consultants to the regulated industry

Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
Woodbridge, VA 22192-575.5
703 590 7337 (Fax 703 580 8637)
Smedley@cfr-scrviccs.com

November 14, 2019

Dr. David Edwards
Director
Division of Animal Feeds (HFV- 220),
Center for Veterinary Medicine,
Food and Drug Administration,
7519 Standish Pl.,
Rockville, MD 20855

Subject: Filing of Animal GRAS Notification
Dried L-Valine Fermentation Product

Notifier: C J CheilJedang Corporation (CJ)
330, Dongho-Ro, Jung-Gu,
SEOUL,04560,KOREA

Dear Dr. Edwards:

On behalf of CJ CheilJedang Corporation (CJ), I am filing an animal Generally Recognized as Safe Notice for the use of Dried L-Valine Fermentation Product (72%). The GRAS substance is produced by a genetically modified *Corynebacterium glutamicum* and the intended use is as a source of L-valine, a nutrient, for use in livestock and poultry diets. The submission is compliant with 21 CFR 570.210-255. The GRAS conclusion is based on scientific procedures.

Should you have any questions on the filing, please contact me directly.

Sincerely,

Kristi O. Smedley
Consultant to CJ CheilJedang Corporation

RECEIVED
NOV 20 2019
By

Cc: Keith Haydon, CJ

ATTACHMENT:

CJ Letter of Representation—Smedley
GRAS Notice L-Valine Fermentation Product

March 5, 2019

David Edwards
Director
Division of Animal Feeds, HFV-220
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Place
Rockville, MD 20855

Subject: CheilJedang Corporation
Authorization of Kristi Smedley as Regulatory Contact
AGRN L-Valine Fermentation Product

Dear Dr. Edwards:

CheilJedang Corporation (CJ) is authorizing Dr. Kristi O. Smedley, Center for Regulatory Services, Inc. 5200 Wolf Run Shoals Road, Woodbrige, VA 22192 (Telephone 703-590-7337), to represent CheilJedang Corporation with respect to the Animal GRAS notice for L-Valine Fermentation Product.

Should you have any questions on this matter, please contact the undersigned.

Sincerely,

Keith D. Haydon, Ph.D.
Director of Technical Services and Marketing

cc: Kristi Smedley, CFR Services

McCrady.Sara님/General Affairs/20190307082₉

Generally Recognized as Safe (GRAS) Notice

for

Dried L-Valine Fermentation Product as a Source of Valine in Livestock and Poultry Feed

Prepared for:
U.S. Food and Drug Administration
Center for Veterinary Medicine
Division of Animal Feeds

Prepared by:
CheilJedang Corporation

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1. PART 1 GRAS Notice

CJ CheilJedang Corporation (hereinafter referred to as "CJ") is submitting a GRAS notice for the substance Dried L-Valine Fermentation Product as a source of valine in livestock and poultry diets.

1.1 Name and Address of Organization

CJ CheilJedang Corporation
Mr. Harry Jang
330, Dongho-Ro,
Jung-Gu, SEOUL, 04560, KOREA
Tel.: +82-2-6740-3940
E-mail: harry.jang@cj.net

CJ BIO America, Inc.
Keith Haydon, PhD
CJ BIO America, Inc.
2001 Butterfield Road, Suite 720
Downers Grove, IL 60515
Tel: (630) 241-0112
E-mail: keith.haydon@cj.net

1.2 Name of the Notified Substance

The common or usual name of the subject substance of this notification is "Dried L-Valine Fermentation Product". It is a source of the essential nutrient L-valine. The level of L-valine in the product is a minimum of 72%. Dried L-Valine Fermentation Product also contains approximately 7% amino acid from biomass (dried *Corynebacterium glutamicum* cell). The trade name of the product is "VAL Pro".

1.3 Intended Conditions of Use

Dried L-Valine Fermentation Product is to be used as an ingredient in animal feed according to current good manufacturing and feeding practice as defined in 21CFR§582.1(b) ("Substances that are generally recognized as safe"). Valine is an essential amino acid that is typically considered to be the fifth limiting amino acid after tryptophan for pigs and as the fourth or fifth limiting amino acid after threonine for poultry. Valine will be incorporated into the diet at levels commensurate with the nutritional requirement. Therefore, the required level will be decided on a case-by-case basis by animal nutritionists, based on good feeding practice for the target species.

1.4 Statutory Basis for GRAS Determination

This GRAS conclusion is based on the scientific procedures as provided in 21CFR§570.30(a) and (b).

1.5 Federal Food, Drug, and Cosmetic Act Premarket Approval Exemption

The submitter has determined that the use of Dried L-Valine Fermentation Product as produced by fermentation with *Corynebacterium glutamicum*, for use as a nutrient (valine) in livestock and poultry feed is Generally Recognized as Safe based on scientific procedure and is thus exempt from the premarket approval requirement of the Federal Food, Drug and Cosmetic Act (21 U.S.C § 301 et.seq.).

1.6 Availability of Information for FDA Review

CJ agrees to make the data and information pertaining to this submission available to FDA.

CJ agrees to both of the following procedures for making the data and information available to FDA:

(A) Upon FDA's request, CJ will allow FDA to review and copy the data and information during customary business hours at the address specified for where these data and information will be available to FDA; and

(B) Upon FDA's request, CJ will provide FDA with a complete copy of the data and information either in an electronic format that is accessible for FDA evaluation or on paper.

1.7 Freedom of Information Act 5 U.S.C 552 Disclosure Exemption

CJ has placed proprietary and confidential information in three appendices: Appendix 1, "Analytical Report; Qualitative and quantitative composition of Dried L-Valine Fermentation Product (CONFIDENTIAL)"; Appendix 3, "Pre-Fermentation Information (CONFIDENTIAL)"; and Appendix 4, "Manufacturing Process (CONFIDENTIAL)".

1.8 Certification of Complete, Representative Submission

To the best of our knowledge and belief, this GRAS notice is a complete, representative and balanced submission that includes unfavorable information, as well as favorable information, known to CJ and pertinent to the evaluation of the safety and GRAS status of the use of Dried L-Valine Fermentation Product produced by fermentation with genetically engineered *Corynebacterium glutamicum* as a source of valine for livestock and poultry feed.

Keith D. Haydon, Ph.D.
Director of Technical Services and Marketing

2. PART 2 GRAS Notice: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Scientific Data and Information that Identifies the Notified Substance

2.1.1 Name and Other Identities

Chemical name according to IUPAC nomenclature	2-amino-3-methylbutanoic acid
Synonyms	(S)-α-Aminoisovaleric acid; L-2-Amino-3-methylbutanoic acid
CAS No.	72-18-4
EC-No.	208-220-0
Appearance	White crystalline powder
Molecular mass	117.15 g/mol
Molecular formula	$C_5H_{11}NO_2$
Structural formula	O, CH_3, HO, CH_3, NH_2

This GRAS notice covers Dried L-Valine Fermentation Product produced by fermentation with *Corynebacterium glutamicum*, with a minimum purity of 72% of L-Valine. L-Valine is the active substance in the Dried L-Valine Fermentation Product. Due to its dedicated chemical properties, L-Valine can only be found as free amino acid, which must not be transformed into a salt to be stable during production, storage and application.

2.1.2 Composition

The majority of the amino acid product is L-valine (≥ 72 %). The product also contains other free amino acids (< 1.0 %), bound amino acids from the biomass (< 7.2 %), sugars (< 0.1 %), organic acids (< 0.2 %), minerals (< 10 %) and moisture (< 5 %), and carrier (< 6 %). As shown in Table 2-1, the analysis of the five batches of Dried L-Valine Fermentation Product demonstrates that the finished

product [illegible] reproducibly manufactured. Refer [illegible] Appendix [illegible] Composition [illegible] Impurity Reports [illegible] additional information regarding [illegible] analytical assessment [illegible] product composition [illegible] carrier [illegible] used [illegible] consistent value [illegible] final product from batch [illegible] batch.

Table [illegible] Chemical Composition Including Impurities

	Units	Method	Batch	Batch	Batch	Batch	Batch	Average
L-valine		HPLC (Appendix Attachment 2)	(b) (4)					[illegible]
Hydrolyzed amino [illegible] insoluble (biomass part) (Total)								6.92
(b) (4)		AOAC 994.12						[illegible]
								[illegible]
								[illegible]
		AOAC 985.28						[illegible]
		AOAC 994.12						[illegible]
		AOAC 988.15						[illegible]
		AOAC 985.28						[illegible]
		AOAC 994.12						[illegible]
[illegible] amino acids (Total [illegible] valine)		AOAC [illegible]						[illegible]
(b) (4)								[illegible]

(b) (4)

(b) (4)			0.06
	%	AOAC 934.01	**1.19**
		ASTM D4327-03	**2.21**
		AOAC 995.13	**0.06**
	%		0.02
			0.04
Organic acids (Total)	%	Korean Feed Standards Codex, 1 of chapter 14	**0.12**
(b) (4)			0.01
			0.04
			0.07
Inorganic anions/cations (Total)		ASTM D4327-03	**9.65**
(b) (4)	%		0.37
			0.95
			0.06
			0.04
			0.17
			0.14
			7.92
	%	AOAC 942.05	2.90
	%		**6.00**
	%		**99.47**

(b) (4)

2.1.3 Fermentation Organism

The fermentation organism is a genetically modified strain of *Corynebacterium glutamicum*. The genetic modification and characterization of the production microorganism can be found in Appendix 3, "Pre-Fermentation Information (CONFIDENTIAL)." The safety of the production microorganism can found in Section 6 of this dossier, Appendix 3 and Appendix 10.

2.2 Manufacturing Process

Dried L-Valine Fermentation Product is produced by fermentation with *Corynebacterium glutamicum* as a production strain (b) (4)

(b) (4) For further information regarding the manufacturing process, refer to Appendix 4, "Manufacturing Process (CONFIDENTIAL)".

CJ purchases raw materials based on feed grade specifications which are suitable for use in the manufacture of feed. Dried L-Valine Fermentation Product is manufactured in accordance to good manufacturing practices as set forth in 21 CFR §507 and meets the requirements of the US Food Safety Modernization Act (FSMA). As part of the facility's FSMA compliance, a Hazard Analysis Risk-Based Preventive Control plan has been implemented and conducted to evaluate the facility, raw materials, processes and product for potential physical, chemical and biological hazards. In order to mitigate potential risks, a hazard analysis was conducted that includes a risk assessment of the raw materials and processing steps with the implementation of appropriate preventive controls to ensure the safety of the product. These control measures are in place to effectively eliminate or reduce hazards to acceptable levels. The facility also uses prerequisite programs such as an approved supplier program to ensure the safety of the raw materials and that the raw materials are appropriate for their intended use and for the manufacture of a feed ingredient. Material suppliers are initially and periodically qualified and verification activities are performed commensurate to the risk of the material. The applicant also declares that no antimicrobial compounds (including antibiotics) were used in the production process.

The pre-fermentation process is provided in Appendix 3, "Pre-Fermentation Information (CONFIDENTIAL)," which includes the genetic engineering process, characterization and assessment of the production microorganism.

The full fermentation process and downstream manufacturing processes are provided in Appendix 4, "Manufacturing Process (CONFIDENTIAL)".

2.2.2 Ingredient Stability (Shelf Life)

Stability testing for Dried L-Valine Fermentation Product was performed using three typical batches. Stability results for zero-time to 24 months are presented in Table 2-2 (25°C, 60%RH).

None of the tested samples showed a significant decrease in the level of the active substance L-Valine at the tested time points. The specified minimum 72 % L-valine content was maintained in all samples over the tested periods. The full report on product stability can be found in Appendix 5, "Stability – 24 months". The data support product stability for twenty four months.

Table 2-2: Shelf life of Dried L-Valine Fermentation Product in % (Target Value is a Minimum 72% L-Valine) at 25°C, 60% RH during Storage of 24 Months

Batch	Measurement	Zero-time		Time in months					
Lot		start value	unit	3	6	9	12	18	24
Batch No. GVAL160407	Valine content	72.12	%	(b) (4)					
	moisture	0.49	%						
Batch No. GVAL160408	Valine content	73.01	%						
	moisture	0.67	%						
Batch No. GVAL160409	Valine content	72.75	%						
	moisture	0.60	%						

The valine levels were stable over the 24 months of testing, demonstrating product stability throughout the testing period at ambient temperatures. This data supports product stability of at least two years.

2.2.2 Stability upon Addition to Animal Feed

A three-month study in broiler mash feed (three batches) was conducted to demonstrate the stability of the product when mixed in a complete feed. The animal feed was assessed every month. The full report can be found in Appendix 6, "Mash Feed Stability of Dried L-Valine Fermentation Product".

Table 2-3: Stability of Dried L-Valine Fermentation Product in Mash Feed for Broilers

	GVAL180404	GVAL180405	GVAL180406
Test Items	Nominal value 0.40 %		
Initial	0.53 %	0.46 %	0.49 %
1 month	(b) (4)		
2 month			
3 month			

This study demonstrated that Dried L-Valine Fermentation Product was a stable source of Dried L-Valine when added to complete mixed feed over a three-month period.

2.3 Specifications

Dried L-Valine Fermentation Product specifications are based on the assay of five batches. The analytical data supporting the specifications is in reported Table 2-1 above and further discussed in Appendix 1, "Analytical Report; Qualitative and quantitative composition of Dried L-Valine Fermentation Product (CONFIDENTIAL)". The product specifications are provided in Table 2-4 below.

Table 2-4: Dried L-Valine Fermentation Product Specifications

Component	Amount	Method
Valine, minimum	72%	HPLC (Appendix 1-Attachment 2)
Moisture, maximum	5%	At 105℃ for 3hr, AOAC 934.01
Ash, maximum	5%	AOAC 942.05

The final product was tested for hazardous substances through appropriate tests such as heavy metals. The heavy metal analysis was carried out with samples of three batches. The following Table 2-5 shows the results and the COA with raw data provided in Appendix 2, "COA of Heavy metals with Raw data". The analysis was performed using ICP/MS, specifically the AOAC Method 2015.01 (AOAC Official Method 2015.01. Heavy metals in food).

Table 2-5. Analysis result of Heavy metals in final product

Batch No.	Test items	Test result	Test method
GVAL180404	Lead(Pb)	< 0.003 mg/kg	ICP/MS (AOAC Official Method 2015.01)
	Arsenic(As)	< 0.003 mg/kg	
	Mercury(Hg)	< 0.000 mg/kg	
	Cadmium(Cd)	< 0.001 mg/kg	
GVAL180405	Lead(Pb)	< 0.003 mg/kg	
	Arsenic(As)	< 0.003 mg/kg	
	Mercury(Hg)	< 0.000 mg/kg	
	Cadmium(Cd)	< 0.001 mg/kg	
GVAL180406	Lead(Pb)	< 0.003 mg/kg	
	Arsenic(As)	< 0.003 mg/kg	
	Mercury(Hg)	< 0.000 mg/kg	
	Cadmium(Cd)	< 0.001 mg/kg	

As a result, the analysis of heavy metals in the final product is below the detection limit and there is no concern about safety due to heavy metals in the animal and human.

2.4 Intended Use (Utility) of Dried L-Valine Fermentation Product

Dried L-Valine Fermentation Product is to be used as L-valine supplemental nutrient in animal feeds in accordance with good manufacturing or feeding practice as defined in 21CFR§582.1(b) Substances that are generally recognized as safe. L-valine is an essential amino acid in all animal species (FEEDAP. 2014. EFSA Journal 2014;12(7):3795). The level of supplementation varies between species and is dependent on the nutritional content of the diet (specifically the amino acids content).

Therefore, the use of supplementation will be determined on a case-by-case basis by animal nutritionists, based on good feeding practice.

Valine is usually the fifth limiting amino acid after tryptophan for pigs and the fourth one after threonine for broilers. As lysine, threonine and tryptophan, valine is an indispensable amino acid for body protein deposition, growth, and maintaining animal health. Thus a dietary deficiency in valine affects the utilization of previous dietary limiting amino acids and consequently animal growth and health status.

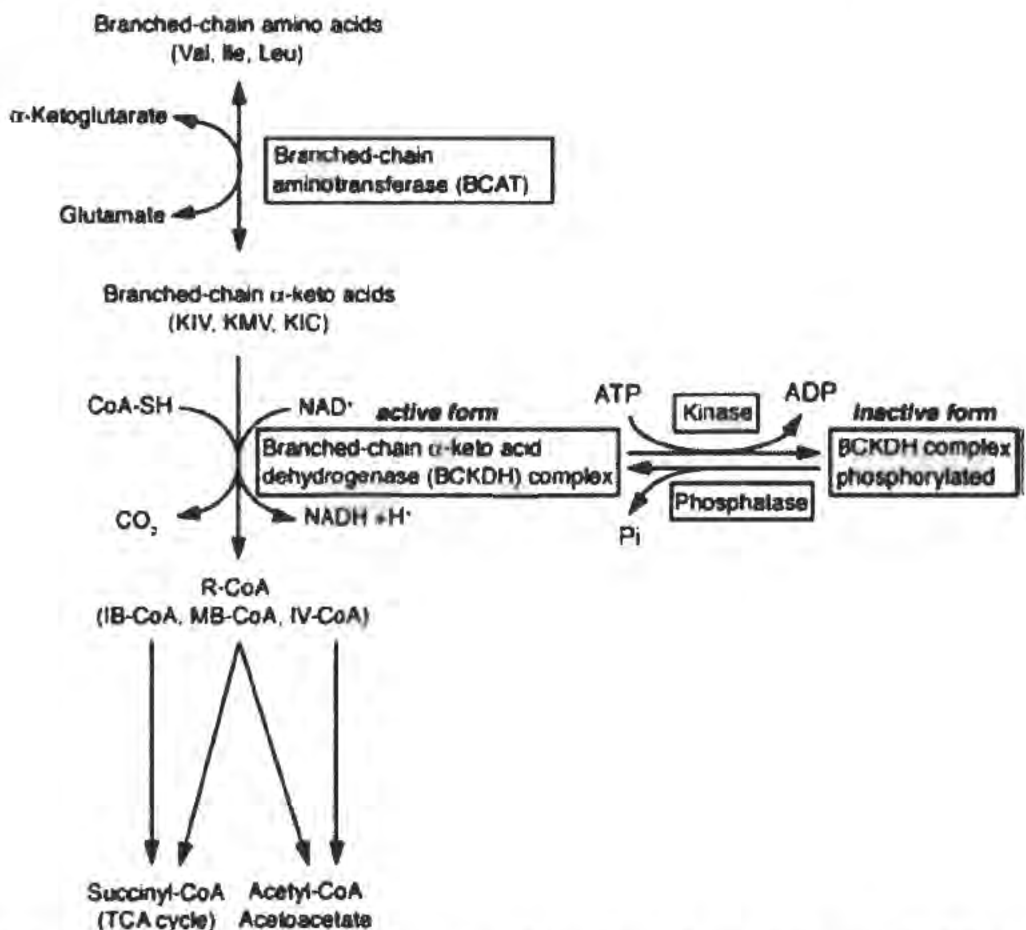


Fig. 2-1. The BCAA catabolic pathway (Brosnan, John T. et al. 2006. Journal of Nutrition 136:207S-211S, 2006)

Valine is included in the branched-chain amino acid (BCAA) group, together with isoleucine and leucine. Due to their common metabolic pathway, some nutritional interactions/antagonisms exist between them. That is why it is very important to meet their individual dietary requirements to ensure that they are neither under- nor over supplied in animal feeds.

Dried L-Valine Fermentation Product can be added directly to the feeding stuffs/complementary feeding stuffs or via premixture. No inclusion levels are proposed as the requirements in quantitative terms depend on the species, the physiological state of the animal, the performance level and the environmental conditions, as well as the amino acid composition of the non-supplemented diet. The formulator of the feed will determine the required level of amino acid supplementation.

Dried L-Valine Fermentation Product is the subject of this GRAS notice application. The active substance is L-valine. Any component of Dried L-Valine Fermentation Product doesn't differ significantly from the constituents of the ordinary diet of the target animal.

The biomass portion of Dried L-Valine Fermentation Product is dried, inactivated *Corynebacterium glutamicum*, which is the same biomass used in the Dried L-Lysine Fermentation product (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.16, 387-388). According to the AAFCO Official Publication (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.16, 387-388), Dried L-Lysine Fermentation product (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.16, 387-388) may be effectively used as an alternative to L-lysine monohydrochloride (L-lysine without biomass product) as a supplemental lysine source in swine diets. The biomass has been demonstrated to not interfere with the lysine availability. This most recently has been confirmed in a publication comparing the bioavailability of L-lysine and Lysine Sulfate (Lysine Fermentation Product) in young swine (Htoo, J. K. et al. 2016. J. Anim. Sci. 2016.94253–256).

Recently, Oliveria Maryane S. F. et al. (Oliveira, Maryane S. F. et al. 2019. Journal of Animal Science, 2019;97(10):4227) conducted a series of experiments with a spray-dried L-Valine fermentation product with biomass from *Corynebacterium glutamicum*. This experimental valine supplement contained 64.4% L-valine. The authors reported that the relative bioavailability by growth assay (ADG, ADFI and FCR) and blood urea nitrogen of the Dried L-Valine Fermentation Product with biomass from *Corynebacterium glutamicum* was 100% as compared to commercial L-Valine (98%) in weanling pigs. Therefore, there is no expectation that the biomass in the Dried L-Valine Fermentation Product with a minimum 72% L-valine, we propose would have a decreased bioavailability of valine.

Additionally, recently Wensley and co-workers (Wensley,. et al. 2019. Trans. Anim. Sci. 2019 doi.org/10.1093/tas/txz163) reported published studies demonstrating the bioavailability on efficacy of three amino acids: Threonine (>75%), Valine (>70%) and Tryptophan (>60%) fed to either broiler chicks or weanling pigs with their respective dried fermentative biomasses produced by CJ. Using growth parameters (ADG and FCR) similar to the approach employed by Oliveira, et al. (2019), it was concluded that the respective amino acids (Thr, Val or Trp) when formulated on an equal amino acid basis were bioequivalent to commercially available forms of the amino acids. Dried L-Valine Fermentation Product the subject of this dossier was the one of the amino acids used in the Wensley, et al. (2019) report. Previous and recently published data clearly demonstrate that there is no

expectation that the biomass will negatively impact the bioavailability of valine from the Dried L-Valine Fermentation Product.

The 28-day broiler utility trial as reported in Wensley et al. (2019) was conducted by Texas A&M University to compare Dried L-Valine Fermentation Product to synthetic valine The trial utilized 2100 Cobb 500 male chicks averaging *39.4* grams. Chicks were blocked on weight and assigned to one of 60 pens (33 chicks/pen). Pens were randomly assigned to one of four dietary treatments. Dietary treatments were: a Positive Control (synthetic AA); a Negative Control (same as Positive Control without synthetic valine); a Negative Control with Dried L-Valine Fermentation Product added at 100% of Positive Control valine level; and a Negative Control with Dried L-Valine Fermentation Product added at 150% of Positive Control valine level. Pen weights and feed disappearance were recorded at day 14 (Starter Phase) and day 28 (Grower Phase). All feed was removed at day 14 and replaced with Grower Phase diets. Growth is a suitable measurement when determining the availability of an essential amino acid, when comparing to a negative control feed.

Table 2-6: Bioavailability Results of Dried L-Valine Fermentation Product Compared to Positive and Negative Control diets as Demonstrated by Growth (Wensley, et al., 2019)

Criteria	Positive Control (PC)	Negative Control (NC)	NC with Dried L-Valine Fermentation Product 100%	NC with Dried L-Valine Fermentation Product 150%	SEM	P-Value
Body Weights, kg						
Day 0	39.4	39.4	39.5	39.3	0.03	0.764
Day 28	1.665[a]	1.551[b]	1.684[a]	1.662[a]	0.0088	<0.001
Feed Intake, g/bird/day						
Day 0 – 28	81.4[a]	78.0[b]	82.4[a]	81.1[a]	0.39	<0.001
Average Daily Gain						
Day 0-28	58.1[a]	54.0[b]	58.7[a]	58.00[a]	0.34	<0.001
Gain to Feed Ratio						
Day 0-28	0.792[a]	0.711[b]	0.730[a]	0.728[a]	0.0031	<0.001

[a-b] Means within columns with non-similar superscripts differ significantly at P≤0.0001.

