamin (thiamin-HCl), 4.0 mg; B6 (pyridoxine), 6.0 mg; B12, 50 µg.

[1] From formulation in AOAC Official Method 960.48. See Appendix 1.
[2] Harlan Research Diets_ Custom Research Diets.pdf. Harlan Laboratories, Inc., Indianapolis, IN, 46250; http://www.harlan.com., tekladinfo@harlan.com.
[3] The vitamin mixes for diets AIN-76A and AIN-93G do not contain choline or a choline salt. Choline bitartrate is added separately at 2.0 g/kg in diet AIN-76A and at 2.5 g/kg in diet AIN-93G. See Harlan Research Diets_Custom Research Diets.pdf. Harlan Laboratories, Inc., Indianapolis, IN, 46250.

Appendix #5. Compositions of the Association of Official Analytical Chemists (AOAC) Official Method 960.48 diet, diets AIN-76A and AIN-93G and National Research Council (NRC) Nutrient Requirements of Laboratory Animals (1978 and 1995).

Nutrient **Sources and amounts**	**AOAC Official Method 960.48 diet** **units/100 g**	**AIN-76A diet** **units/100 g**	**AIN-93G diet** **units/100 g**	**NRC (1978) nutrient requirements for rats** **units/100 g**	**NRC (1995) nutrient requirements for rats** **units/100 g**
Reference	**(1)**	**(2, 3)**	**(4)**	**(5)**	**(6)**
Protein, g	ANRC Reference Casein (10%) (N x 6.25)	Casein, 20	Casein (≥ 85% protein), 20	12	15
Fat g	Cottonseed oil, ~8% (per formula)	Corn oil, 5	Soybean oil, 7	5.0	5
Carbohydrate, g	Sucrose or corn starch (per formula)	Cornstarch, 15; Sucrose, 50	Corn sugar, 39.75; granular sugar, 10; dextrin, 13.2	-[1]	-
Fiber, g	Cellulose, ~1	Cellulose, 5	Cellulose, 5	-	-
Minerals					
Calcium, g	0.763	0.52	0.52	0.5	0.5
Phosphorus, g	0.443	0.4	0.20	0.4	0.3
Magnesium, g	0.058	0.05	0.05	0.04	0.05
Sodium, g	0.274	0.1	0.11	0.05	0.05
Potassium, g	0.559	0.36	0.38	0.36	0.36
Chloride, g	0.423	0.16	0.22	0.05	0.05
Sulfur, g[2]	0.093	0.033	0.03	0.03	0.03 may be beneficial
Iron, mg	27.1	3.7	5.20	3.5	3.5
Zinc, mg[3]	0.624	2.9	3.68	1.2	1.2
Manganese, mg	6.52	5.9	1.13	5.0	1
Copper, mg	0. 61	0.6	0.67	0.5	0.5
Iodine, µg	3,025	20	21	15	15
Selenium, µg	-	16	18	10	15
Cobalt, µg	29	-	-	-	-
Chromium, µg	-	200	100	30	-
Molybdenum, µg	-	-	15	-	15
Fluoride, µg	-	-	100	100	-

Nutrient **Sources and amounts**	**AOAC Official Method 960.48 diet** **units/100 g**	**AIN-76A diet** **units/100 g**	**AIN-93G diet** **units/100 g**	**NRC (1978) nutrient requirements for rats** **units/100 g**	**NRC (1995) nutrient requirements for rats** **units/100 g**
Vitamins					
A (retinol), mg or IU (1 IU = 0.3 µg retinol)	2000 IU	400 IU	400 IU	400 IU	230 IU (0.07 mg)
D (cholecalciferol), mg or IU (1 µg cholecalciferol = 40 IU)	200 IU	100 IU	100 IU	100 IU	100 IU (0.0025 mg)
E *(RRR-α*-tocopherol), mg or IU (1 IU = 0.667 mg *d-α*-tocopherol)	10 IU	5 IU	7.5 IU	3 IU	2.7 IU (1.8 mg)
K, mg	0.5 (menadione)	0.05 (menadione)	0.09 (phylloquinone)	0.005 (phylloquinone) or 0.05 (menadione)	0.10 (phylloquinone)
Biotin (*d*-biotin), mg	0.04	0.02	0.02	-	0.02
B12, µg	3	1	2.5	5	5
Folic acid, mg	0.2	0.2	0.2	0.10	0.1
Niacin (nicotinic acid), mg	4	3	3	2.0	1.5
Pantothenate (Ca-*d*-pantothenate), mg	4	1.6	1.5	0.8	1
B6 (pyridoxine), mg	0.5	0.7	0.6	0.6	0.6
B2 (riboflavin), mg	0.8	0.6	0.6	0.3	0.3
B1 (thiamin), mg	0.5	0.6	0.5	0.4	0.4
Choline, mg	200 (choline)	200 (choline bitartrate)	250 (choline bitartrate)	100 (choline)	75 (free base)
Inositol, mg	10	-	-	-	-
***p*-Aminobenzoic acid, mg**	10	-	-	-	-

Nutrient Sources and amounts	AOAC Official Method 960.48 diet units/100 g	AIN-76A diet units/100 g	AIN-93G diet units/100 g	NRC (1978) nutrient requirements for rats units/100 g	NRC (1995) nutrient requirements for rats units/100 g
Amino acids					
***DL*-methionine + cysteine, g**	-	-	-	-	0.98
***DL*-methionine, g**	-	0.3	-	-	-
***L*-cystine, g**	-	-	0.3	-	-
Antioxidants					
***t*-butyl-hydroquinone, g**	-	-	0.0014	-	-
Ethoxyquin, g	-	0.001	-	-	-
Other constituents					

[1] In otherwise adequate diets, glucose, sucrose, maltose, and fructose support similar levels of performance. In rats fed diets low in protein or water-soluble vitamins, insoluble carbohydrates such as starch or dextrin promote more growth than do soluble carbohydrates such as sucrose or glucose (NRC (1978) Nutrient Requirements of Laboratory Animals, Third Revised Edition, National Academy Press, Washington, DC). http://nap.nationalacademies.org/20047.

[2] Sulfur: Calculated from composition of mineral salts. In diet AIN-76-A, the mineral mix contains 52.0 g potassium sulfate (18.39% sulfur)/kg mix and is used at 3.5 % of the diet. Potassium sulfate contributes 0.033 g sulfur/100 g diet. In diet AIN-93G, the mineral mix contains 46.6 g potassium sulfate (18.39% sulfur)/kg mix and is used at 3.5% of the diet. Potassium sulfate contributes 0.0299 g sulfur/kg diet (46.6 x 0.1839 = 8.5697 g sulfur/kg salts x 3.5% = 0.0299 g sulfur/100 g). In the mineral mixtures for diets AIN-76A and AIN-93G, Mg is provided as its oxide and Fe is provided as its citrate salt. Mn, Zn, and Cu are provided as their carbonate salts.

[3] Zn: If rats are housed in galvanized cages, no more than 0.2-0.4 mg Zn/100 g is required (NRC, 1978). http://nap.nationalacademies.org/20047.

References:

(1) Official Methods of Analysis of AOAC International, 18th edition, 1995, AOAC Official Method 960.48 Protein Efficiency Ratio, Rat Bioassay, Section 45.3.04. Note: *p*-Aminobenzoic acid (PABA) is included in the AOAC PER diets, but not in the AIN diets. PABA is an intermediate in the synthesis of folate by bacteria, plants and fungi. It may have been included in the AOAC diets to facilitate synthesis of folate by intestinal bacterial. Rats do not require PABA.

(2) American Institute of Nutrition, 1977, Report of the American Institute of Nutrition *Ad Hoc* Committee on Standards for Nutritional Studies, Journal of Nutrition 107: 1340-1348 (AIN-76™ Purified Diet).

(3) American Institute of Nutrition, 1980, Second report of the *ad hoc* committee for experimental animals. Journal of Nutrition 110: 1726.

(4) Reeves, P.G., Nielsen, F.H. and Fahey, G.C., Jr. ,1993, AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition *Ad Hoc* Writing Committee on the Reformulation of the AIN-76A Rodent Diet, Journal of Nutrition 123: 1939-1951.

(5) National Research Council, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board of Agriculture, 1978, Nutrient Requirements of Laboratory Animals, Third Revised Edition, National Academy Press, Washington, DC. http://nap.nationalacademies.org/20047.

(6) National Research Council, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board of Agriculture, 1995, Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, National Academy Press, Washington, DC. http://nap.nationalacademies.org/4758.

Appendix #6. Matching the mineral, vitamin, and amino acid compositions of PER study test and reference diets.

One approach to matching the mineral and vitamin compositions of the PER study test and reference diets is described below. The values shown in Table 1 are for illustrative purposes only and should not be interpreted as target values. Columns 1-7 in Table 1 can be completed with the following information:

Column 1. List all minerals and vitamins, including their forms and units. Minerals and vitamins should be presented as, for example, Ca (from CaCO3); Vitamin K (as phylloquinone). All entries should be expressed in appropriate units.