Broiler performance was negatively impacted with the reduction of valine level in the diet as body weight and feed intake were reduced and feed conversion ratio was increased in the NC fed broilers as compared to the PC fed broilers. Increasing the digestible valine level with Dried L-Valine Fermentation Product in the NC diet to equal levels of the PC diet, increased body weight and feed intake and reduced feed conversion ratio compared to the NC diet to levels similar to the PC fed broilers. Feed conversion ratio during the starter phase in the broilers fed the Dried L-Valine Fermentation Product at the equivalent level of the PC diet actually had an observed improved lower feed conversion ratio compared to the PC which may be associated with the additional nutrients

contributed with the biomass. Increasing the amount of Dried L-Valine Fermentation Product to 150% the level of L-valine in the PC diet did not have any negative impact on broiler performance.

This study published peer-review study (Wensley, R. et al. 2019)) demonstrates the L-valine bioavailability from Dried L-Valine Fermentation Product in animal feed. It also confirms, as previously demonstrated with the Lysine, Threonine and Tryptophan Fermentation Products, the *Corynebacterium glutamicum* biomass does not impact bioavailability of the amino acid.

3. Part 3 GRAS Notice: Target Animal and Human Exposures

3.1 Target Animal Exposure

L-valine is an essential amino acid in all animal species (FEEDAP. 2014. EFSA Journal 2014;12(7):3795), including livestock and poultry (NRC. 1994. National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition and NRC. 2012. National Research Council. 2012. Nutrient Requirements of Swine). The level of supplementation varies between species and is dependent on the nutritional content of the diet (specifically the amino acids content). Therefore, the use of supplementation will be determined on a case-by-case basis by animal nutritionists, based on good feeding practice.

Based on the overall level of supplementation in the most fortified diets, (for example broilers, egg layers and swine), the maximum level of use for valine would, in normal feeding practices, be approximately from 0.01 % to 0.30 % of the layers feed and approximately 0.01 - 0.40 % of the broilers feed (NRC. 1994. National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition). In swine feeds L-valine supplementation levels range from 0.01 % to 0.15 % depending on production phase and feed ingredients used in the diet (NRC. 2012. National Research Council. 2012. Nutrient Requirements of Swine: Eleventh Revised Edition). Other species would be similar.

Therefore, although the level of use of Dried L-Valine Fermentation Product in the formulated feed will be based on the valine content naturally occurring in the feed, a maximum would be considered 0.5 % of the feed.

The impurities of Dried L-Valine Fermentation Product are all either essential nutrients or typical components of feed (amino acids, minerals and organic acids) and are consistent with normal components of feed, as such would not be a source of residues beyond that found in animal food products from traditionally fed animals.

3.2 Human Food Exposure

The valine requirement is nutritionally important in the human, since it used for muscle growth, tissue repair and energy source. Valine is an essential amino acid, hence it must be ingested, as a component of proteins usually obtained from soy, cheese, fish, meats and vegetables.

Dried L-Valine Fermentation Product is intended for use in animal feed only as a nutritional source of the essential amino acid valine. Therefore, dietary intake of valine by animal is significantly below the amount which causes physiological imbalance and adverse effects. The other components of the ingredient are nutritional and available for uptake, metabolism and growth. Therefore, the

composition of the milk, meat, and eggs from animals fed Dried L-Valine Fermentation Product, should be no different than from animals fed a nutritionally complete diet. There is no expectation of a residue from the feeding of Dried L-Valine Fermentation Product.

Also, in general, amino acids cannot be stored by the organism. Free amino acids, whether ingested in form of additives or released after the digestion of proteins by proteolytic enzymes, are absorbed through the intestinal mucosa to enter the blood. After absorption, alpha amino acids are directly used in protein synthesis or rapidly metabolized into intermediates in the citric cycle as evidenced by the presence of only trace amounts of alpha amino acids in the plasma.

Thus it can be concluded that there will be no additional exposure to L-valine above the natural basal content for the consumer raised by digested meat produced from animals fed with compound feed supplemented by Dried L-Valine Fermentation Product

4. Part 4 GRAS Notice: Self-Limiting Levels of Use

There is no self-limiting use information specific to this substance.

5. Part 5 GRAS Notice: Experience Based on Common Use in Food Before 1958

The GRAS determination is not based on common use in animal feed prior to 1958.

6. Part 6 GRAS Notice: Narrative

6.1 Safety of Corynebacterium glutamicum – Production Organism

Corynebacterium glutamicum is a gram positive bacteria belonging to the family of *Corynebacteriaceae*. These bacterial strains are scientifically recognized as safe and provide no negative impact on human and the environment. Additionally, these strains have a long history of safe use in industrial production (Eggeling, Lothar et al. 2005. Handbook of *Corynebacterium glutamicum*. CRC Press). In addition, *Corynebacterium glutamicum* is a GRAS microorganism and has a "Qualified Presumption as Safe" (QPS) status (BIOHAZ. 2011. EFSA Journal 2011; 9(12):2497). A description and summary of the QPS review of *Corynebacterium glutamicum* is provided in Appendix 10, "Literature Review *Corynebacterium glutamicum*," Section 2.

Corynebacterium glutamicum is an authorized source for a number of feed ingredients. It is listed in the AAFCO OP (2018). It is the source organism for Condensed Extracted Glutamic Acid Fermentation Product (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.1, 384-385). It is also the source organism for Dried L-lysine Fermentation Product (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.16, 387-388) as well as Liquid L-lysine Fermentation Product (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.17, 388). As recent as 2014, the US Food and Drug Administration, Division of Animal Feeds (OS&C/FDA) had reviewed the safety assessment of this source organism for the use in animal feed. Based on that recent review, CJ was recommended to review the recent literature after 2003 to assure the assessment was complete. Appendix 10, Section 3 of this GRAS notice provides results of this extensive literature review. Overall, no studies were retrieved either in the electronic literature search (ELS) or follow-up selective searches that contained information indicating potential safety issues or hazards associated with *Corynebacterium glutamicum*. This is consistent with the previous safety assessment completed by the US FDA, Division of Animal Feeds.

6.2 Safety Considerations due to the Nature of Modification to Corynebacterium glutamicum

The production microorganism used to produce Dried L-Valine Fermentation Product is a genetically altered strain of *Corynebacterium glutamicum*. The full genetic modification process, safety assessment, and stability assessment is provided in Appendix 3, "Pre-Fermentation Information (CONFIDENTIAL)." The production strain is deposited in the Korean Culture Center of Microorganisms (KCCM). As shown in Appendix 3 of this notice, the assessment of the genetic engineering process demonstrates that there is no hazard imparted due to the engineering process. This data is summarized in the sections below.

6.2.1. Safety for humans and animals

Dried L-Valine Fermentation Product is intended for use as a nutrient for animal consumption. Ordinarily, a GRAS notice will address the potential human dietary consumption of a component of animal feed due to consumption of animal products and tissues in which the component may be present. In this case, however, there is no need to determine the estimated daily intake (EDI) of the Dried L-Valine Fermentation Product for human consumption. The Dried L-Valine Fermentation Product and any of the described biomass (see above) will be metabolized when the animal consumes and digests its food (like all feed). Dried L-Valine Fermentation Product derived from the genetically modified *Corynebacterium glutamicum* will be indistinguishable from other sources, as will be the potential impurities, which are all normal components of animal feed.

1) Information on any toxic, allergenic or other harmful effects on human or animal health

The genetic modifications made, resulting in strain *Corynebacterium glutamicum* KCCM80058, exclusively correspond to the over-expression of existing metabolic enzymes or the elimination of several enzymes. The initial parental strain *Corynebacterium glutamicum* is about the most used bacterium industrially. It has been used for the manufacturing of feed additives for many years and is generally accepted as safe. We conducted ORF analyses of the deleted genes and the homologous sequences which also include the junction sequences. The assessment for the presence of open reading frames not associated with intended genetic changes and potential for spill-over effects were assessed and found not to provide any safety concern (Appendix 3).

In addition, the fermentation of the wild type strain and production strain were carried out under the same conditions as actual production. It is considered that other components have no safety concerns for humans and animals as the amount of other components is extremely small. In addition, the analysis of organic acids shows the same pattern between wild type strains and production strains, and it is considered that organic acids have no safety concerns for humans and animals as the amount of organic acids production is extremely small (Appendix 3).

2) Potential for DNA transfer or any capacity for enhanced gene transfer

To limit any potential transfer of genetic material to other organisms, the strategy of construction for *Corynebacterium glutamicum* KCCM80058 strain was based on procedures described below.

2-1) Any genetic material including plasmid to be autonomously replicable was not used.
2-2) All the genetic modifications were done on the chromosome.

3) The resistance of antibiotics of Production strain

This study is to determine the antibiotic minimum inhibitory concentration (MIC) for the Dried L-Valine Fermentation Product production strain. The broth tube dilution method was used to determine the susceptibility of the production strain *Corynebacterium glutamicum* KCCM80058. In regards to antibiotic resistance, to the knowledge of CJ, *Corynebacterium glutamicum* wild-type strains have not been reported to have any antibiotic resistance. This was confirmed by the test report,

"Determination of antibiotic minimum inhibitory concentration (MIC) of *Corynebacterium glutamicum* KCCM80058" provided in Appendix 3, Attachment 4. *Corynebacterium glutamicum* KCCM80058 showed the same antibiotic MIC as the *Corynebacterium glutamicum* wild-type. These results indicated that there are no possible antibiotic resistance genes in the chromosome of the *Corynebacterium glutamicum* KCCM80058. The full test report is included in Appendix 3, Attachment 4.

4) The absence of viable cell in final product

CJ conducted the test of the residually viable production strain in the final product. In this test, all conditions used for testing were in accordance with the European Food Safety Authority guidance (FEEDAP et al. 2018. EFSA Journal 2018;16(3):5206). A complex agar plate was used for viable cell counting. The complex medium is consisted of glucose, beef extract, polypeptone, yeast extract, NaCl, urea and agar. Glucose is a primary source of energy for living organisms. Beef extract and yeast extract are used as a nutrient source in various culture media.

All experiments were conducted in accordance to sterile technique. All media and equipment used for testing were sterile and all experiments were conducted in a clean bench.

For the detection of viable cells in the final product, Dried L-Valine Fermentation Product samples were suspended in sterile saline solution, spread onto plates, and colonies were counted. Spread plates allowed for the even spreading of bacteria onto a petri dish and the isolation of individual colonies, for counting. A bacterial colony is defined as a visible cluster of bacteria growing on the surface of or within a solid medium, presumably cultured from a single cell.

6.3 Safety Considerations for L-Valine

Dried L-Valine Fermentation Product is a source of nutritional valine that can be safely used in the production of proteins like all other sources of valine.

Valine is codified as a Generally Recognized as Safe amino acid for the use in animal feed (21CFR§582.5925). Valine is an essential amino acid, as discussed in Section 2 of this notice and is formulated in diets that are deficient in naturally occurring valine.

The European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has recently reviewed the safety and efficacy of L-valine when used in animal diets (FEEDAP. 2014. EFSA Journal 2014;12(7):3795). According to this report, L-valine additives in animal feed is incorporated into proteins of tissues and/or products of target animal species, and L-valine that exceeds the valine requirement of the animal is excreted as urea/uric acid and carbon dioxide. Consequently, no free L-valine occurs or accumulates in target animal tissues. L-Valine is an essential amino acid for humans. Free valine is not a residue issue. Therefore, Dried L-Valine Fermentation Product presents no exposure risk to humans consuming tissues or products from the target animal.

6.4 Safety Considers of Dried L-Valine Fermentation Product

As seen in Table 2-1 in this dossier and in Appendix 1, "Analytical Report; Qualitative and quantitative composition of Dried L-Valine Fermentation Product (CONFIDENTIAL)", there are no substances in the product that are not typical components of animal feed. And as seen in Table 2-5 in this dossier and in Appendix 2, "COA of Heavy metals with Raw data", there is no concern about safety due to heavy metals in the animal and human.

To corroborate the safety assessment, CJ conducted an acute toxicity study in rats as seen in Appendix 7, "Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)". The starting dose was set at 300 mg/kg, and sighting study was conducted in which 1 female rat per group was sequentially administered at a dose level of 300 and 2000 mg/kg according to the fixed dose procedure. After a sighting study, a main study was conducted in which 4 female rats were administered at a dose level of 2000 mg/kg.

Mortalities, clinical signs, and body weight changes were monitored for 15 days, and then all animals were sacrificed and necropsy findings were observed. The results were summarized as follows:

1. There were no mortalities.
2. There were no test article-related clinical signs.
3. There were no test article-related body weights changes.
4. No macroscopic abnormalities were observed at necropsy.

Based on the above results, when Dried L-Valine Fermentation Product was dosed to Sprague-Dawley rats by acute oral fixed dose procedure, the acute oral median lethal dose (LD_{50}) was estimated to be 2000 mg/kg ~ 5000 mg/kg body weight according to GHS category which is classification '5/unclassified'.

In the Bacterial Reverse Mutation Assay (OECD 471) that was performed on Dried L-Valine Fermentation Product, Dried L-Valine Fermentation Product was found to be non–mutagenic. The assay results can be found in Appendix 8, "Bacterial Reverse Mutation Assay with Granule Valine (Dried L-Valine Fermentation Product)". These studies corroborate the safety assessment.

6.5 Safety Assessment of Known Impurities and/or Potential Contaminants

Based on the known composition of the product, there are no known impurities or contaminants introduced in the manufacture of the product that could raise safety concerns. The product is 72 % Dried L-Valine and the specifications permit for 5 % water and 10 % inorganic compounds. The use levels of valine in the diet are small enough that these impurities cannot be considered nutritional source of minerals or free amino acids as there are found at ppm levels (Table 6-1). Section 3 of this notice suggests the maximum level of use in the diet as 0.5 % of feed.

Table 6-1: Feed Levels of Dried L-Valine Fermentation Product -Impurities

Substance	Average level in Dried L-Valine Fermentation Product, %	Feed Level when Dried L-Valine Fermentation Product incorporated at 0.5 %, ppm in the diet
Ammonium	2.21	(b) (4)
Sodium	0.37	
Potassium	0.95	

Magnesium	0.06		(b) (4)
Calcium	0.04		
Chloride	0.17		
Phosphate	0.14		
Sulfate	7.92		
Malic Acid	0.01		
Succinic Acid	0.04		
Lactic Acid	0.07		
Glucose	0.02		
Trehalose	0.04		
Lysine	0.01		
Glycine	0.06		
Alanine	0.18		
Threonine	0.03		
Isoleucine	0.08		
Leucine	0.05		
Phenylalanine	0.19		
Histidine	0.06		

The levels of impurities are consistent with conventional feedstuffs, and none of the levels in the complete feed would be a concern.

6.6 Safety Assessment for Human Consumption

Dried L-Valine Fermentation Product is intended for use as a nutrient for animal consumption. Ordinarily, a GRAS notice will address the potential human dietary consumption of a component of animal feed due to consumption of animal products and tissues in which the component may be present. In this case, however, there is no need to determine the estimated daily intake (EDI) of the Dried L-Valine Fermentation Product for human consumption. Valine (Dried L-Valine Fermentation Product) and any of the described impurities shown in Table 6-1 above will be metabolized when the animal consumes and digests animal feed containing Dried L-Valine Fermentation Product. Dried L-Valine Fermentation Product derived from the genetically modified *Corynebacterium glutamicum* will be indistinguishable from other valine sources, as will be the potential impurities, which are all normal components of animal feed. Non-valine components of Dried L-Valine Fermentation Product are all typical feed components, mostly nutrients and will not be a concern for residues.

This same determination was made by the FDA in their support of the AAFCO definition 36.16 Dried L-Lysine Fermentation Product (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.16, 387-388), 36.17 Liquid L-Lysine Fermentation product (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.17, 388) and 36.1 Condensed Extracted Glutamic Acid Fermentation Product (AAFCO. 2018. AAFCO 2018 Official Publication CHAPTER SIX, 36.1, 384-385).

In this regard, the European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has recently reviewed (FEEDAP. 2014. EFSA Journal 2014;12(7):3795) the safety and efficacy of L-valine produced by *Corynebacterium glutamicum* for use in the diets of all animal species. According to this report, L-valine additives in animal feed will be incorporated into proteins of tissues and/or products of target animal species. Also, doses exceeding the L-valine requirement of the animal will be excreted as urea/uric acid and carbon dioxide. Consequently, no free L-valine occurs or accumulates in target animal tissues and the only form of valine that humans will be exposed to from its use in animal feed is in the form of protein that will be digested, absorbed, and metabolized consistent with human nutrient needs. The absence of residual L-valine in the tissues of animals consuming L-valine in its diet will, therefore, not result in a subsequent human exposure or safety issue. As indicated by the analytical values displayed in Table 2-1, Appendix 1, and Table 3-1, residual components of Dried L-Valine Fermentation Product' are at levels too low to present any risk of humans consuming the tissues of food animals fed the nutrient. All residual constituents are common metabolites or minerals and will be either excreted or metabolized. Therefore, they present no exposure risk to humans consuming tissues or products from the target animal. A review of the publicly available literature does not reveal information demonstrating that any of these residual constituents appears to present a risk of accumulation or harm to humans at the levels that would be consumed from animal tissue (Otten, Jennifer J. et al. 2006. Dietary Reference Intake, NAS/NAP). It should also be noted that L-valine is an essential amino acid for human nutrition is approved for direct addition to human food (21CFR§172.320).

In the Bacterial Reverse Mutation Assay (OECD 471), Dried L-Valine Fermentation Product was not mutagenic in this bacterial assay system (Appendix 8). The results indicate that the test article, Dried L-Valine Fermentation Product, was not mutagenic in this bacterial assay system.

The final product was assayed for potential valine derivatives (i.e., alpha-aminobutyric acid, alpha-hydroxyvaline, thiazole alanine, and norvaline) and these derivatives were not found above the limit of detection (Appendix 9).

Table 6-2. Analysis result of valine derivatives in final product

Batch No.	Test items	Result	Test method
GVAL180404	α-Aminobutyric acid	< 1.44 mg/L	LC-MS
	α-hydroxyvaline	< 2.58 mg/L	
	Thiazole alanine	< 1.88 mg/L	
	Norvaline	< 1.83 mg/L	
GVAL180405	α-Aminobutyric acid	< 1.44 mg/L	
	α-hydroxyvaline	< 2.58 mg/L	
	Thiazole alanine	< 1.88 mg/L	
	Norvaline	< 1.83 mg/L	
GVAL180406	α-Aminobutyric acid	< 1.44 mg/L	

	α-hydroxyvaline	< 2.58 mg/L	
	Thiazole alanine	< 1.88 mg/L	
	Norvaline	< 1.83 mg/L	

As such there is no hazard specific to these potential derivatives nor any other compounds as assessed by CJ in the full description of the GRAS substance (Table 2-1) and heavy metals (Table 2-5). Therefore, there is no concern for target animal safety and human food safety.

6.7 Safety Conclusion

Based on the documentation provided in this GRAS Notification and as discussed above, CJ has concluded that Dried L-Valine Fermentation Product produced by fermentation with *Corynebacterium glutamicum* is generally recognized as safe via scientific procedures as a nutrient for animal consumption. The notifier has reviewed the available data and information and is not aware of any data and information that is, or may appear to be, inconsistent with your conclusion of GRAS status.

7. Part 7 GRAS Notice: List of Supporting Data and Information

7.1 Confidential Information

The only information that is considered confidential in this GRAS Notice is the information specific to the production of the genetically modified organism, the manufacturing process, and the documentation of the assays specific for the composition of the marketed product. None of the information to support the safety narrative, Section 6 of this notice, is considered to be confidential. All this information is provided in a summary basis in the body of the submission, as required by 21 CFR§570 Subpart E. Therefore, the summary of the manufacturing process, with the full disclosure of the safety assessment, are consistent with the general recognition standards.

7.2 Supporting data information

All submitted data and reports were tested with samples produced on a pilot scale in CJ R&D center. The production process is the same for both the pilot scale and the commercial scale, ensuring that the identity of the final product is the same regardless of the scale.

7.3 Publically Available References

AAFCO, 2018, 36.1 Condensed, Extracted Glutamic Acid Fermentation Product, Page 384-385

AAFCO, 2018, 36.16 Dried L-Lysine Fermentation Product. Page 387-388

AAFCO, 2018, 36.17 Liquid L-Lysine Fermentation Product, Page 388

AOAC Official Method 2015.01 Heavy Metals in Food

EFSA Panel on Biological Hazards (BIOHAZ). 2011. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2011 update). EFSA Journal 2011;9(12):2497.

Brosnan, John T. and Margaret E. Brosnan. 2006. American Society for Nutrition. Branched-Chain Amino Acids: Enzyme and Substrate Regulation. Journal of Nutrition 136:207S-211S, 2006

Eggeling, Lothar and Michael Bott. 2005. (eds). Handbook of Corynebacterium glutamicum. CRC Press, Taylor & Francis Group, 6000 Broken Sound Parkway NW, Suite 3000, Boca Raton, FL.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guido Rychen, Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace, Pieter Wester, Boet Glandorf, Lieve Herman, Sirpa Kärenlampi, Jaime Aguilera, Montserrat Anguita, Rosella Brozzi and Jaume Galobart. 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206

FEEDAP. 2014. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific Opinion on the safety and efficacy of L-Valine(ValAMINO®) produced by Corynebacterium glutamicum(DSM 25202) for all animal species, based on a dossier submitted by Evonik Industries AG. EFSA Journal 2014;12(7):3795

Htoo, J. K., J. P. Oliveira, L. F. T. Albino, and M. I. Hannas, N. A. A. Barbosa, and H. S. Rostagno. 2016. Bioavailability of l-lysine HCl and l-lysine sulfate as lysine sources for growing pigs. J. Animal Science 94:253

NRC. 1994. Nutrient Requirements of Poultry, Ninth Revised Edition. National Research Council, National Academy Press, Washington, D.C. Pages 27-29.

NRC. 2012. Nutrient Requirements of Swine: Eleventh Revised Edition. Washington, DC: The National Academies Press. Pages 15-44.

OECD 471. Bacterial Reverse Mutation Test

Oliveira, Maryane S. F. de, John K. Htoo, J. Caroline González-Vega, and Hans H. Stein. 2019. Bioavailability of valine in spray-dried L-valine biomass is not different from that in crystalline L-valine when fed to weanling pigs. Journal of Animal Science. 97(10):4227

Otten, Jennifer J., Jennifer Pitzi Hellwig, Linda D. Meyers. 2006. Dietary Reference Intake: The Essential Guide to Nutrient Requirements. NAS/NAP

Wensley, Madie, R., Jason C. Woodward, Joel M. DeRouchey, Steve S. Dritz, Mike D. Tokach, Robert D. Goodband, Hunter G. Walters , Bryce A. Leopold , Craig D. Coufal , Keith D. Haydon, and Jason T. Lee. 2019. Effects of amino acid biomass or feed grade amino acids on growth performance of growing swine and poultry. Translational Animal Science, txz163, https://doi.org/10.1093/tas/txz163

Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
Woodbridge, VA 22192-575.5
703 590 7337 (Fax 703 580 8637)
Smedley@cfr-services.com

November 19, 2019

Dr. David Edwards
Director
Division of Animal Feeds (HFV- 220)
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Pl.
Rockville, MD 20855

Subject: Animal GRAS Notification
Dried L-Valine Fermentation Product
APPENDIX 10 REFERENCES

Notifier: C J CheilJedang Corporation (C J)
330, Dongho-Ro, Jung-Gu,
SEOUL,04560,KOREA

Dear Dr. Edwards:

On behalf of CJ CheilJedang Corporation (CJ), I am providing the reference material in support of Appendix 10 of the animal Generally Recognized as Safe Notice for the use of Dried L-Valine Fermentation Product (72%).