Column 2. List all mineral and vitamin components in the infant formula in units/100 g or units/kg. The infant formula should be identified by name or associated numbers and a Certificate of Analysis or Product Analysis Certificate should be included. The Certificate of Analysis will usually provide the information needed to develop an estimate of the composition of the PER study test diet. These Certificates will often include a list of the test methods by which all nutrients were determined. Such information may indicate the sensitivity and specificity of individual methods.

Column 3. Calculate the contribution of the infant formula to the PER study test diet based on the use rate or incorporation rate (e.g., 85%, 90%) of the infant formula in the test diet. Review the NRC (1995) nutrient requirements for rats (Column 7) to determine the adequacy of the composition of the test diet at this stage.

Column 4. Identify minerals or vitamins that do not meet the NRC (1995) specifications. The NRC specifications are minimal and do not include a margin of safety. Calculate the amounts of minerals and vitamins that need to be added to meet the NRC (1995) levels with a margin of safety (e.g., ~ 10%). Identify those that may be near or at a potentially toxic level based on information provided by the NRC (1995). Several worked examples are shown in the Table. Formulate mineral or vitamin mixtures that are needed to bring deficient levels, if any, in Column 3 to NRC (1995) levels. List the contributions to the test diet from these mineral or vitamin mixtures in Column 4.

Column 5. Add Columns 3 and 4 to obtain the estimated final composition of the PER study test diet. Review the composition against the NRC (1995) nutrient specifications for rats to confirm the adequacy of the estimates.

Column 6. The composition of the PER study reference diet is developed from the estimates in Column 5. For each mineral and vitamin, calculate a range that is ±20% around the estimated value in Column 5. Compare the low and high ends of the calculated range against NRC (1995) values for nutrient. Formulate vitamin and mineral mixtures needed for the reference diet and list their contributions in column 6. All estimates should fall within the ranges identified in Column 5 (that is, the estimates for the reference diet should fall within ±20% of the final estimates for the test diet in Column 5). Compare all estimates with NRC (1995) levels and adjust as needed to reduce the possibility of potentially deficient or toxic levels of specific minerals or vitamins.

Fiber (see Section IV.B.1.g of the guidance): Infant formulas frequently contain non-digestible carbohydrates such as fructo-oligosaccharides (FOS) or galacto-oligosaccharides (GOS). Our current thinking is that it is not necessary to add cellulose to the infant formula test diet or to its matched casein control diet when the infant formula includes non-digestible carbohydrates such as FOS and/or GOS. When FOS or GOS are present in the infant formula, then they should be included in the matched casein control diet. Due to our uncertainty as to the outcome of PER studies conducted in completely fiber-free diets, we further recommend that 1% cellulose be added to the test and matched casein control diet when the infant formula does not contain a source of non-digestible carbohydrate.

Vitamin E and Polyunsaturated Fatty Acids (PUFA) (see Section IV.B.1.c of the guidance): The need for vitamin E increases with an increase in consumption of PUFA and with the degree of unsaturation of dietary PUFAs. The following adjustment takes this into consideration: The vitamin E and PUFA contents of the PER study test and reference diets can be estimated from the Certificates of Analysis or other information. The ratios of vitamin E:PUFA expressed as mg vitamin E/g PUFA can be calculated. The ratio of 0.48 ± 0.28 mg of vitamin E per gram of PUFA is used as a general guideline for the relationship between these nutrients. Modifications in the concentration of vitamin E in the reference diet can be made as needed. See the Table below for an example.

Inorganic Sulfur: While there is uncertainty regarding an appropriate concentration of inorganic sulfur to add to the PER study casein reference control diet, we are recommending that the concentration of inorganic sulfur in the control diet be adjusted to provide the inorganic sulfur content set forth in the AOAC Method (i.e., 0.964 g/kg diet).

Sulfur Amino Acids (methionine, cystine) (see Section IV.B.1.h of the guidance) (Table 2): Diets containing 8-10% protein from casein are limiting in sulfur amino acids methionine and cystine. Sponsors may want to consider matching the concentrations of (methionine + cystine) in the PER study test and reference casein diets if there is concern about a potential failure of the casein reference group because of low concentrations of cystine in casein. This matching is optional and can be performed at the discretion of the sponsor. Sponsors who choose to prepare the (methionine + cystine)-matched diet described below may want to include it as a second casein reference control group in their PER study.

The matching may be performed as follows: The infant formula and the casein for the control diet should be analyzed for their methionine and cystine content prior to diet preparation. The concentrations of (methionine + cystine) should be calculated from the results of these analyses. If the concentration of cystine estimated to be present in the control diet is <80% of the value for cystine in the test diet, then cystine can be added to the control diet so that the concentrations of (methionine + cystine) are similar in both diets. The final concentration of (methionine + cystine) in the control diet should be ±20% of the value for (methionine + cystine) in the test diet. Addition of methionine alone is not an appropriate remedy for a deficit of cystine in the casein control diet. See the Table below for an example.

In cases in which the sum of (methionine + cystine) in the test diet is less than the sum of (methionine + cystine) in the control diet, and the amount of cystine in the control diet is < 80% of the value for cystine in the test diet, then cystine can be added to the control diet so that the concentrations of cystine are the same in both the control and reference diets.

Use of NRC (1995) <u>Nutrient Requirements of Laboratory Animals (Reference 1)</u>: The NRC (1995) values are expressed on an as-fed basis for diets containing 10% moisture and 3.8-4.1 kcal metabolizable energy/g and should be adjusted for diets of differing moisture and energy concentrations. In addition, the NRC (1995) levels for growth are for diets containing 15% protein and 5% fat. Unless otherwise specified, the listed nutrient levels represent minimal values and do not include a margin of safety.

We consider that most nutrient levels would be significantly lower for the 10% PER study diets than the NRC (1995) values for 15% protein diets, but we are not able to determine the reductions that might be appropriate for each nutrient. While we are unable to provide specific guidance in this area, we consider that the NRC (1995) values should be used to identify and correct a PER study diet that may be deficient in a required mineral or vitamin. The same consideration applies to identifying and correcting a PER study diet that may be formulated to contain a potentially toxic level of a specific mineral or vitamin.

The values shown below are for illustrative purposes only and should not be interpreted as target values. The composition of each infant formula must be evaluated independently in order to determine how to formulate appropriate PER study test and reference diets.

Table 1. Example of an approach for matching mineral and vitamin compositions of PER study test and reference diets.

Column 1 Mineral or vitamin Sources and amounts	Column 2 Composition of infant formula, units/100 g	Column 3 Contribution of infant formula to test diet Rate of use (%), 90 units/100 g	Column 4 Additions to test diet units/100 g	Column 5 Test diet, final units/100 g	Column 6 Reference diet = test diet ± 20% units/100 g	Column 7 NRC (1995) nutrient requirements units/100 g
Minerals						
Calcium ($CaCO_3$), g	0.50	0.45	0.05	0.50	0.40-0.60	0.5
Phosphorus, g	0.40	0.36	none	0.36	0.29-0.43	0.3
Magnesium, g	0.06	0.054	none	0.054	0.043-0.065	0.05
Sodium, g	0.22	0.20	none	0.20	0.16-0.24	0.05
Potassium, g	0.67	0.60	none	0.60	0.48-0.72	0.36
Chloride, g	0.33	0.30	none	0.30	0.24-0.36	0.05
Sulfur, g	-	-	-	-	-	0.03
Iron, mg	20.00	18	none	18	14.4-21.6	3.5
Zinc, mg	0.90	0.81	0.39	1.20	0.96-1.44	1.2
Manganese, mg	1.67	1.5	none	1.5	1.19-1.80	1
Copper, mg	0.67	0.60	none	0.60	0.48-0.72	0.5
Iodine, µg	88.90	80	none	80	64-96	15
Selenium, µg	22.20	20	none	20	16-24	15
Cobalt, µg	-	12		12	-	Not required
Chromium, µg	-	-		-	-	Not required
Molybdenum, µg	16.70	15	none	15	12-18	15
Fluoride, µg	-	-	-	-	-	Not required
Vitamins						
A (retinol), mg or IU; 1 IU = 0.3 µg retinol	5,000 IU	4500 IU	none	4500 IU	3600-5400 IU	230 IU (0.07 mg)
B-carotene	-	-	-	-	-	-
D3 (cholecalciferol), mg or IU; 1 µg D3 = 40 IU	680 IU	612 IU	none	612 IU	490-734 IU	100 IU (0.0025 mg)
E *(RRR-α*-tocopherol), mg or IU; 1 IU = 0.667 mg *d-α*-tocopherol	40 IU	36 IU	none	36 IU	29-43 IU	2.7 IU (1.8 mg)
K (phylloquinone), mg	0.08	0.072	0.028	0.10	0.08-0.12	0.10