This file folder was inadvertently not copied to the CD that was provided for filing. We are requesting the file to support AGRN for Dried L-Valine Fermentation Product amended to include this information.

Should you have any questions on this request, please contact me directly.

Sincerely,

Kristi O. Smedley
Consultant to CJ CheilJedang Corporation

Cc: Keith Haydon, CJ

ATTACHMENT:

GRAS Notice L-Valine Fermentation Product –Appendix 10 Reference material—CD

ANALYTICAL REPORT

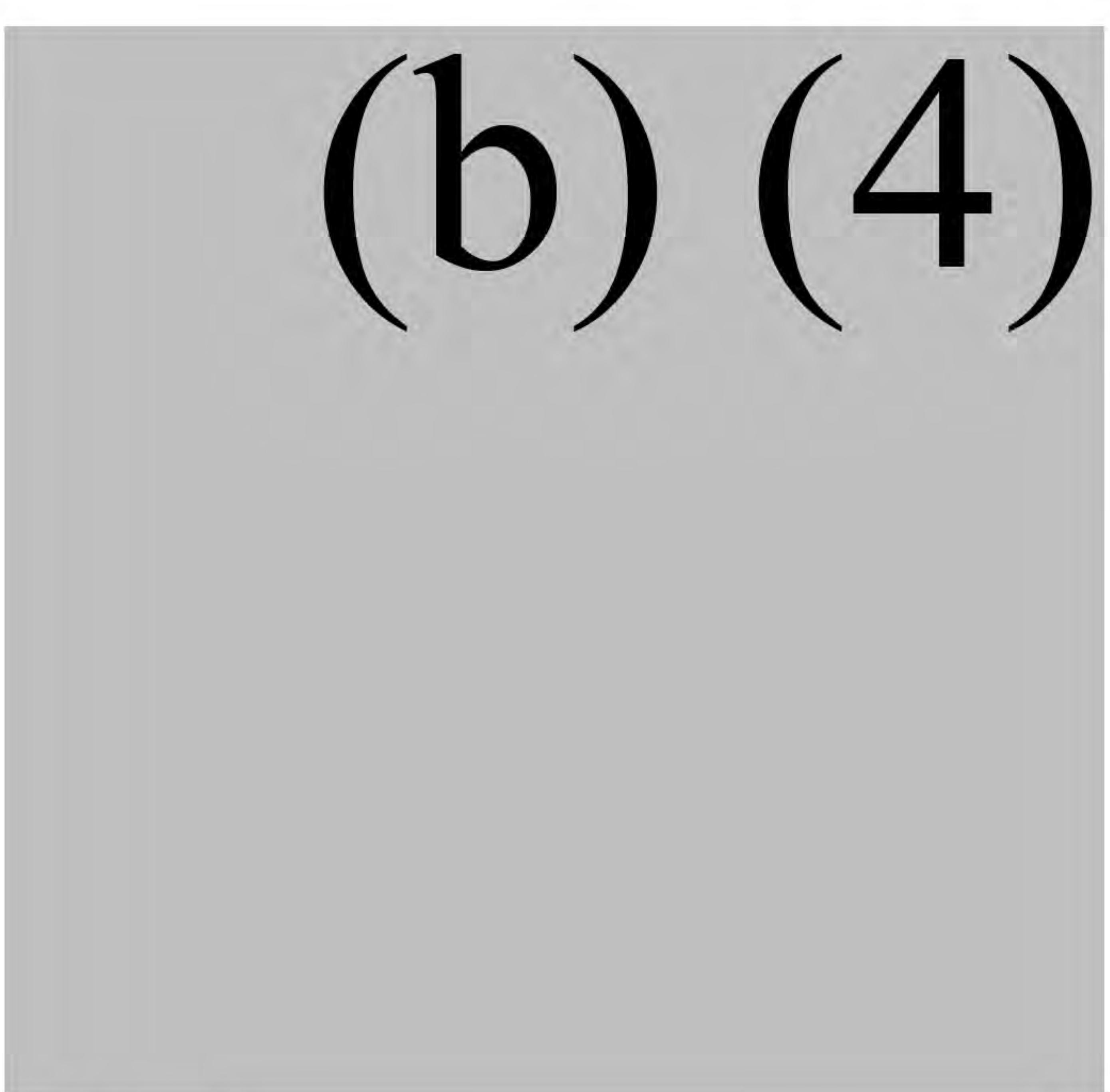
(CONFIDENTIAL)

Qualitative and quantitative composition of

Dried L-Valine Fermentation Product

(Document No.: BA18020)

CJ Research Institute of Biotechnology



11. Attachments 43

12. List of References 44

Dried L-Valine Fermentation Product

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Dried L-Valine Fermentation Product

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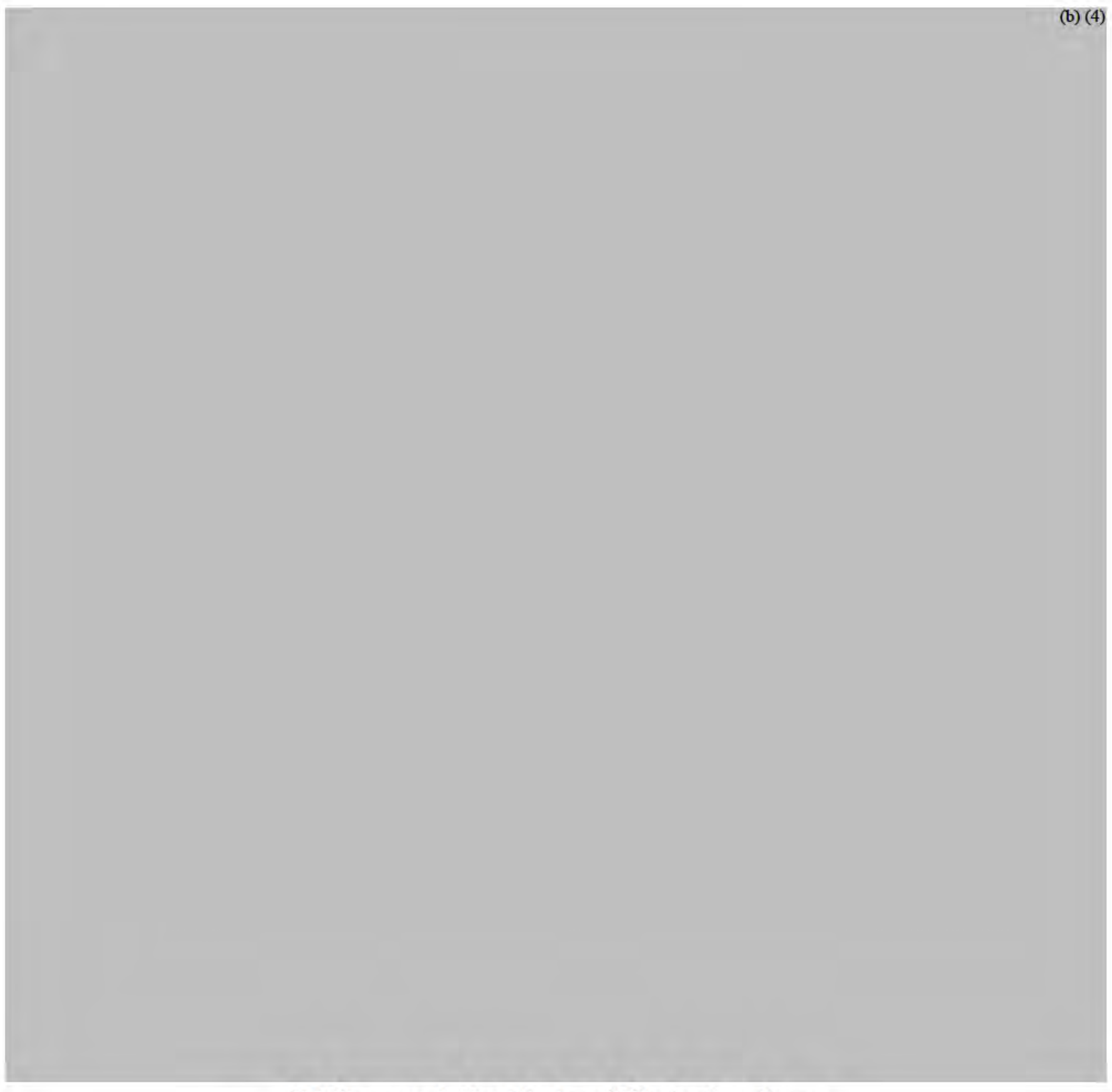
REPORT



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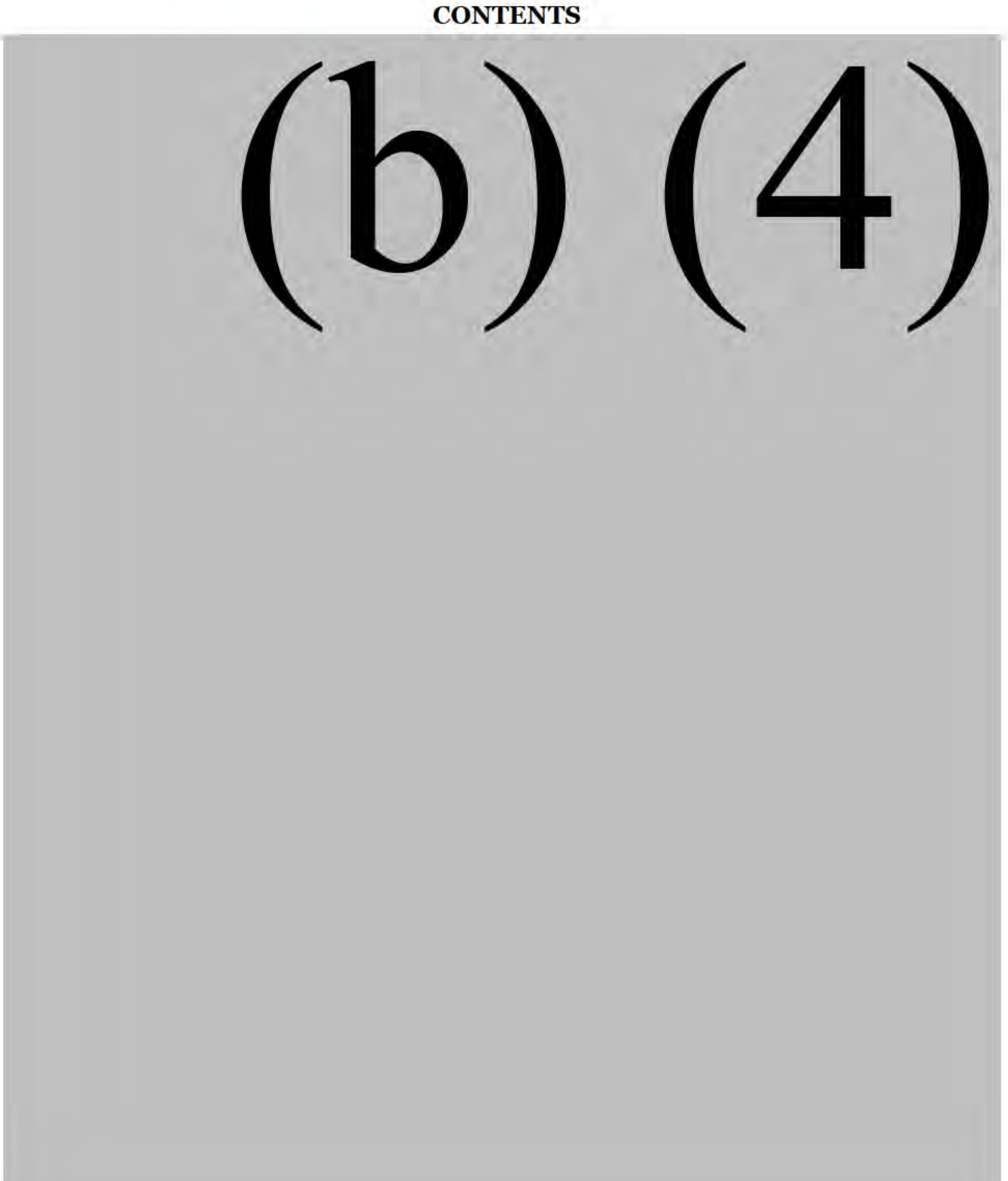

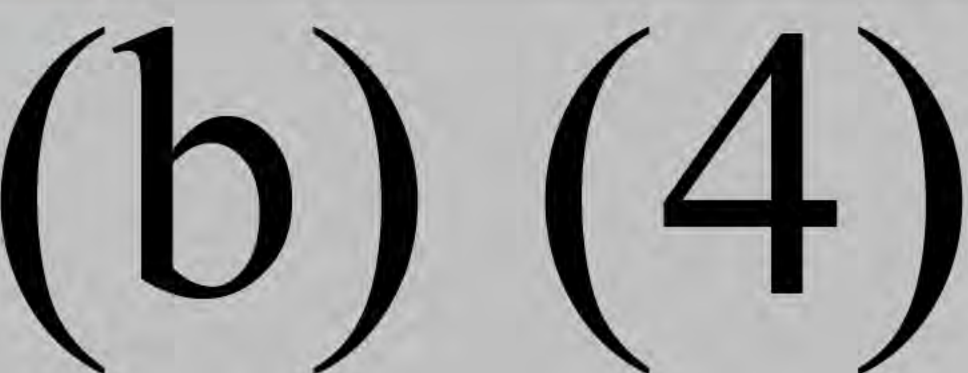
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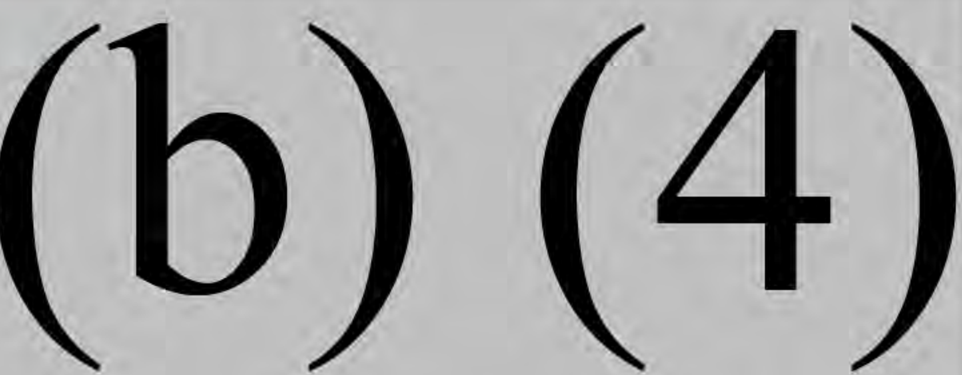
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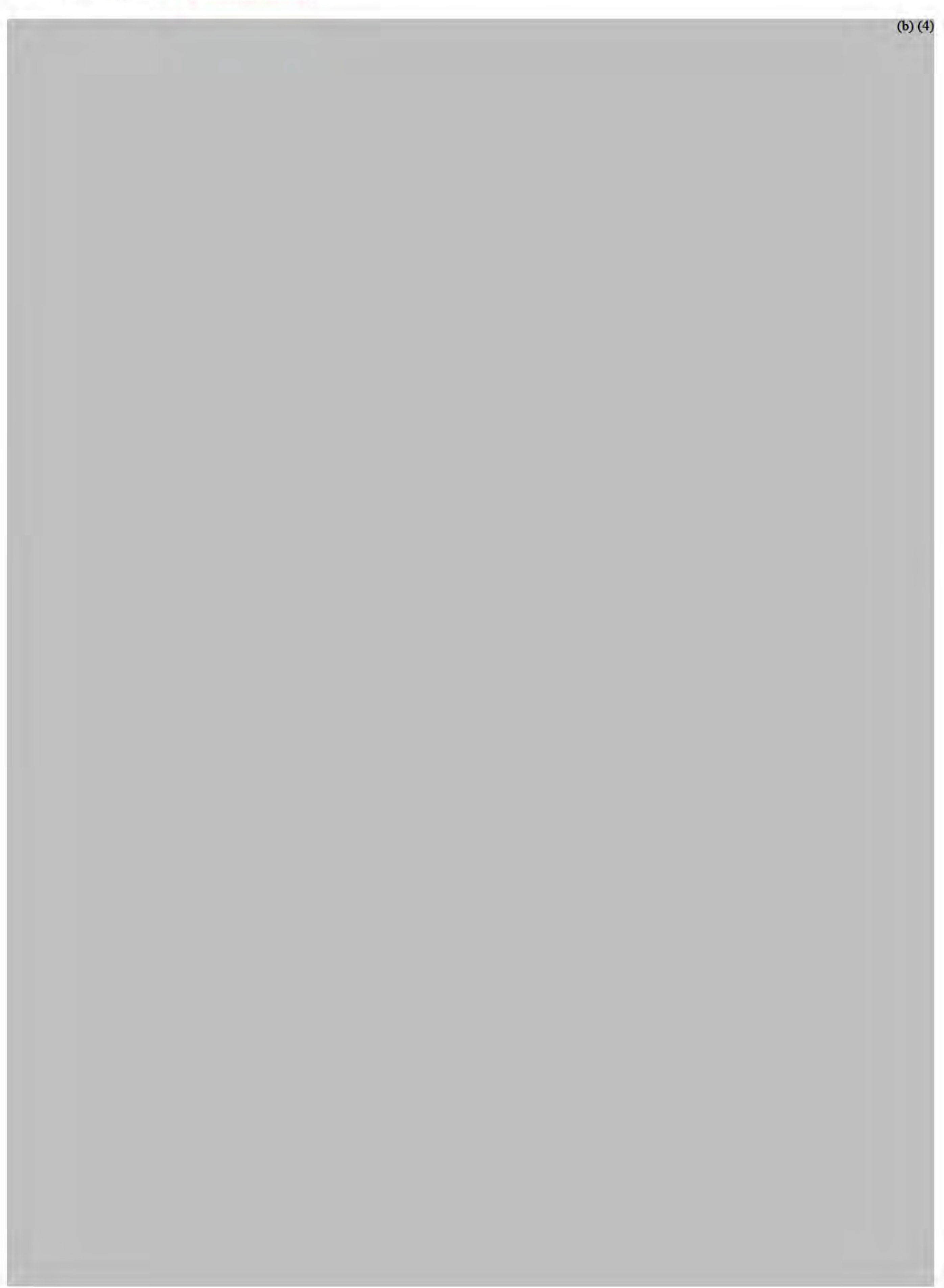
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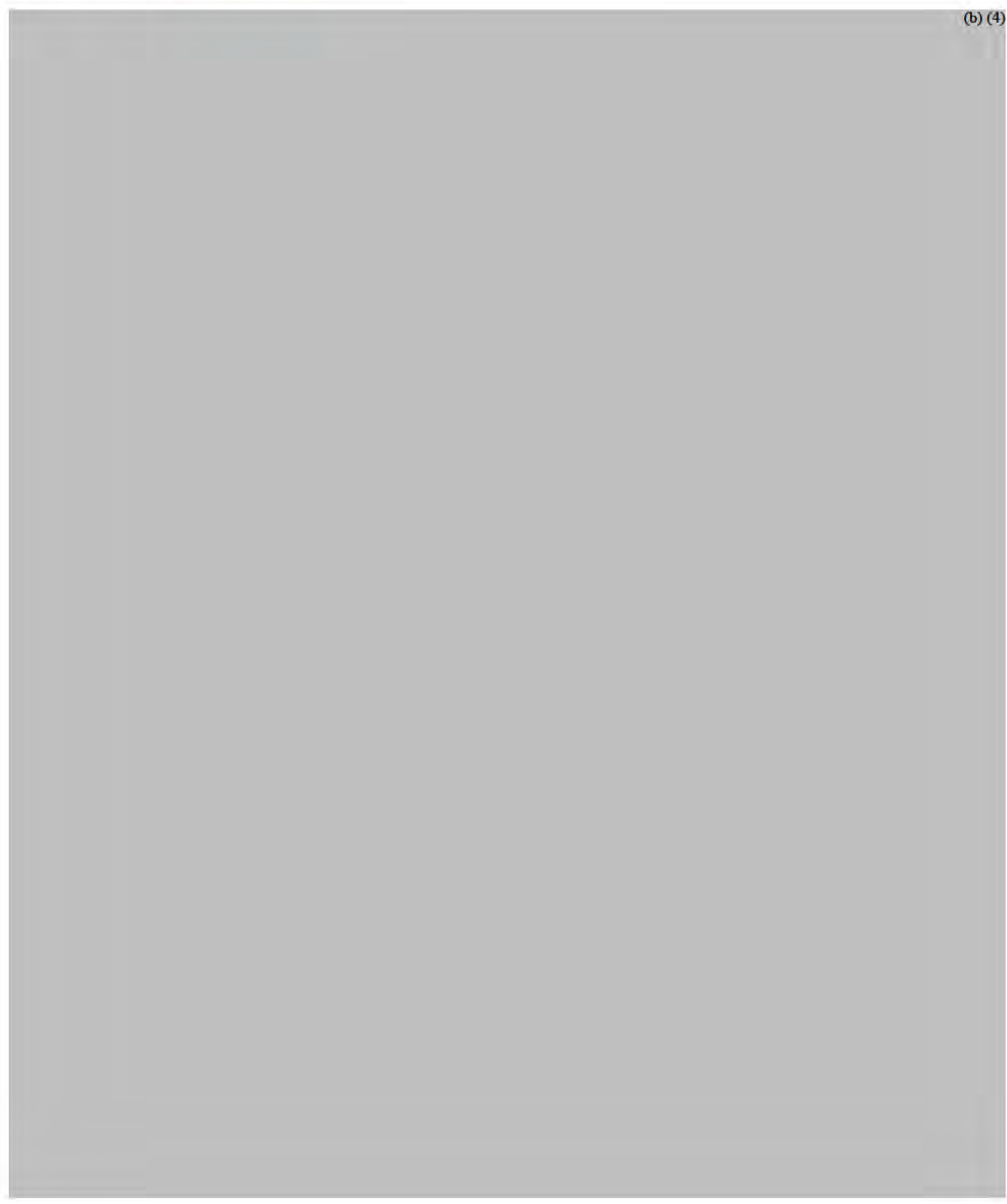
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REPORT

Analytical Method Validation of Dried L-Valine Fermentation Product using HPLC
(Confidential)