Column 1 Mineral or vitamin Sources and amounts	Column 2 Composition of infant formula, units/100 g	Column 3 Contribution of infant formula to test diet Rate of use (%), 90 units/100 g	Column 4 Additions to test diet units/100 g	Column 5 Test diet, final units/100 g	Column 6 Reference diet = test diet ± 20% units/100 g	Column 7 NRC (1995) nutrient requirements units/100 g
Biotin (*d*-biotin), mg	0.03	0.027	none	0.027	0.022-0.032	0.02
B12, µg	5.0	4.5	0.50	5.0	4.00-6.00	5
Folic acid, mg	0.22	0.20	none	0.20	0.16-0.24	0.1
Niacin (nicotinic acid), mg	7.78	7.0	none	7.0	5.60-8.40	1.5
Pantothenate (Ca-*d*-pantothenate), mg	5.00	4.5	none	4.5	-	1
B6 (pyridoxine), mg	0.90	0.81	none	0.81	0.65-0.97	0.6
B2 (riboflavin), mg	1.11	1.0	none	1.0	0.80-1.20	0.3
B1 (thiamin), mg	1.00	0.90	none	0.90	0.72-1.08	0.4
Choline, mg	3.89	350	none	350	280- 420	75 (free base)
Inositol, mg	33.3	30	none	30	24-36	Not required
***p*-Aminobenzoic acid, mg**	-	-	-	-	-	Not required
Vitamin C, mg	166.67	150		150	120-180	Not required
Fiber	See Narrative Section above	-	-	-	-	-

Column 1 Mineral or vitamin Sources and amounts	Column 2 Composition of infant formula, units/100 g	Column 3 Contribution of infant formula to test diet Rate of use (%), 90 units/100 g	Column 4 Additions to test diet units/100 g	Column 5 Test diet, final units/100 g	Column 6 Reference diet = test diet ± 20% units/100 g	Column 7 NRC (1995) nutrient requirements units/100 g
Vitamin E, mg **PUFA, g**	5.0 7.0	4.5 6.3	none	4.5 6.3	3.6-5.4 5.04-7.56	The mean value of 0.48 mg vitamin E/g PUFA (or 48 mg vitamin E/100 g PUFA) is a general guideline for the minimum ratio of the two nutrients.
Ratio of mg vitamin E/g PUFA		0.71 mg/g		0.71 mg/g	Estimated range of ratios: 3.6/5.04=0.71 3.6/7.56=0.48 5.4/5.04=1.07 5.4/7.56=0.71 ~0.48 - 1.07 mg/g	
Ratio of mg vitamin E/100 g PUFA		71 mg/100 g		71 mg/100 g	~48-107 mg/100g	

"none": The contribution of the infant formula to the test diet meets or exceeds the NRC (1995) (Reference 1) specifications. No addition is needed.

Table 2. Optional inclusion of a second reference casein control group with diet matched to cystine + methionine content of the infant formula-based test diet.

Amino acid	Composition of infant formula g/100 g	Contribution of infant formula to test diet Rate of use (%), 90 g/100 g	Addition to test diet g/100 g	Test diet final g/100 g	Addition to reference diet g/100 g	Variability in reference diet (%)
Cystine **Methionine** **Sum**	0.243 0.268 0.511	0.219 0.241 0.460	N/A N/A	0.219 0.241 0.460	0.037+0.084 0.339 0.460	Value for SAA in the reference diet should be ± 20% of the value for SAA in the test diet.

Cystine/methionine: In this example, assume that casein provides per 100 g: 0.37 g cystine and 3.39 g methionine for a total of 3.76 g/100 g sulfur amino acids. Cystine provides 9.8% of the SAA in casein (0.037/0.376) x 100 = 9.8%). When used at 10% of the reference diet, casein provides per 100 g of diet: 0.037 g cystine and 0.339 g methionine. In this example, cystine provided by casein in the reference diet is 16.9% of the amount of cystine provided in the infant formula diet ((0.037/0.219) x 100 = 16.9%).

The amount of cystine to be added to the casein reference diet is calculated as follows (g/100 g): (sum of cystine + methionine in test diet) less (sum of cystine and methionine in casein reference diet) = 0.460 – 0.037 – 0.3339 = 0.084 g cystine/100 g to be added to the reference diet. When this addition is accomplished, the test diet and reference diet have the same concentrations of SAA. An example of this approach is found in Hoskin, 2022; https://doi.org/10.1093/jaoacint/qsac110.

Cystine contributes 47.6% (0.219/0.046 = 0.476 x 100 = 47.6%) of the total SAA in the infant formula test diet and 26.3% ((0.037 + 0.084)/0.460 = 0.263 x 100 = 26.3%) of the total SAA in the casein reference diet.

"N/A": Not applicable.

Reference:

(1) Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995, Subcommittee on Laboratory Animal Nutrition, National Research Council, National Academies Press, Washington, DC, Chapter 2, pages 11-79. http://nap.nationalacademies.org/4758.