Original Final report date: Aug 28, 2018

CJ Research Institute of Biotechnology

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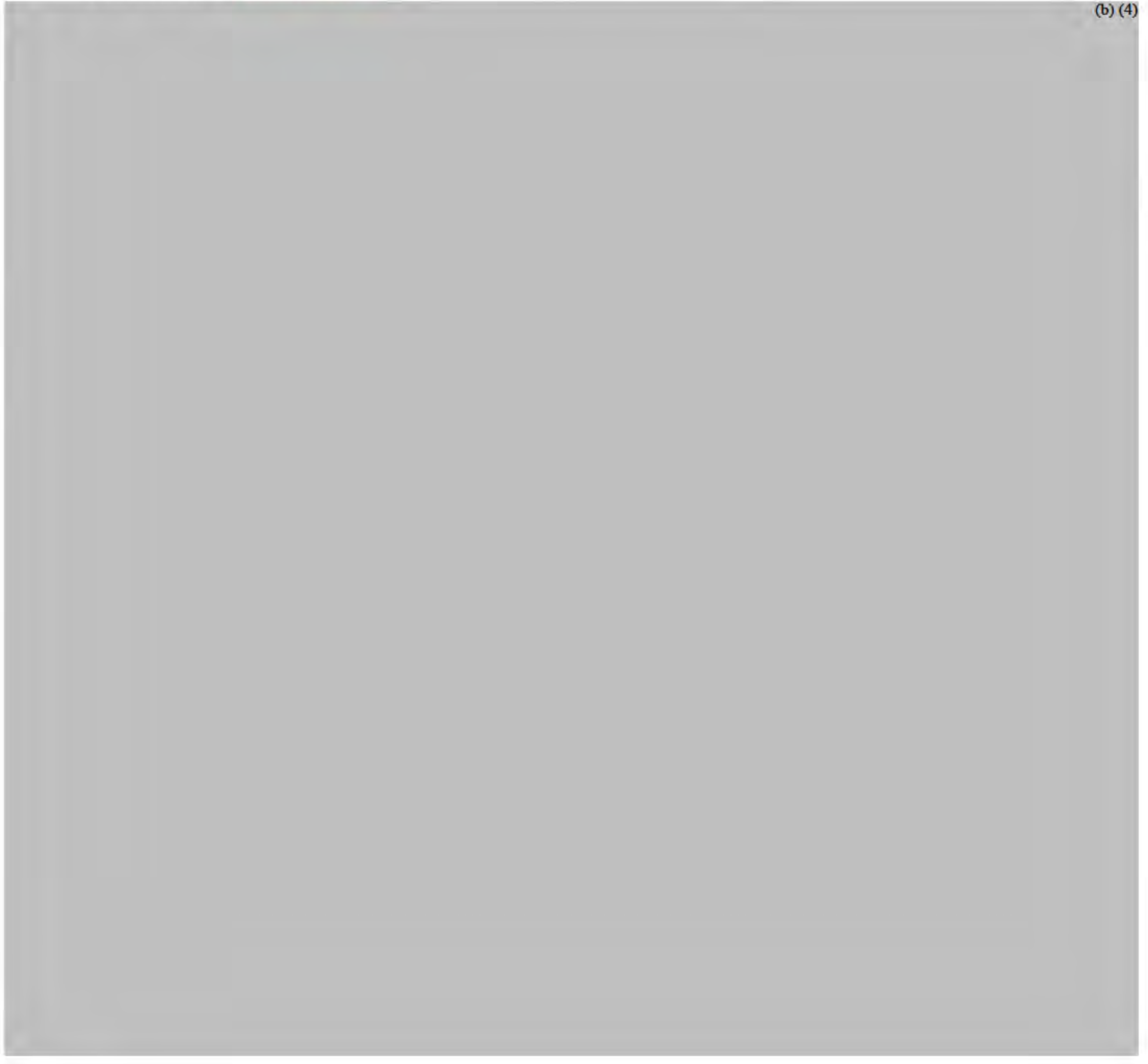
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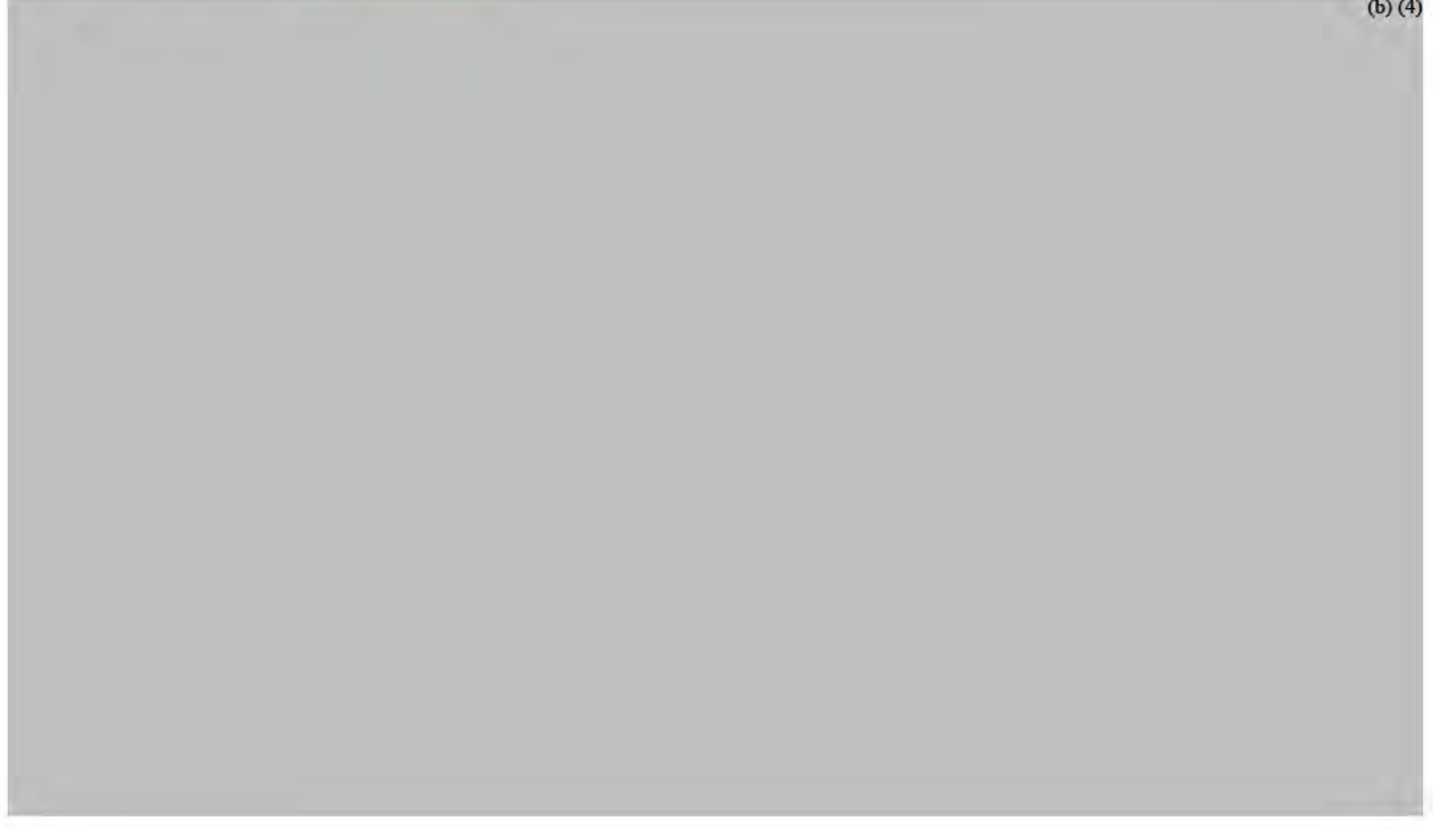
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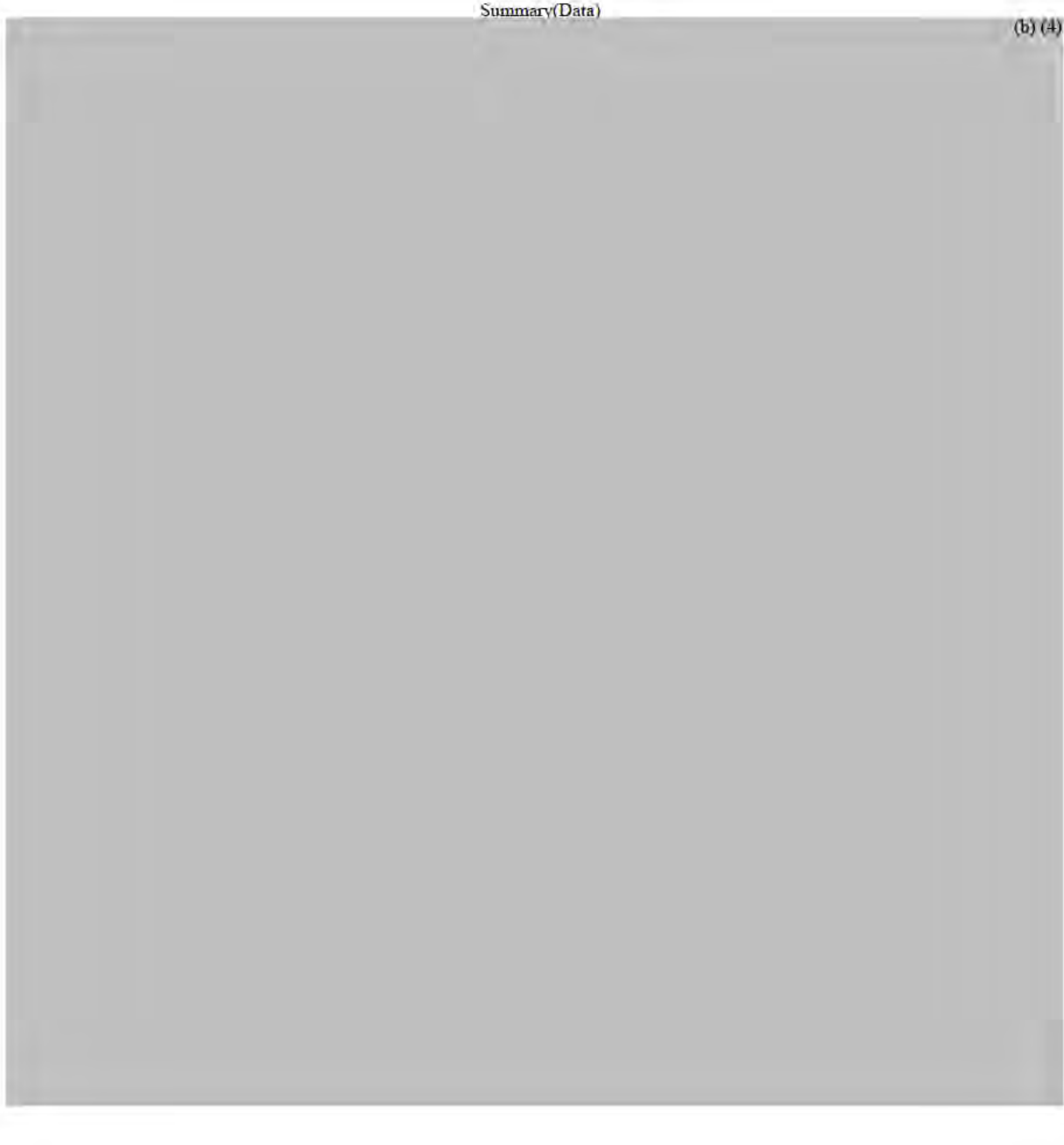

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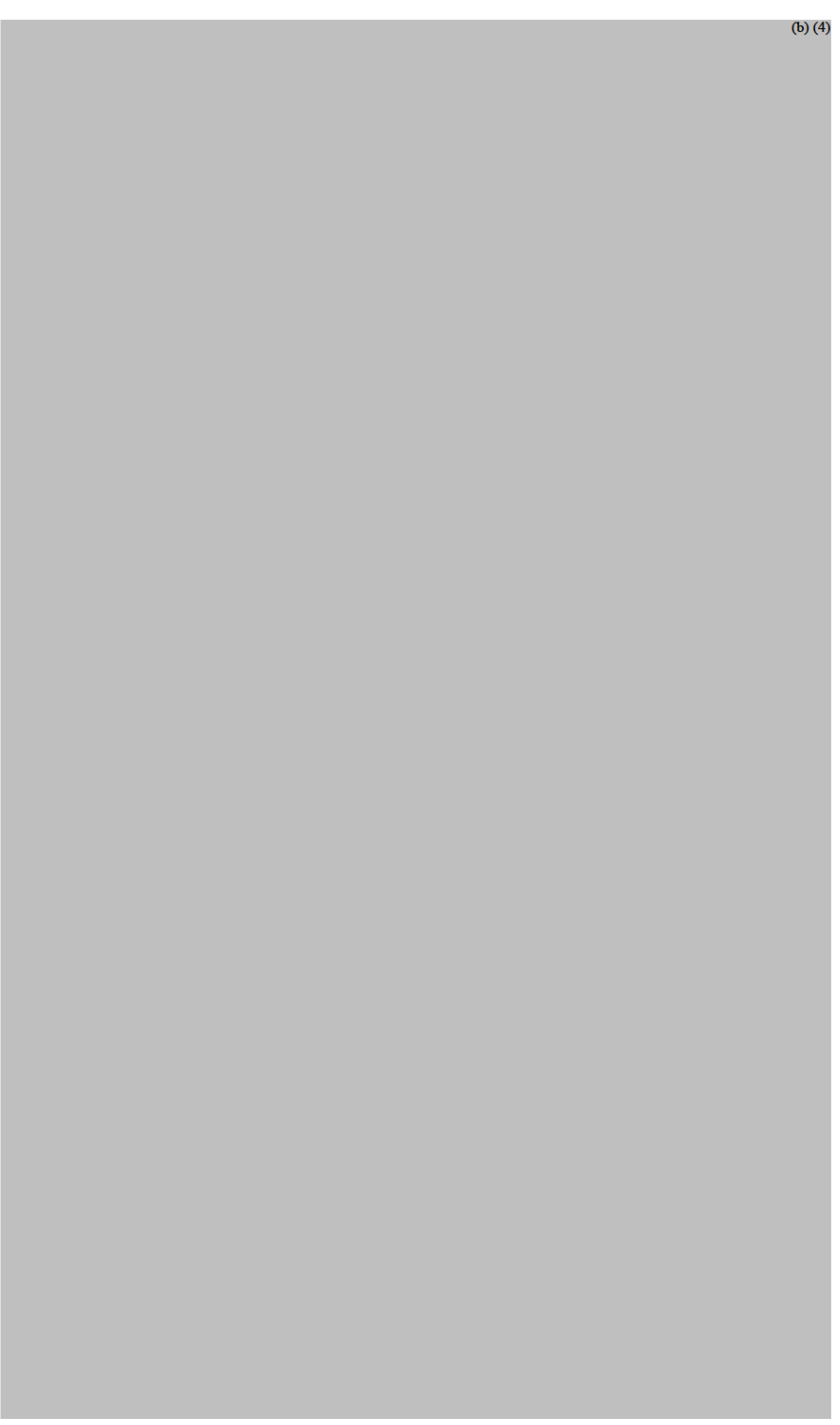
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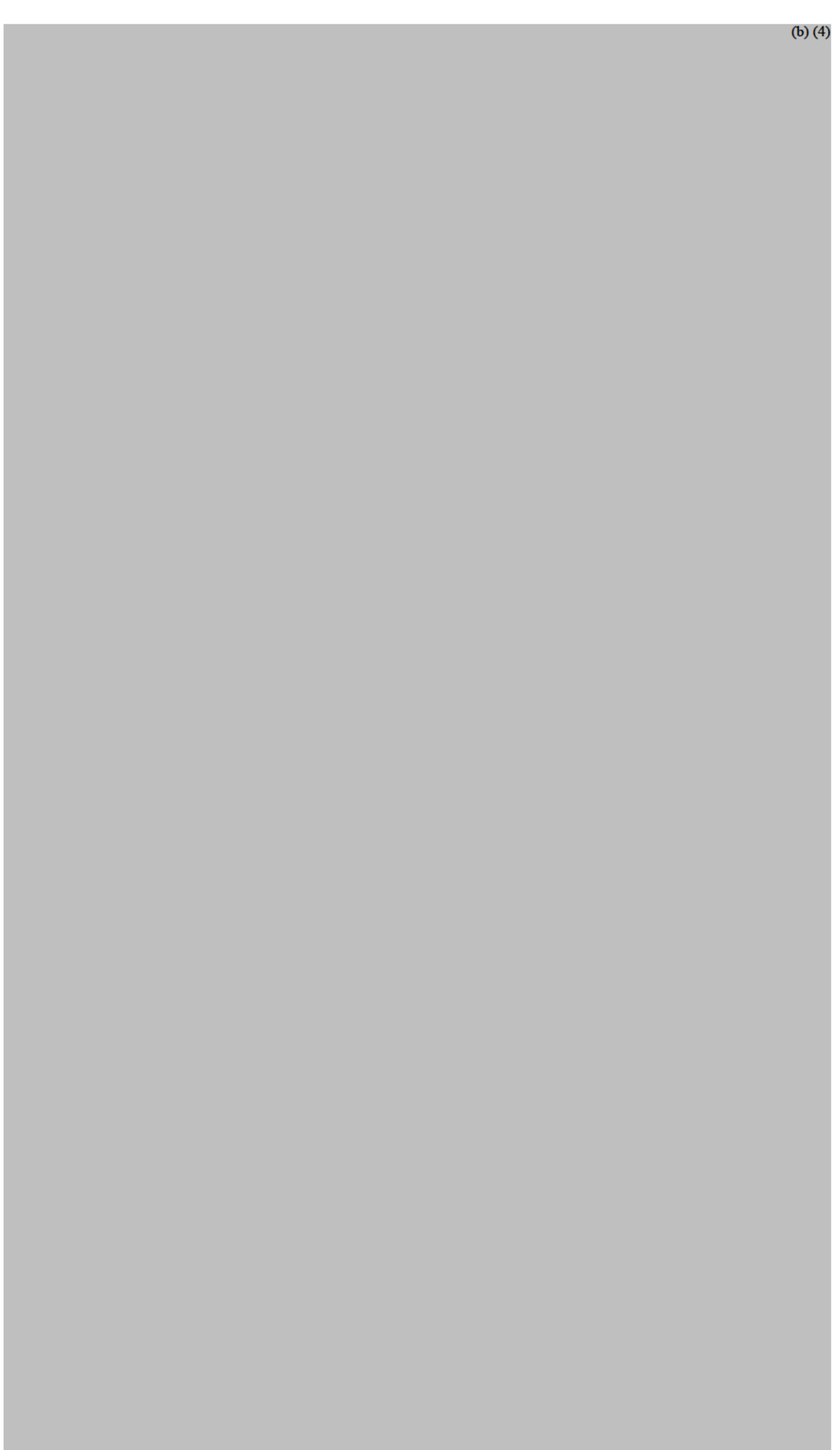
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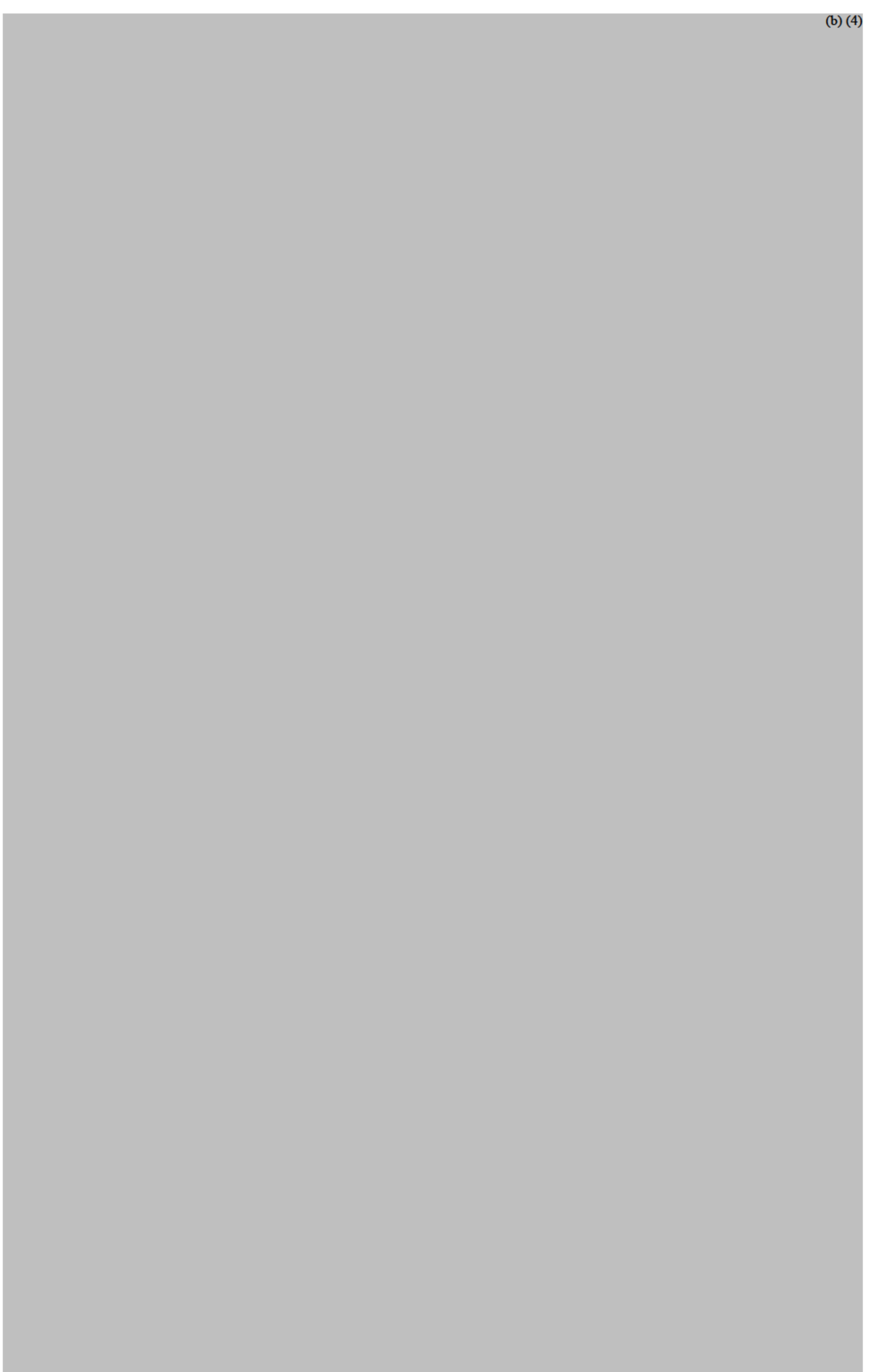
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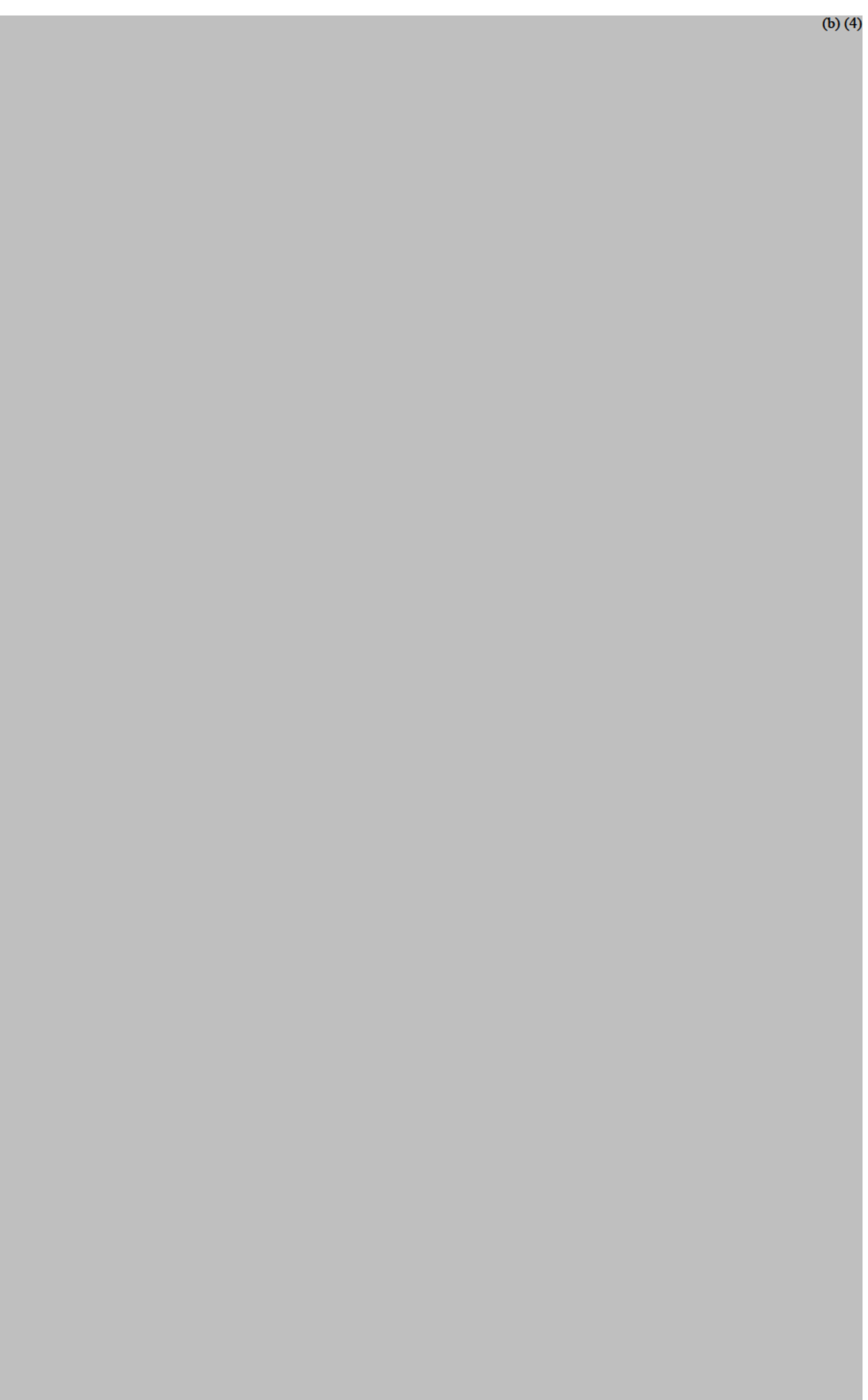
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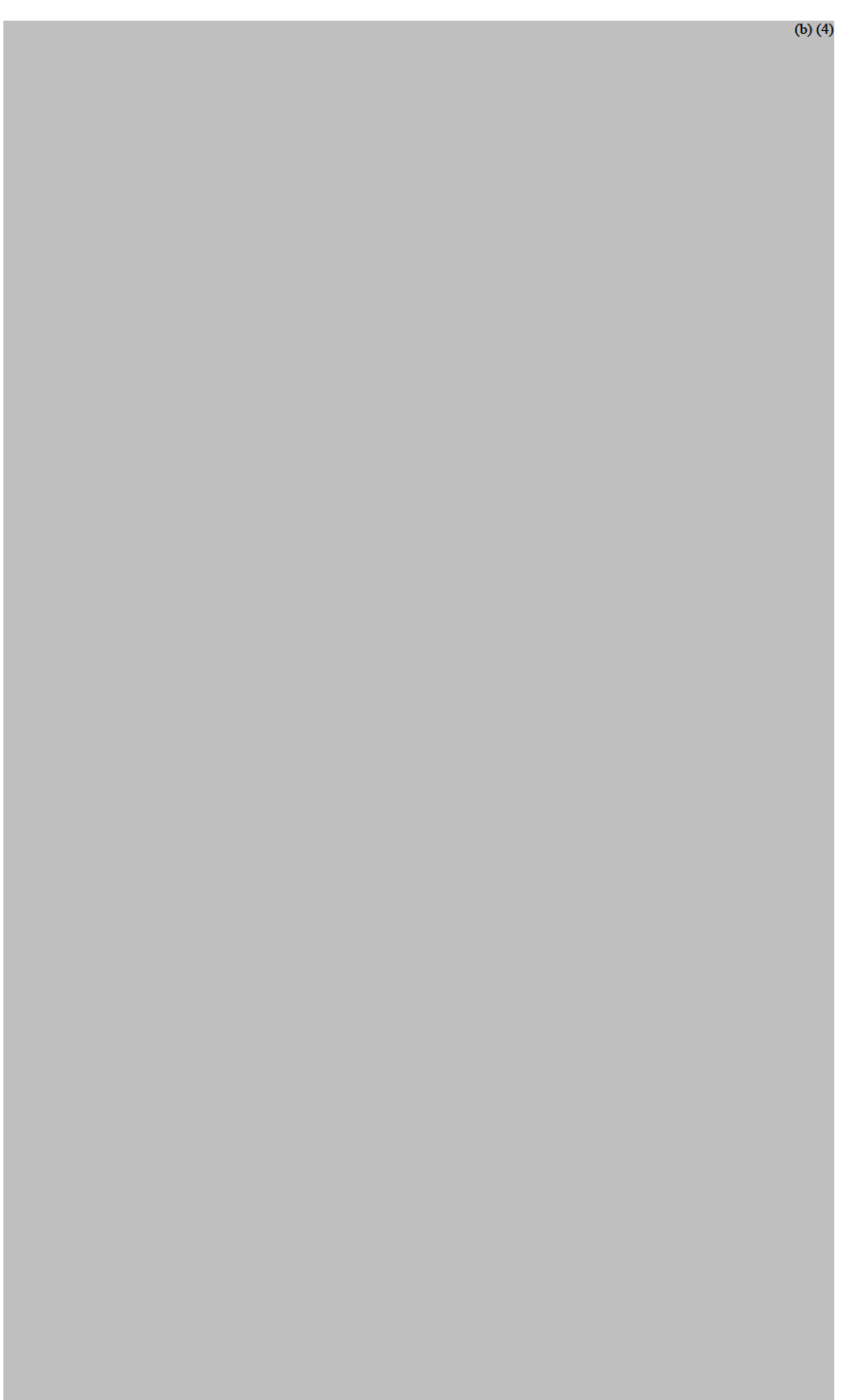
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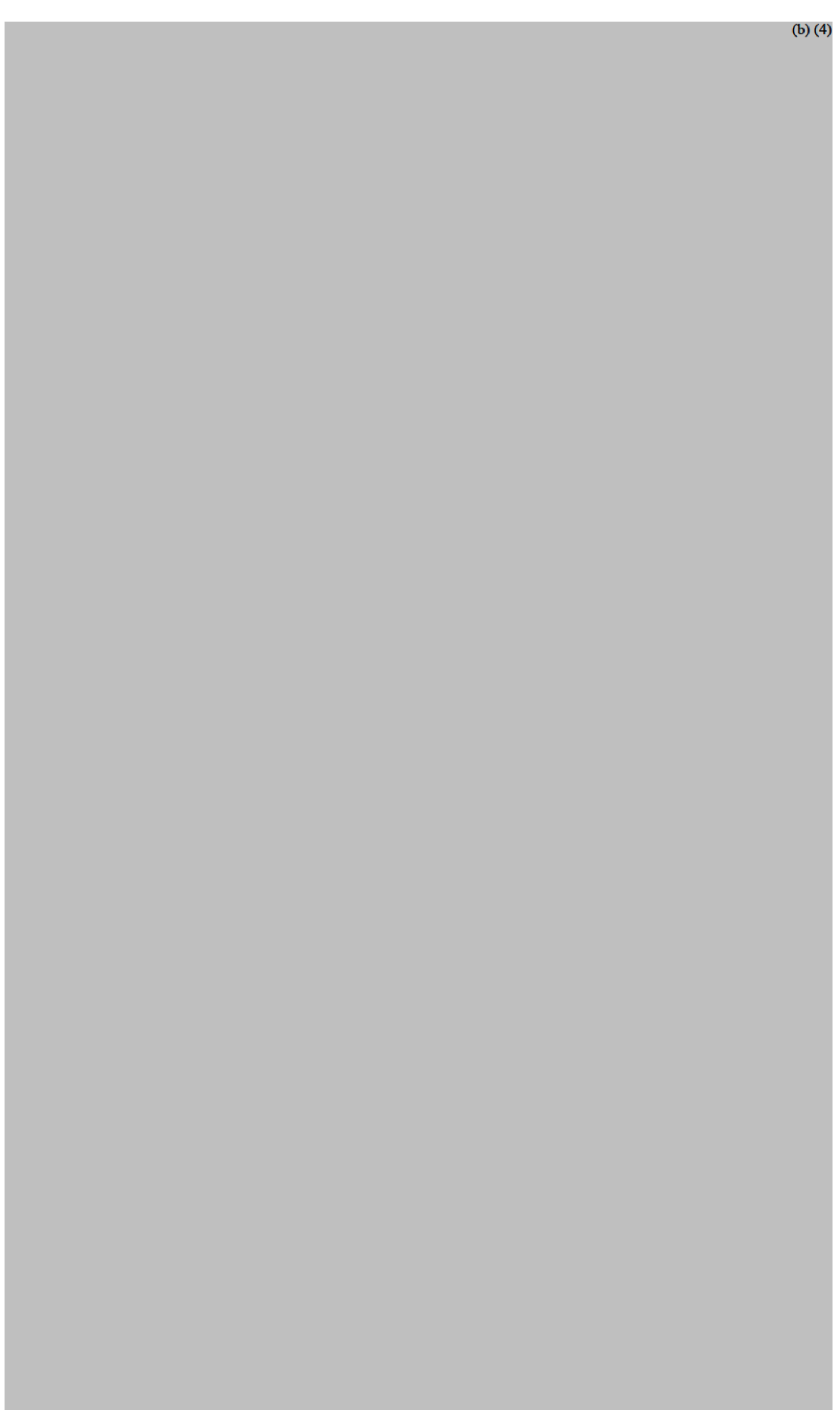
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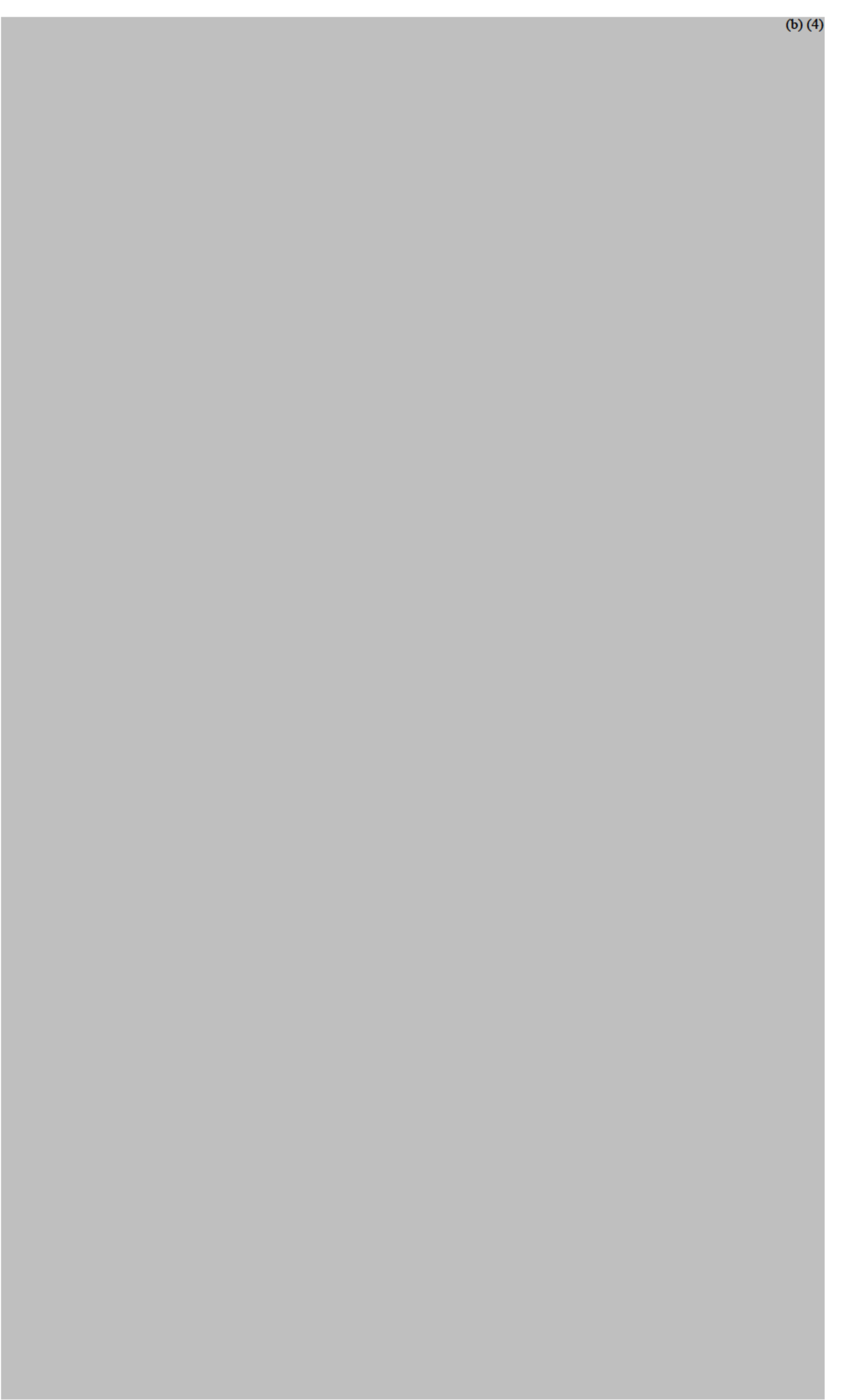
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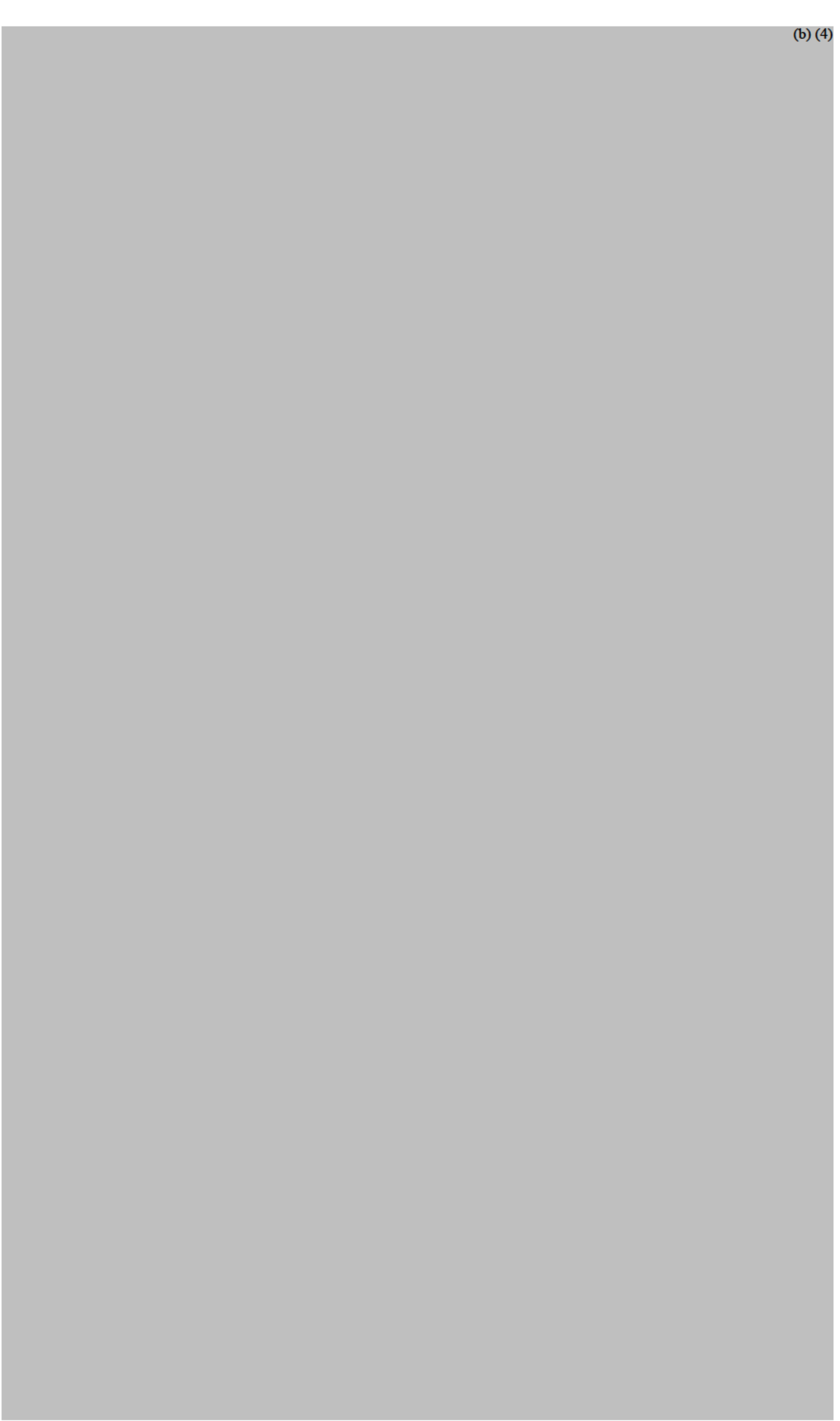
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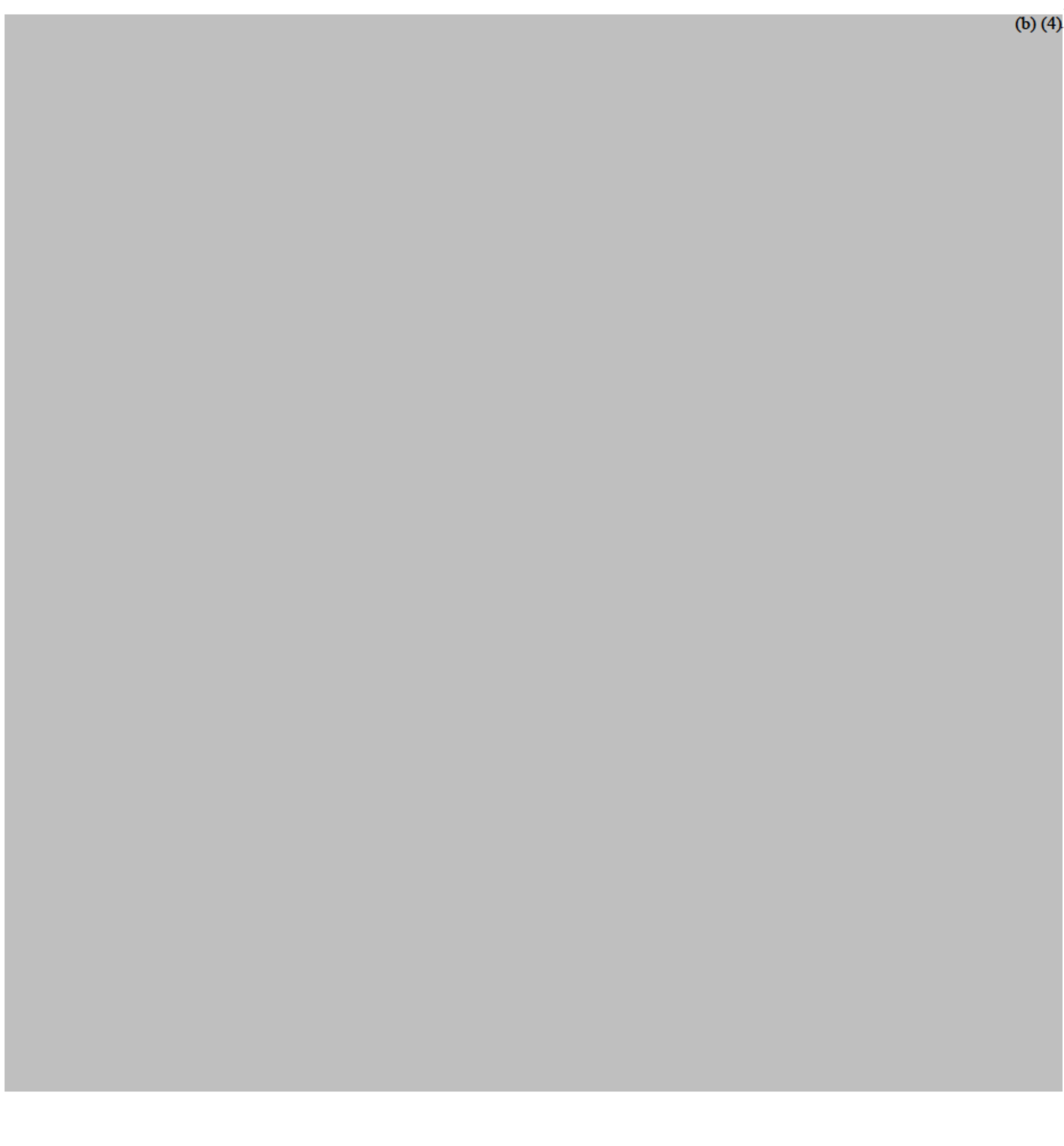
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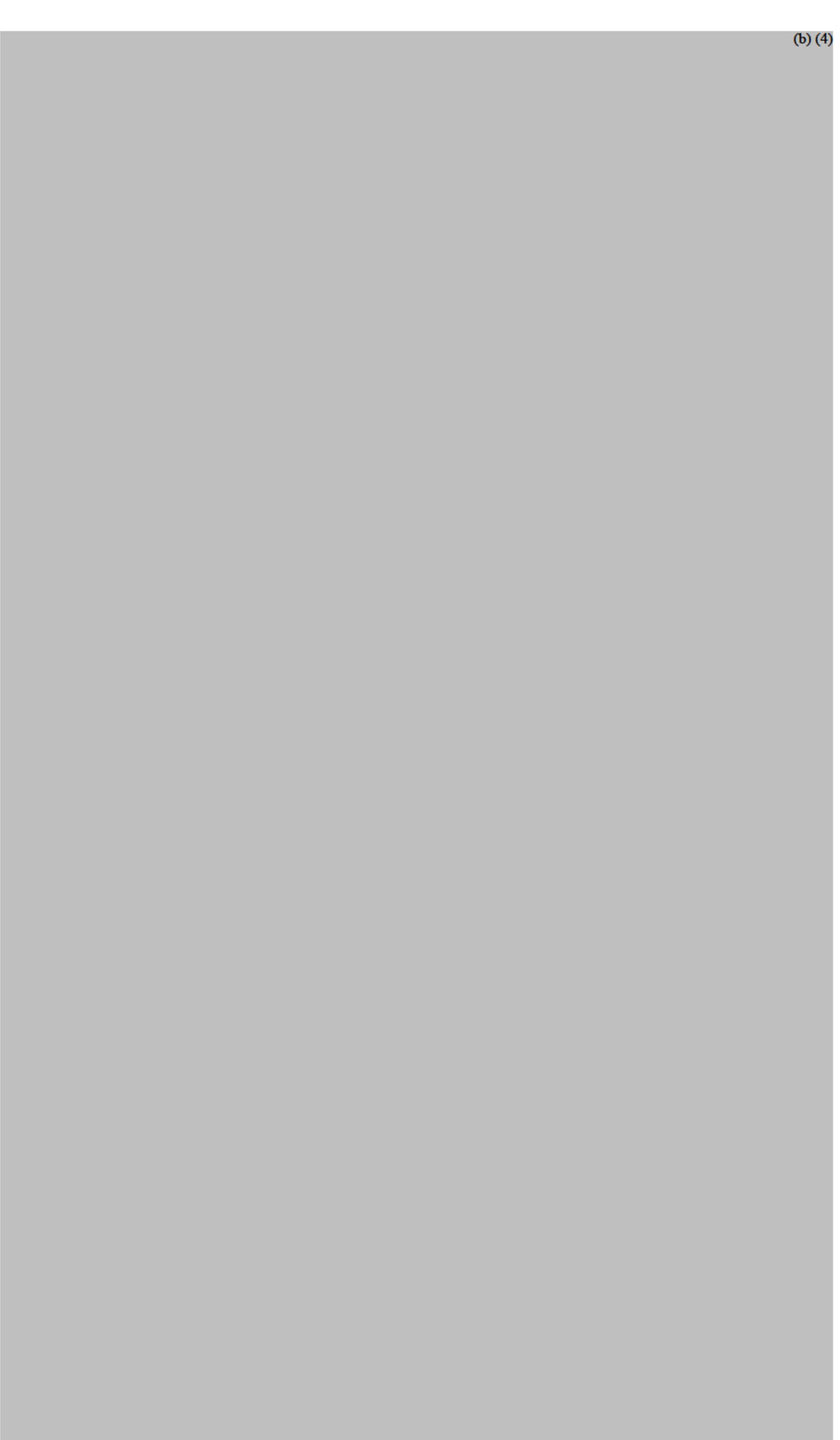
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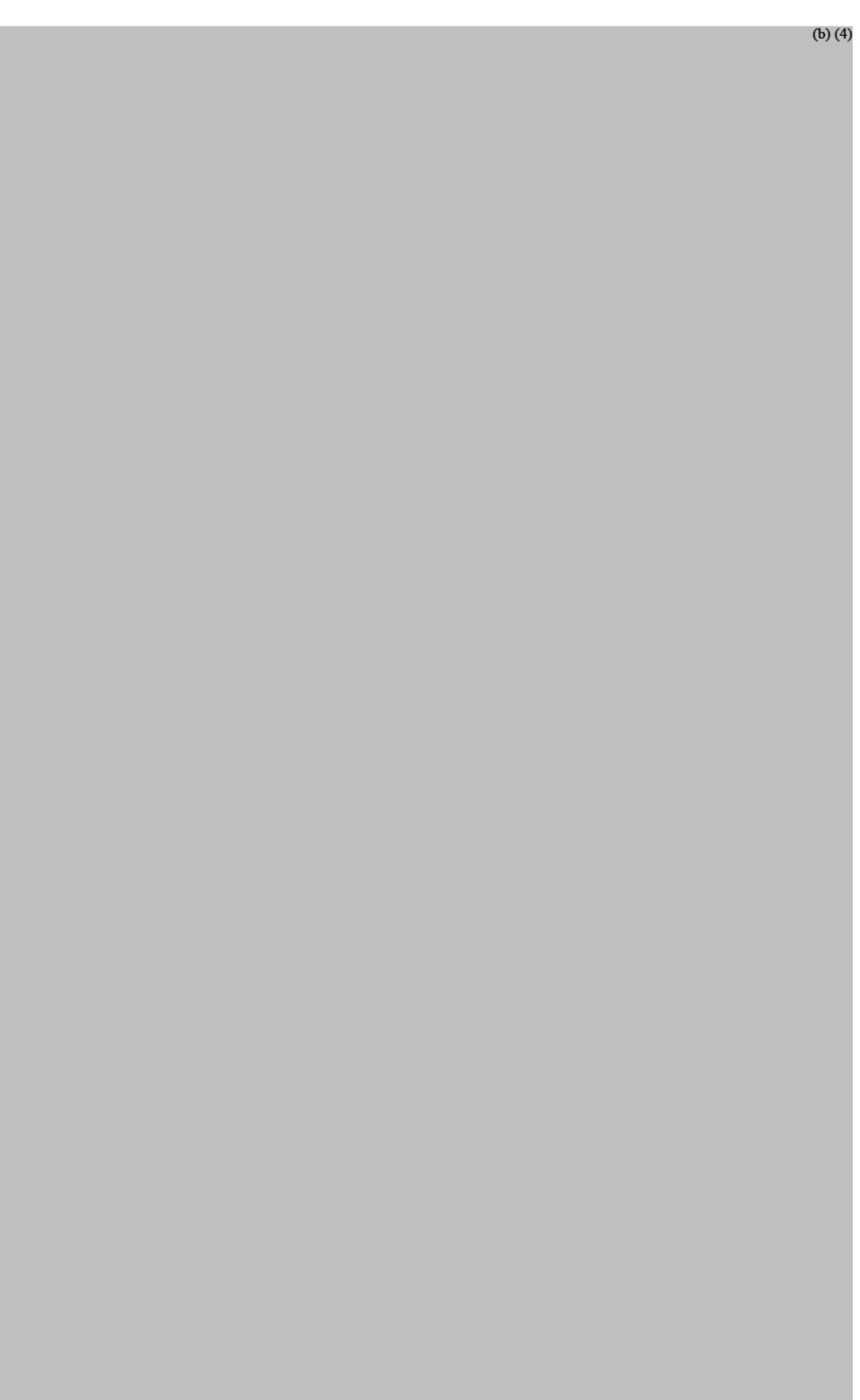
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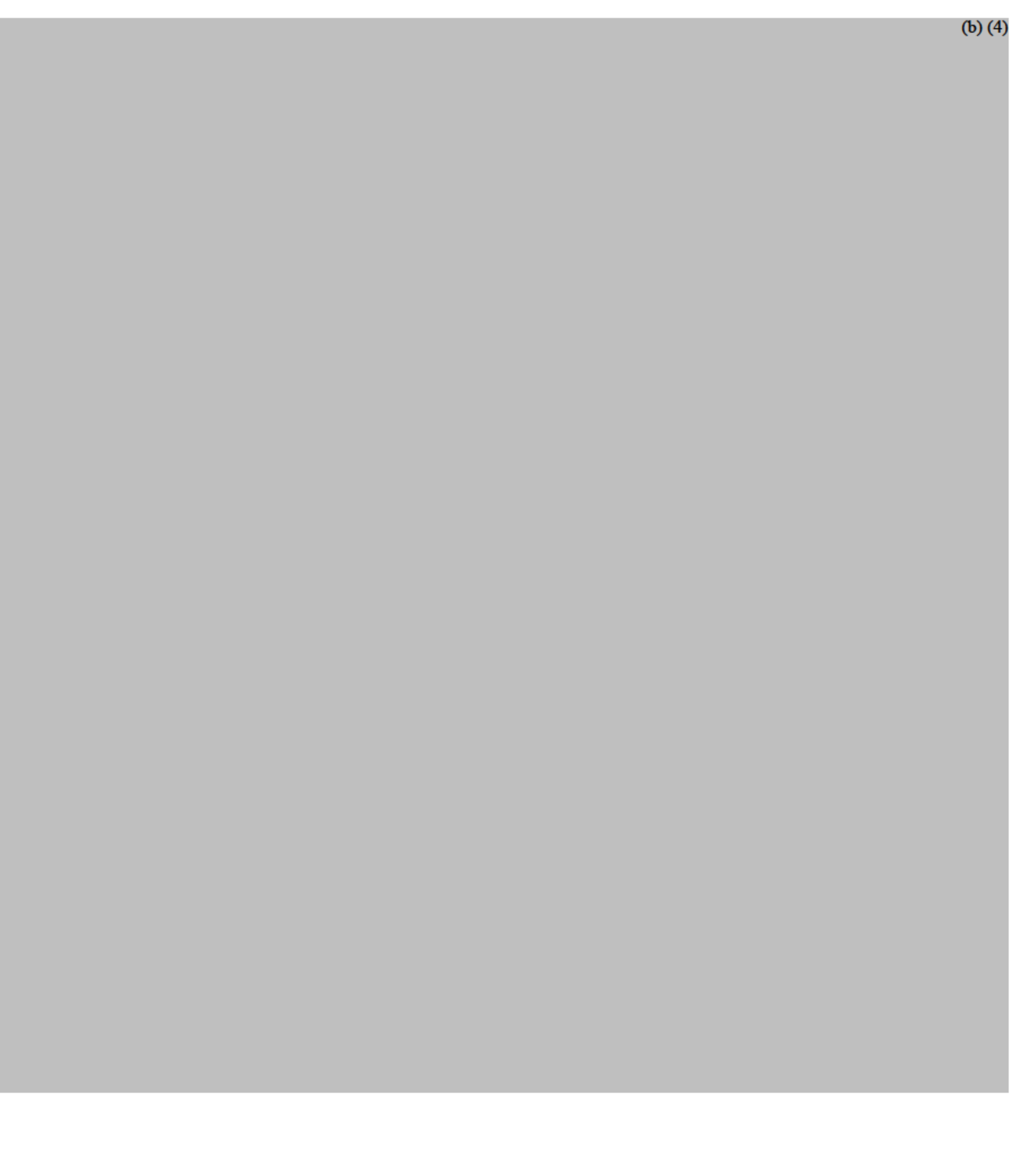
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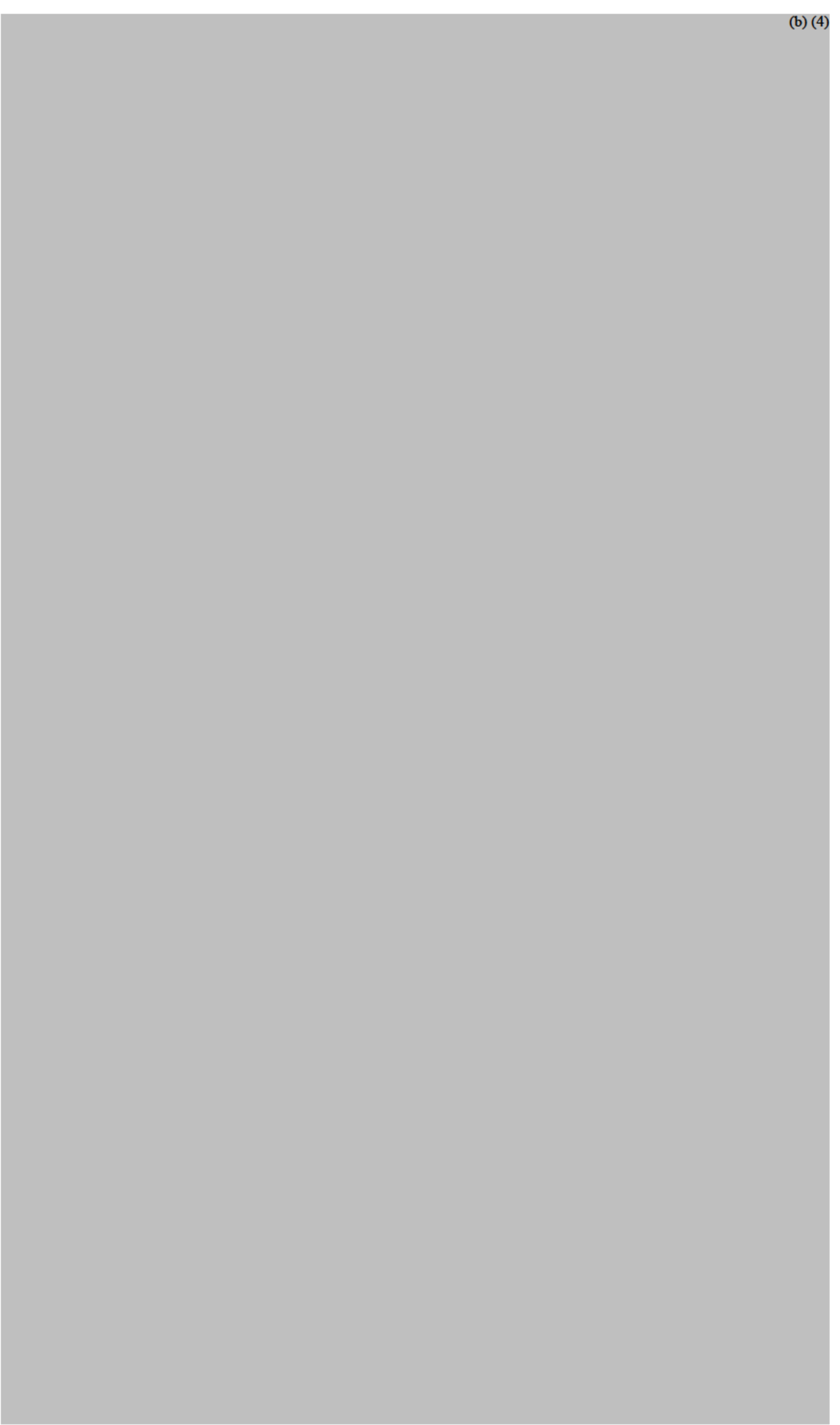

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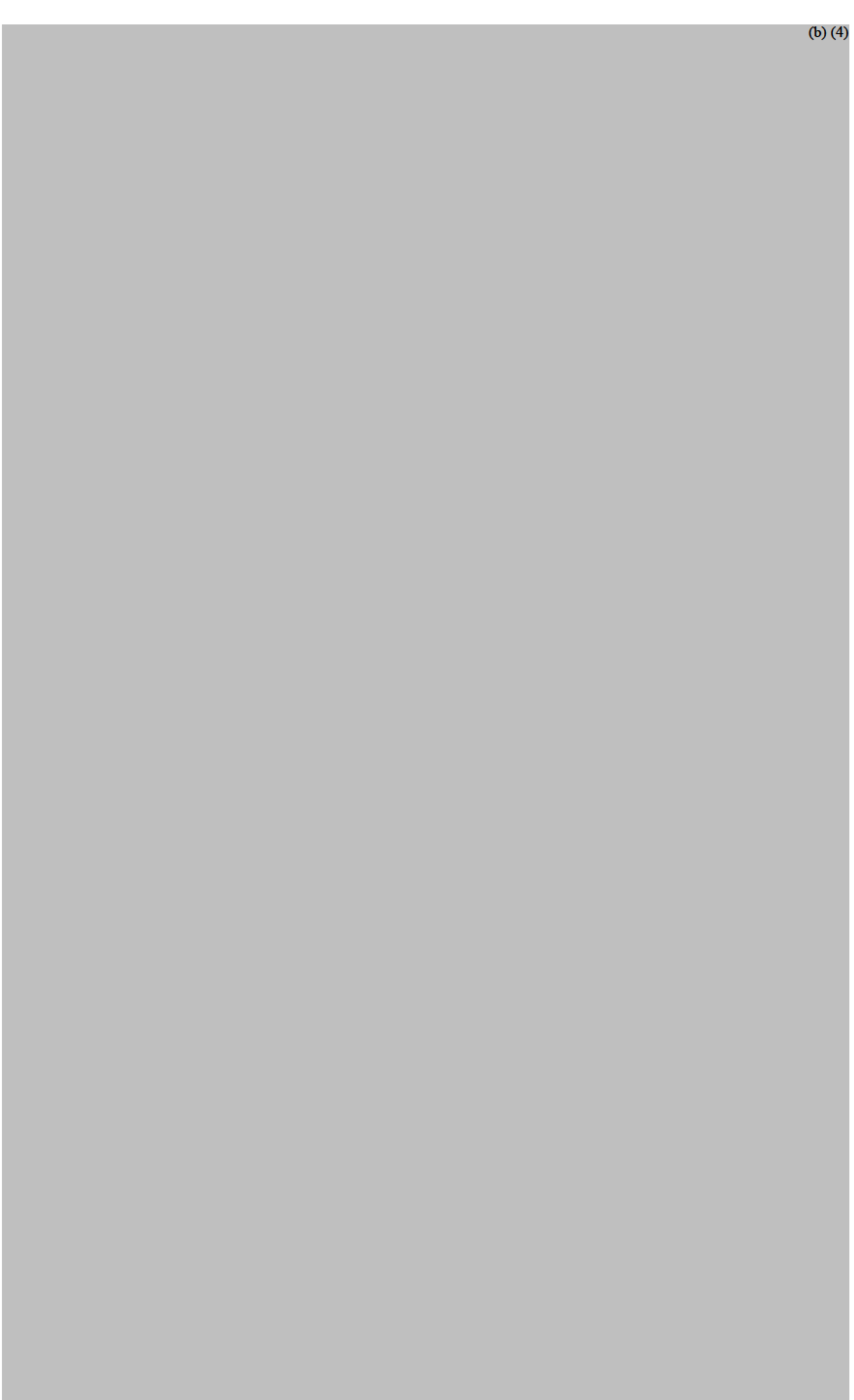
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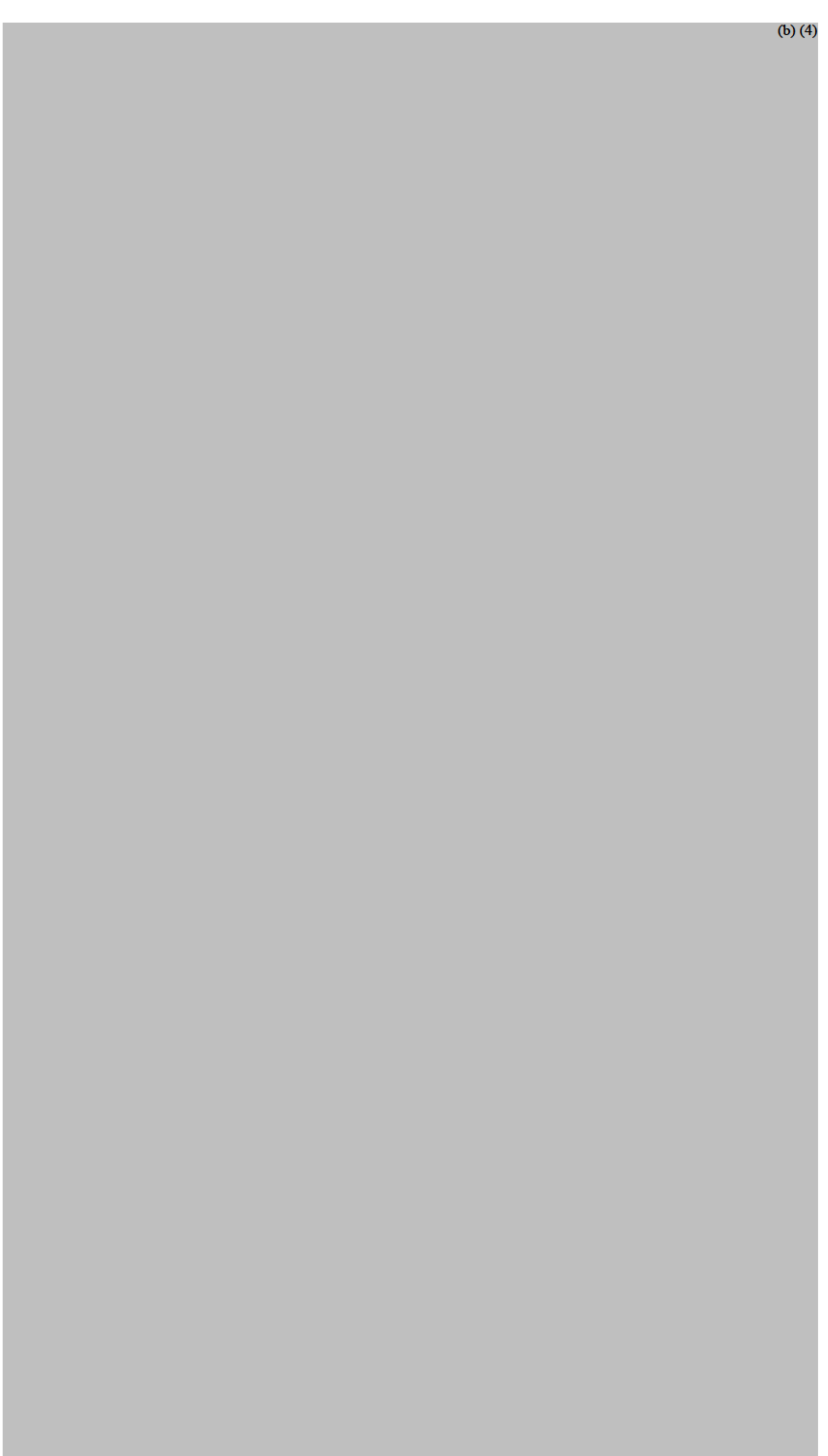

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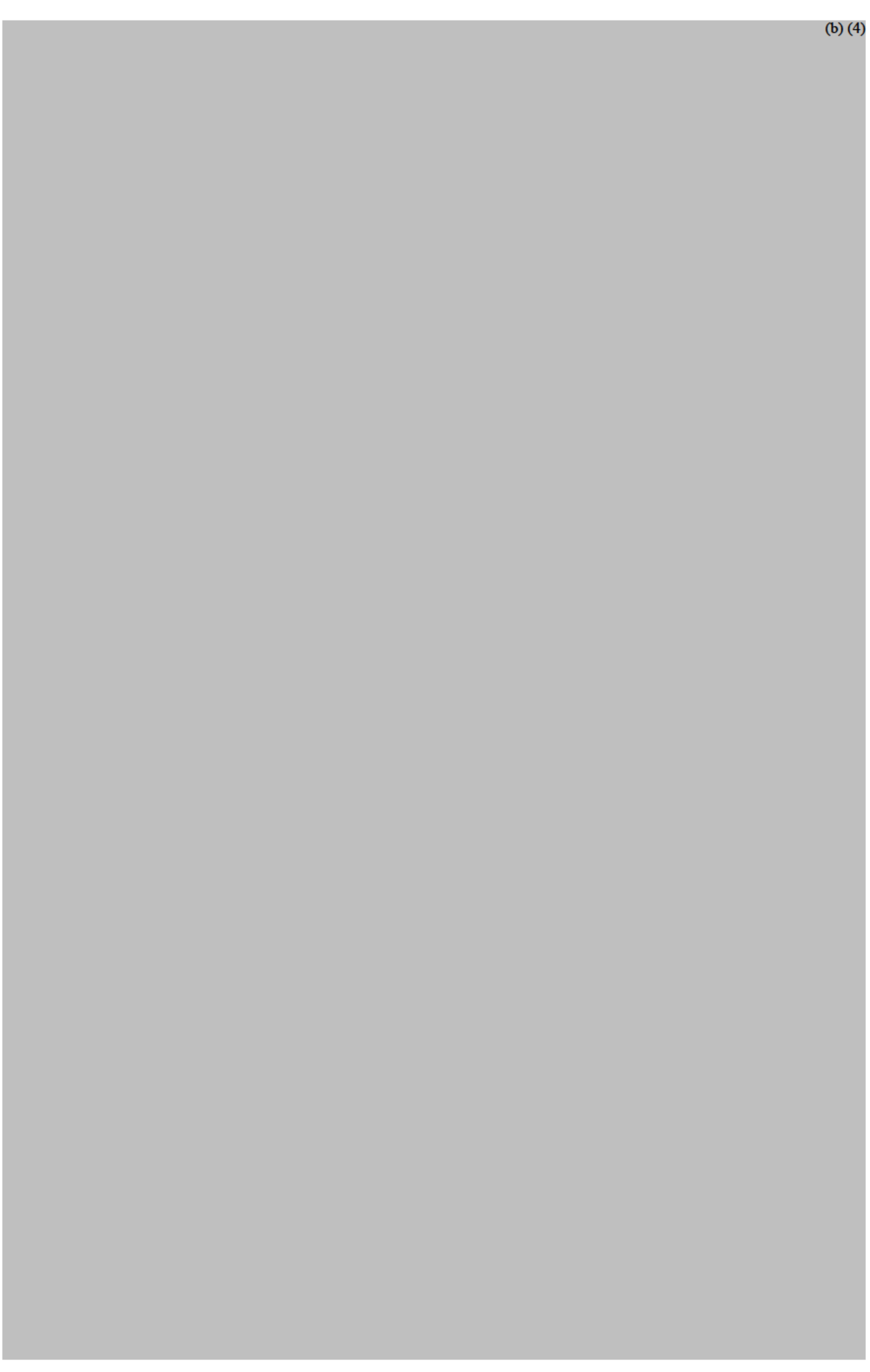
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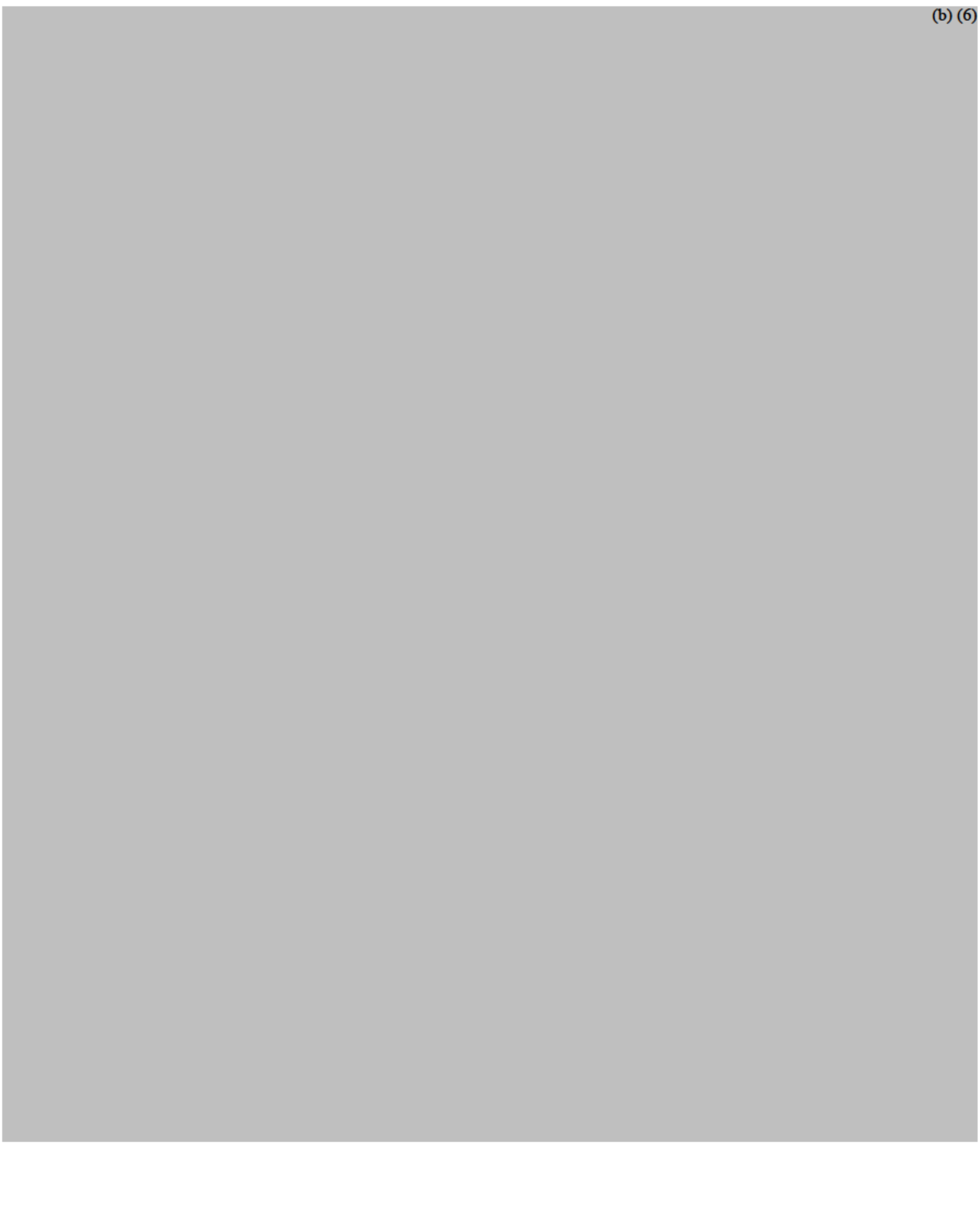
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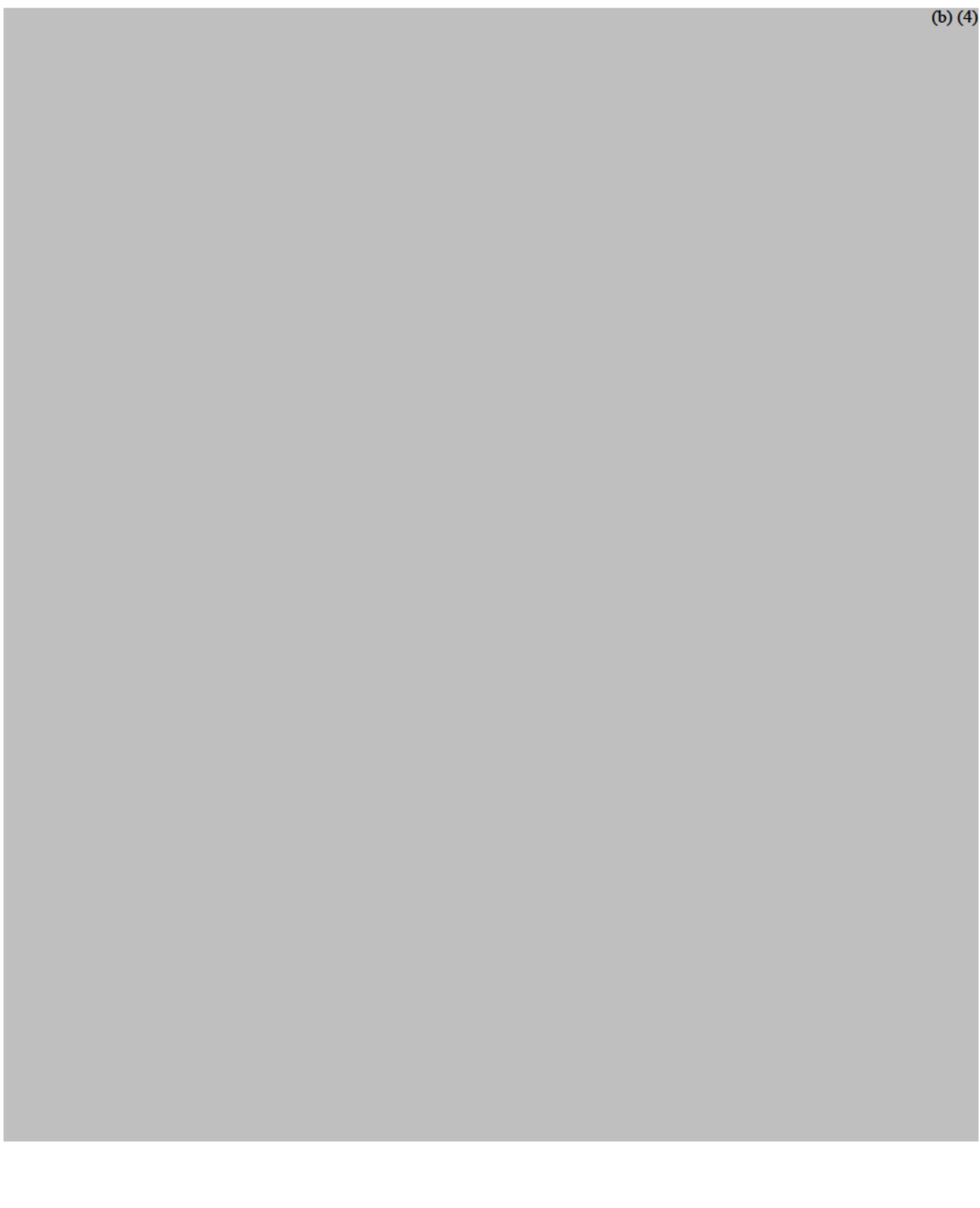
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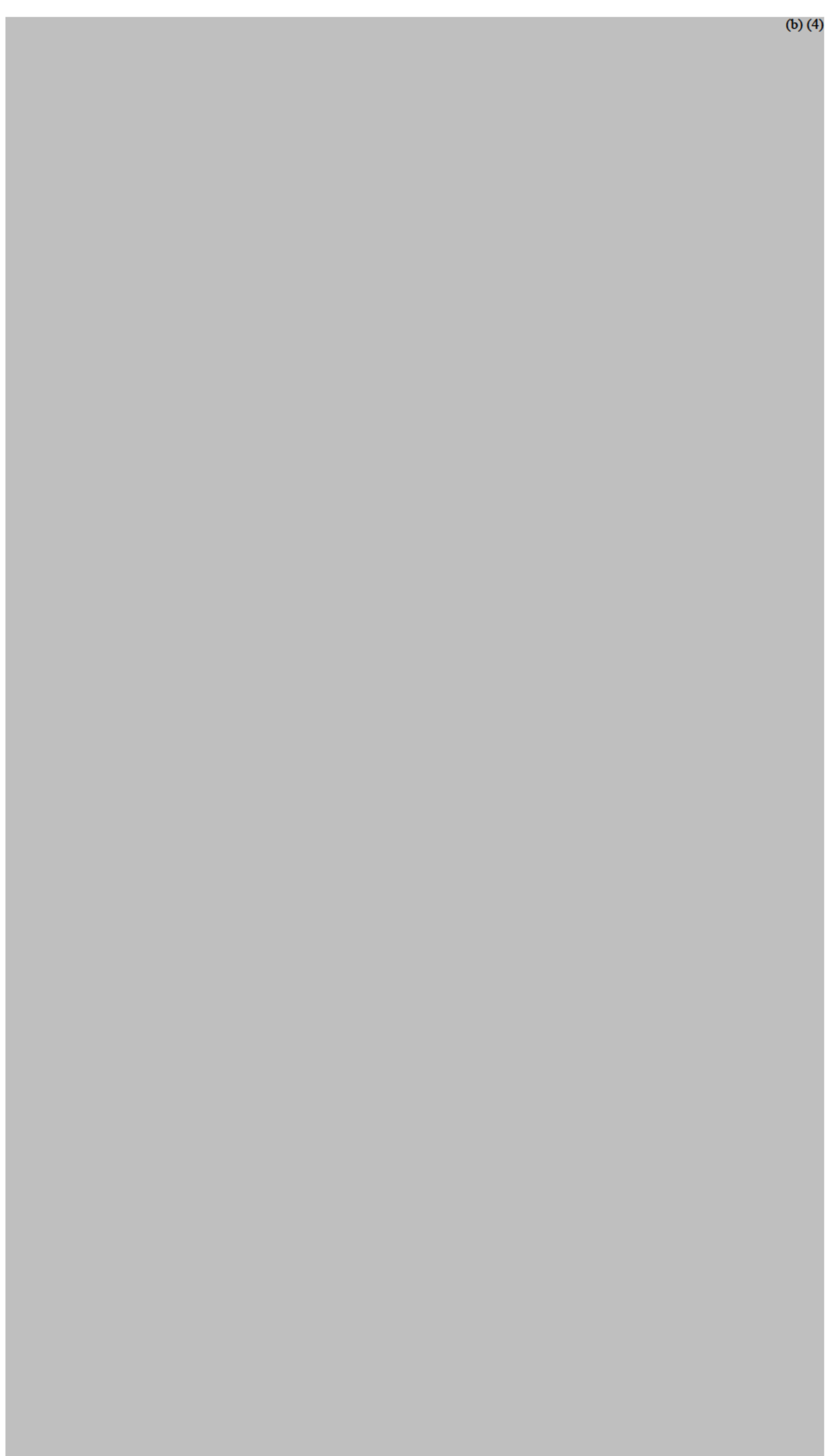
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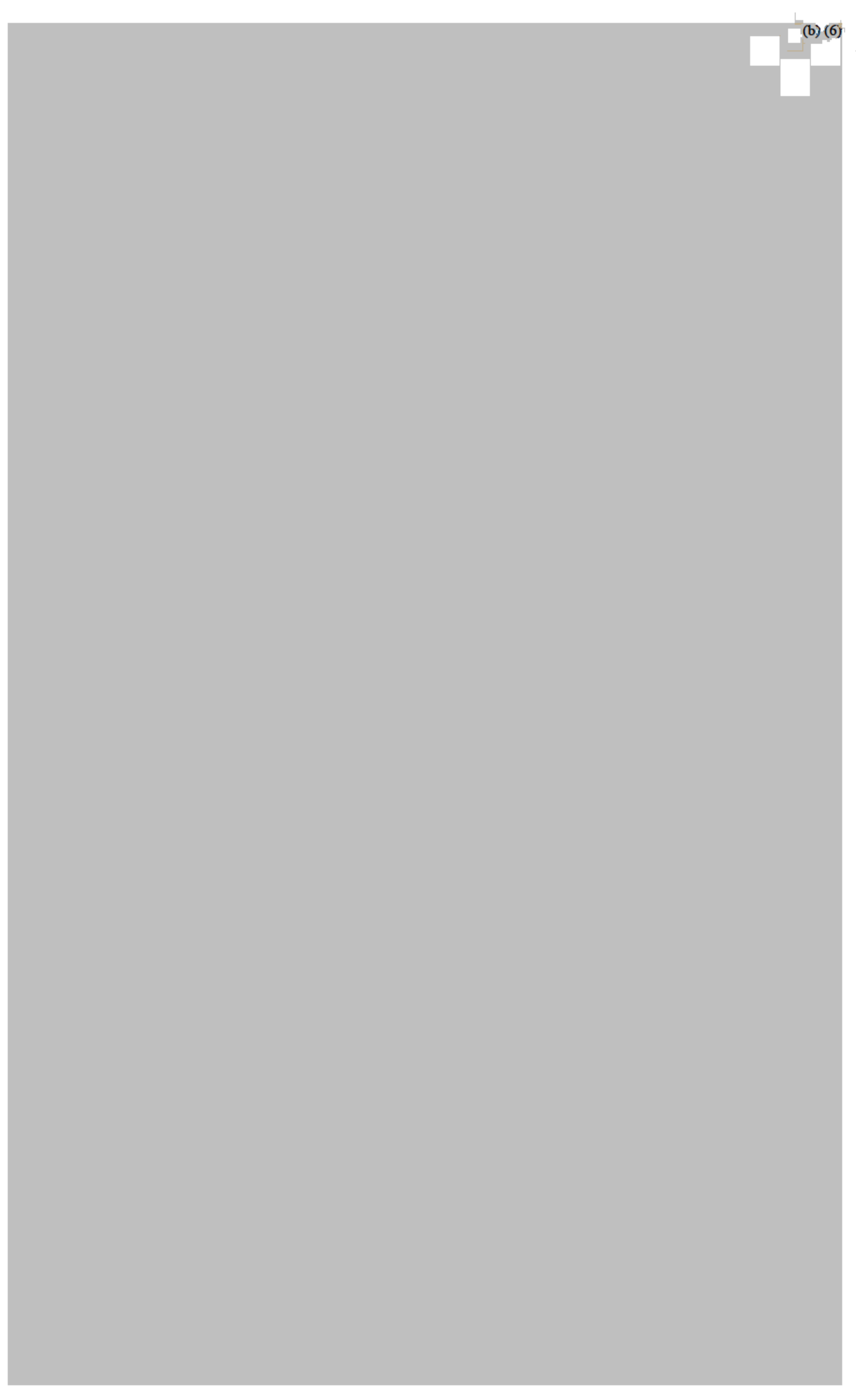
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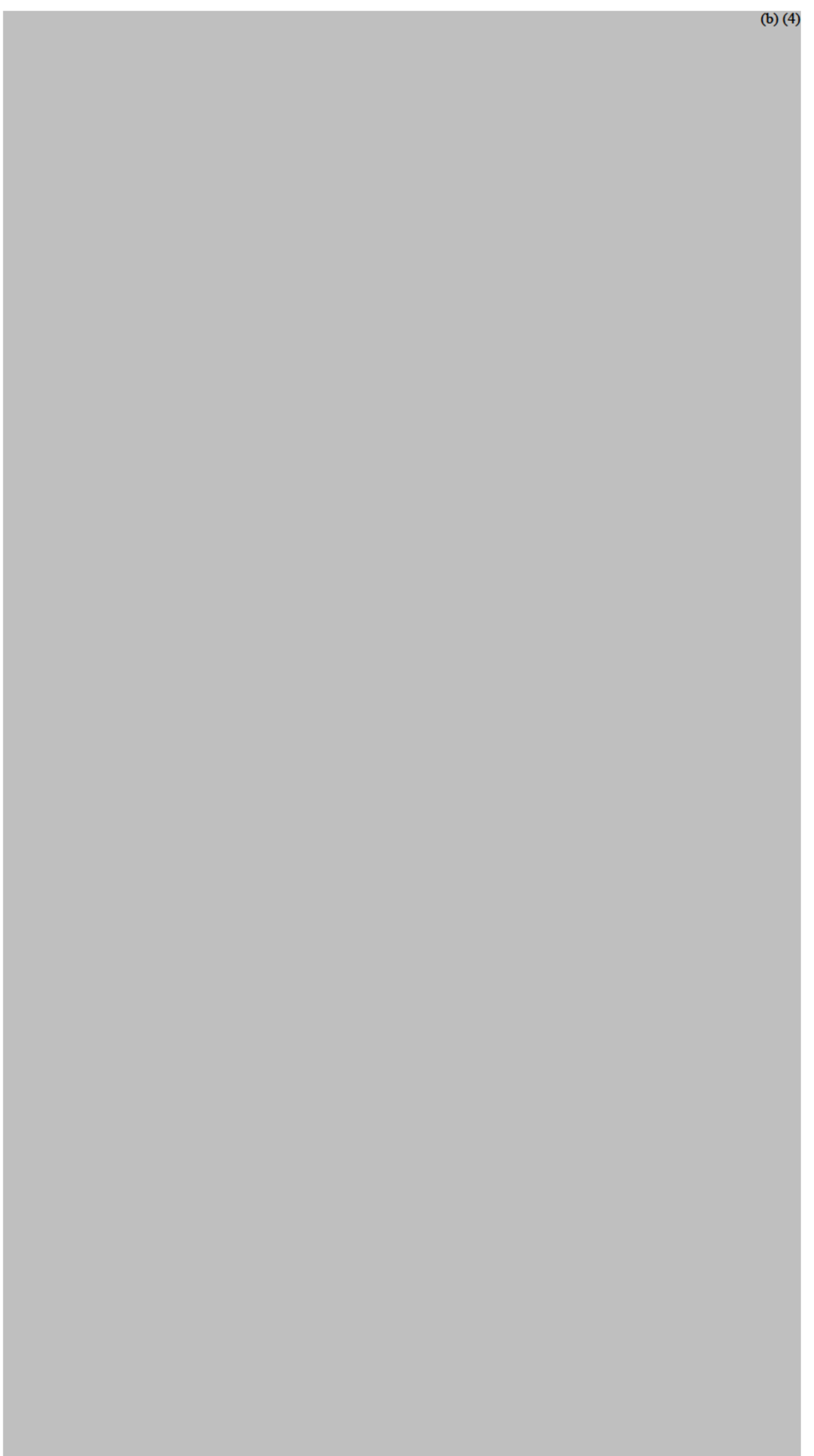
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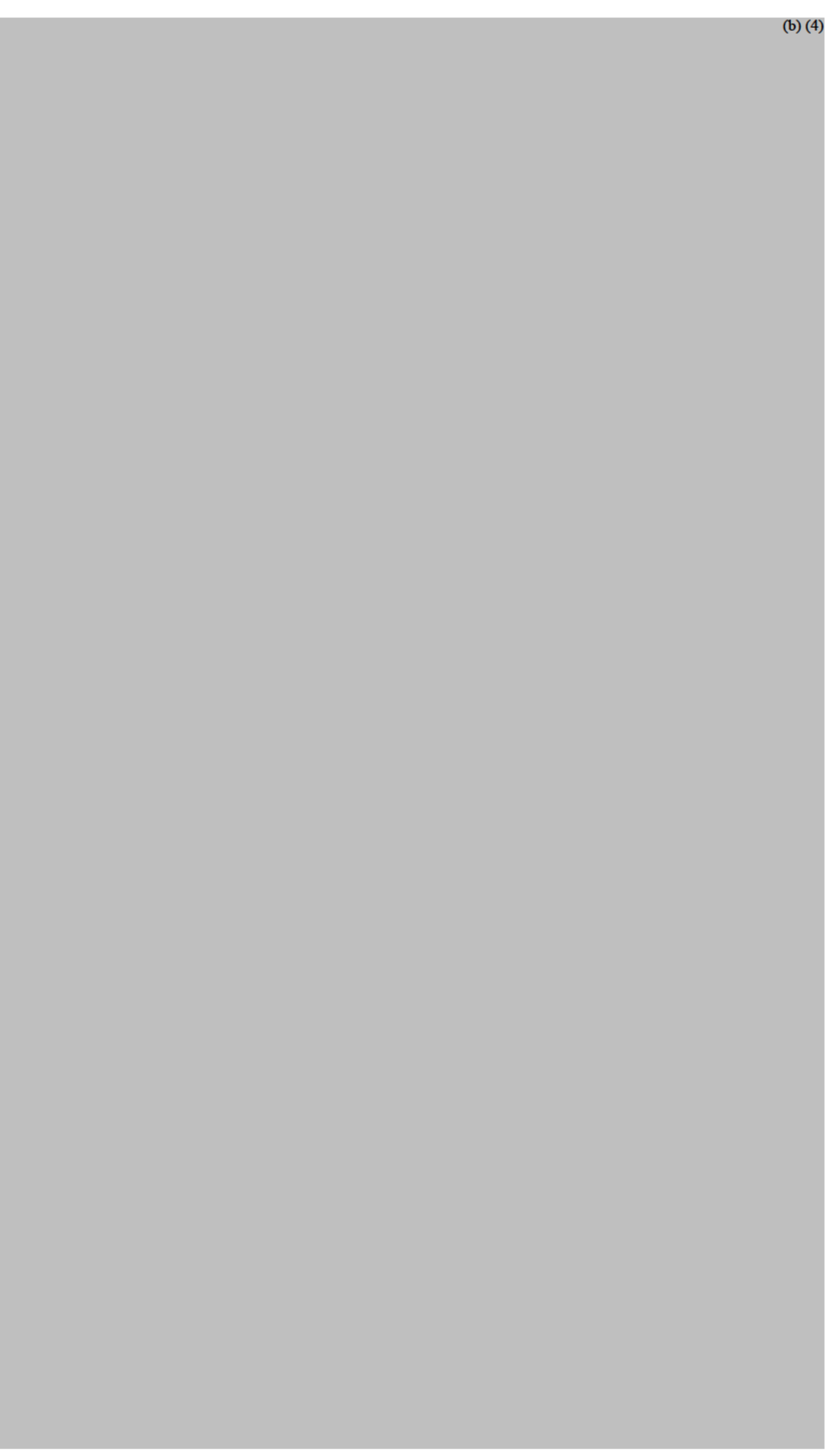
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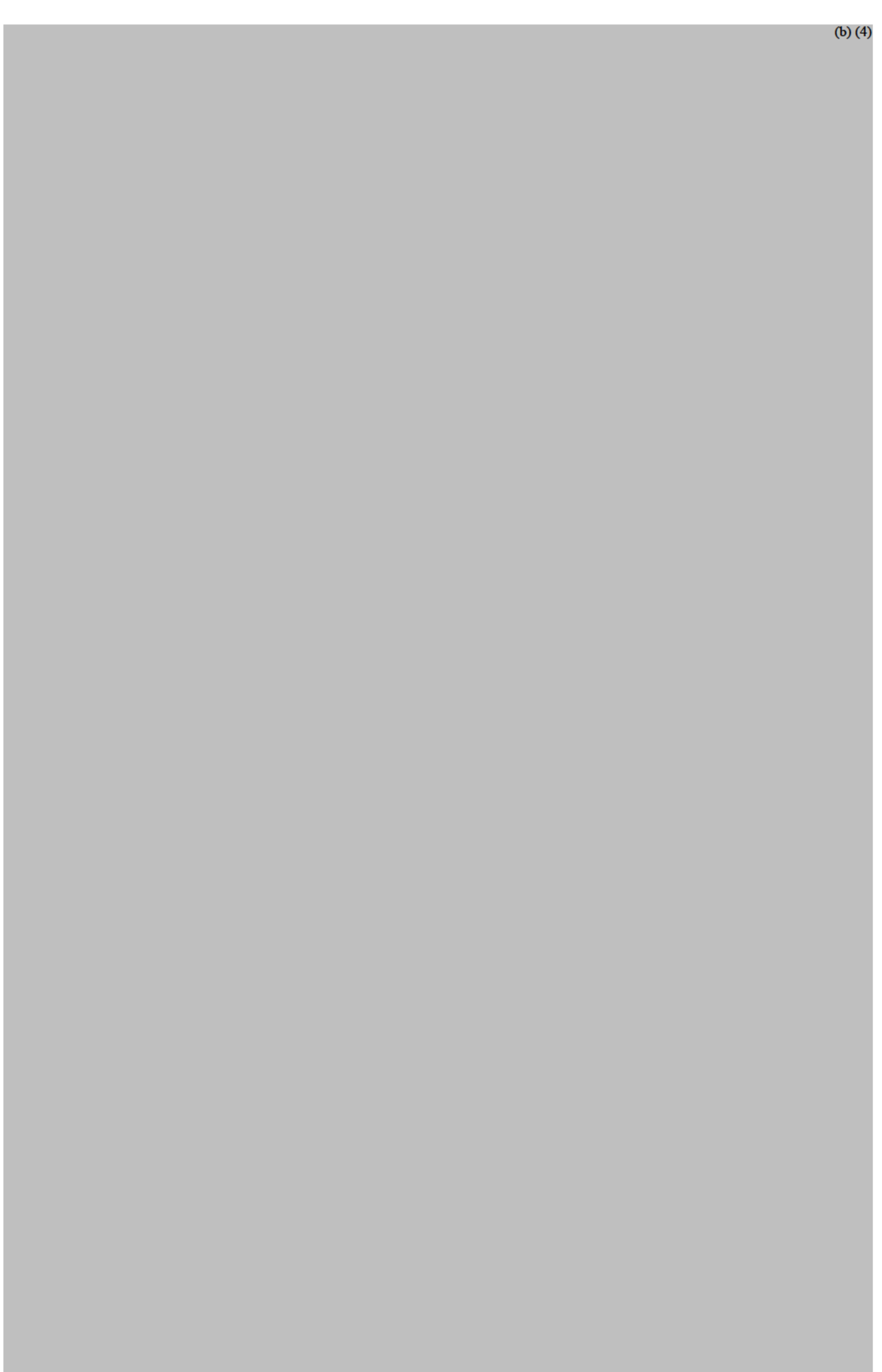
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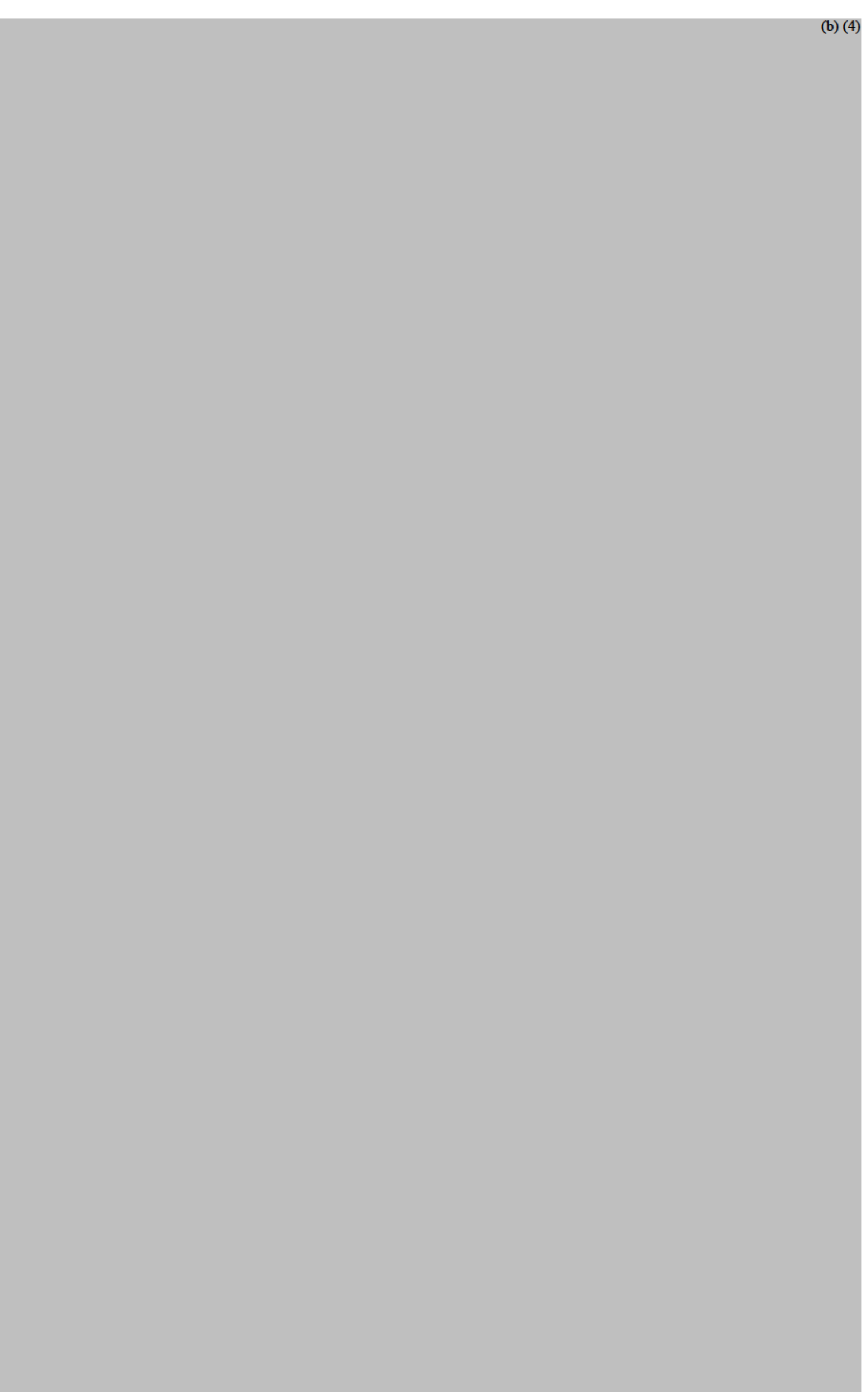
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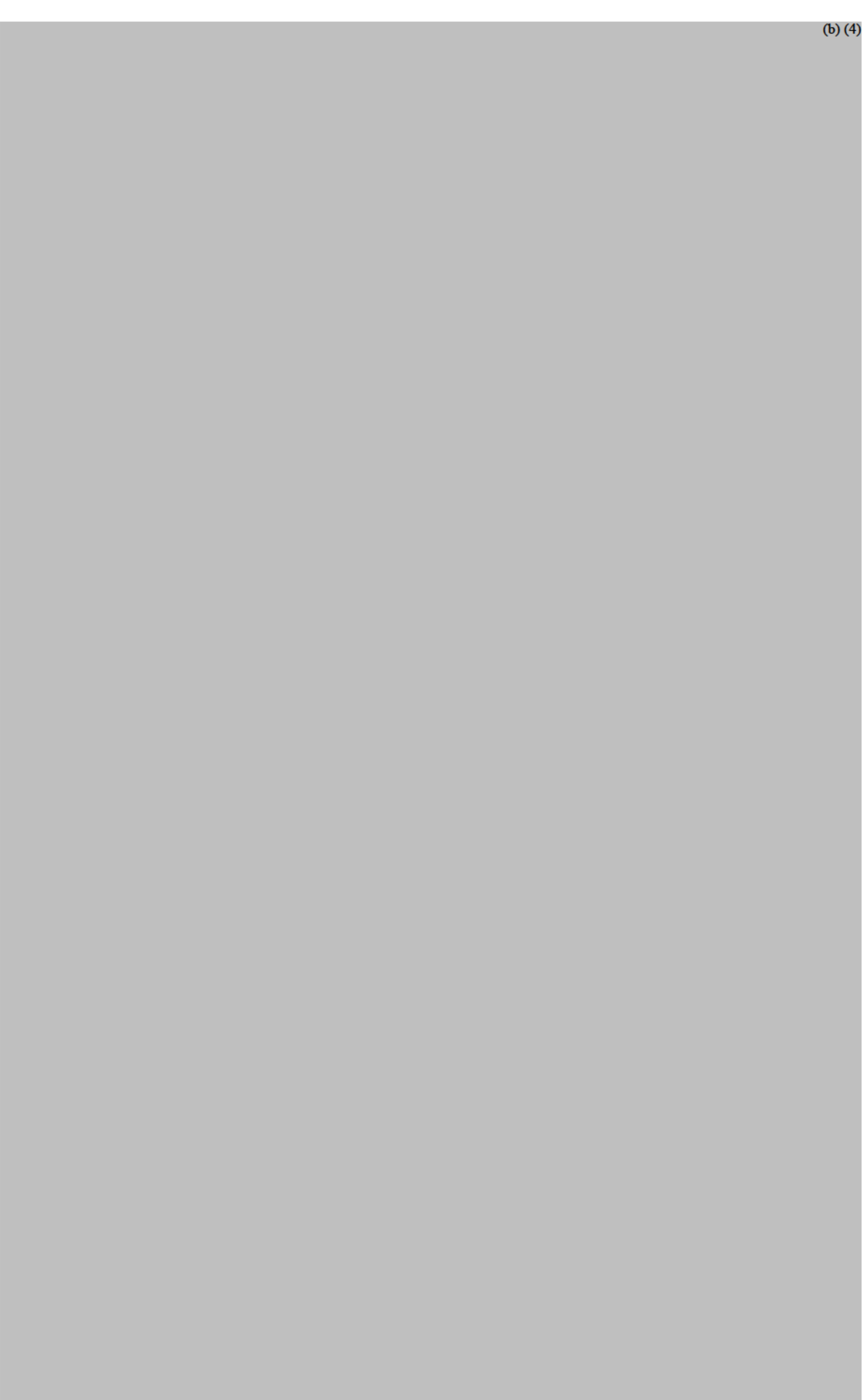

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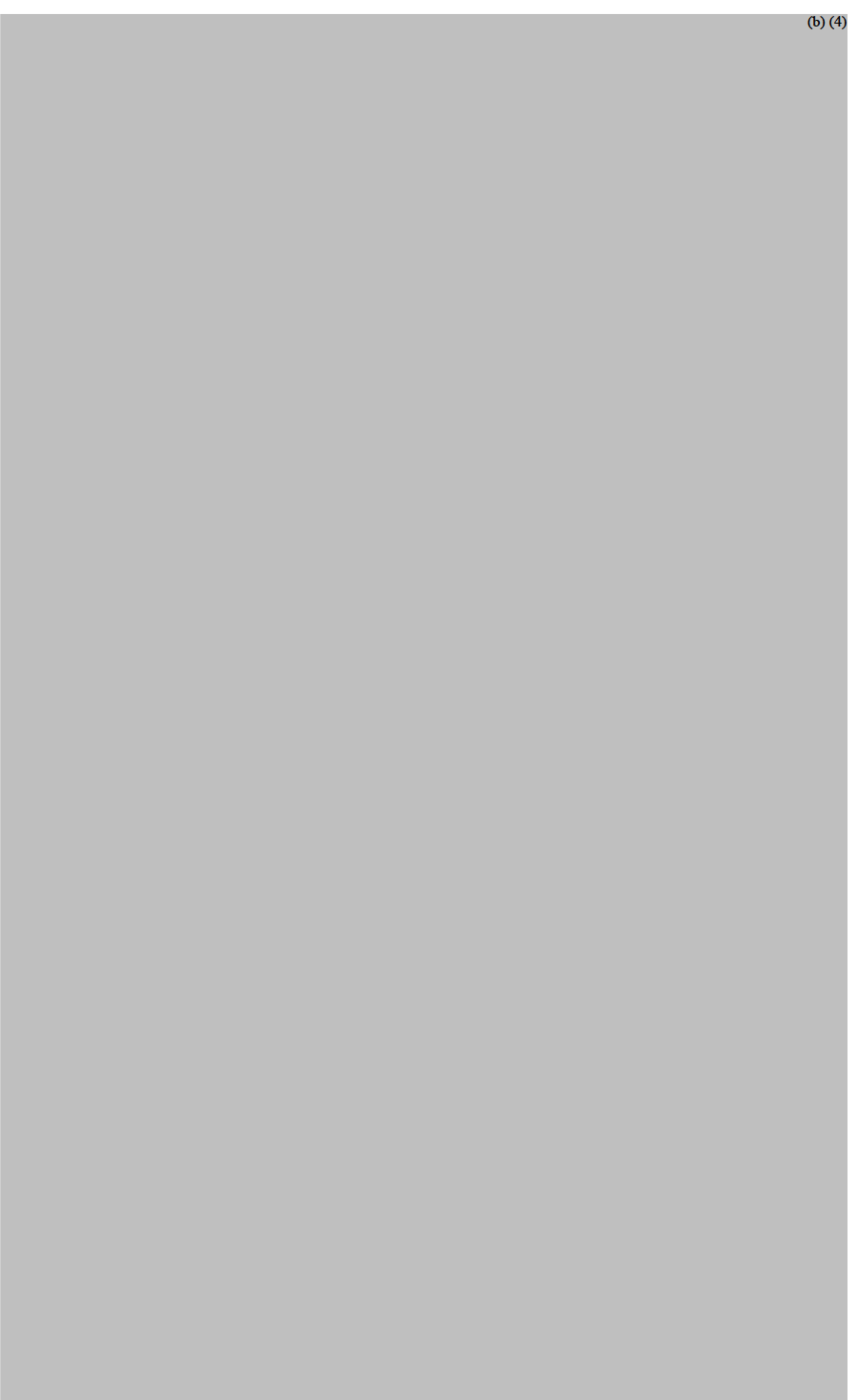
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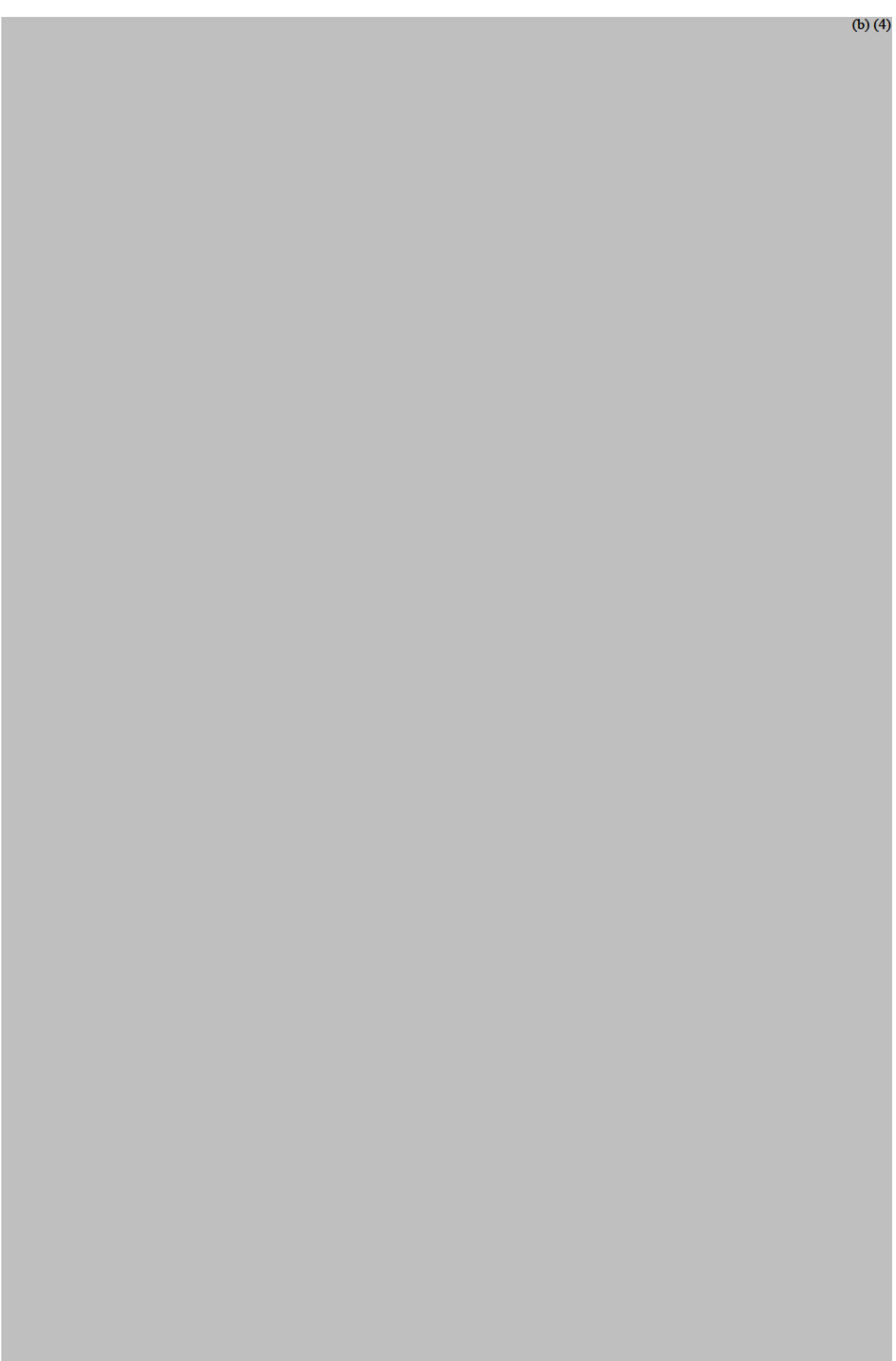
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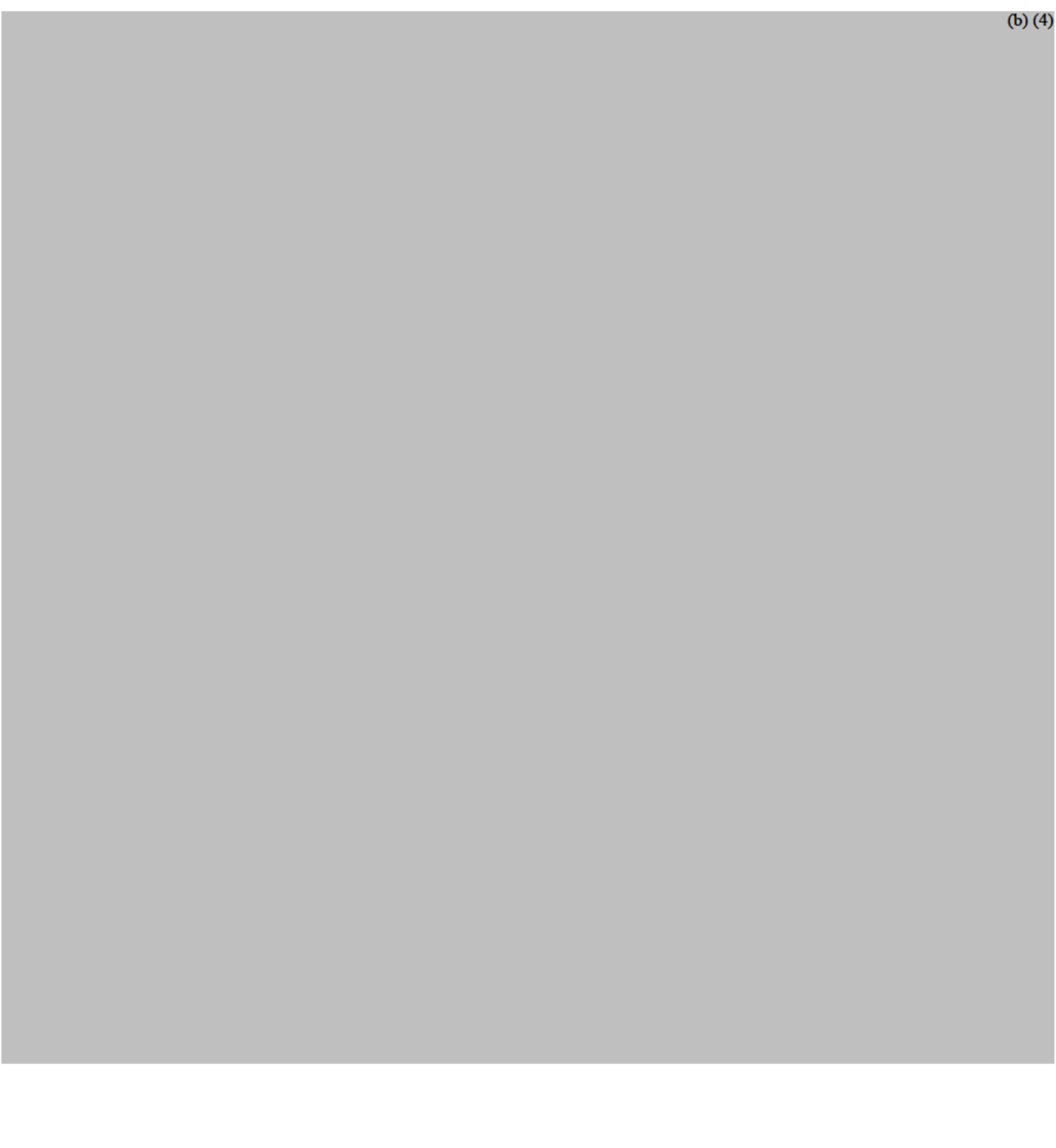
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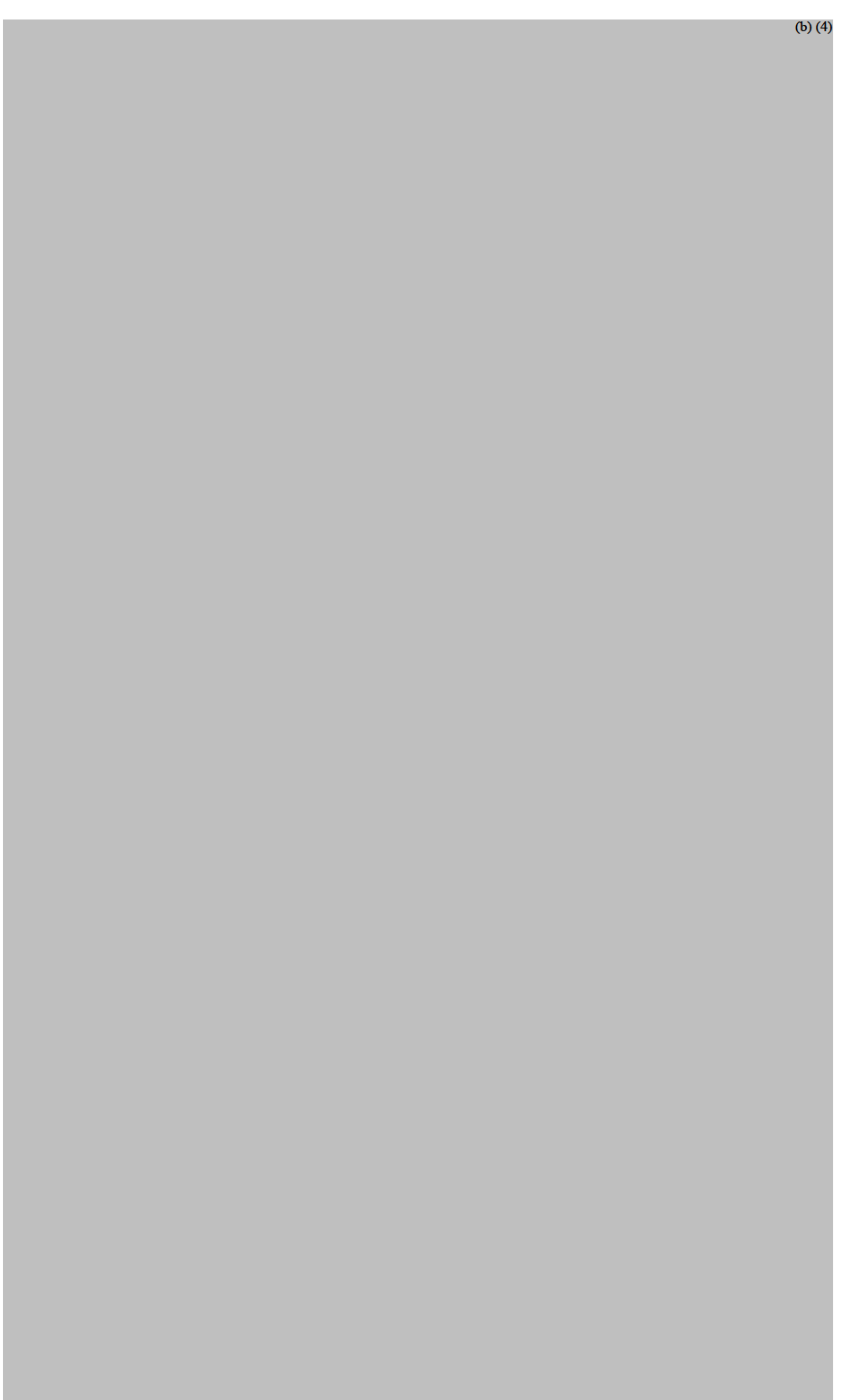
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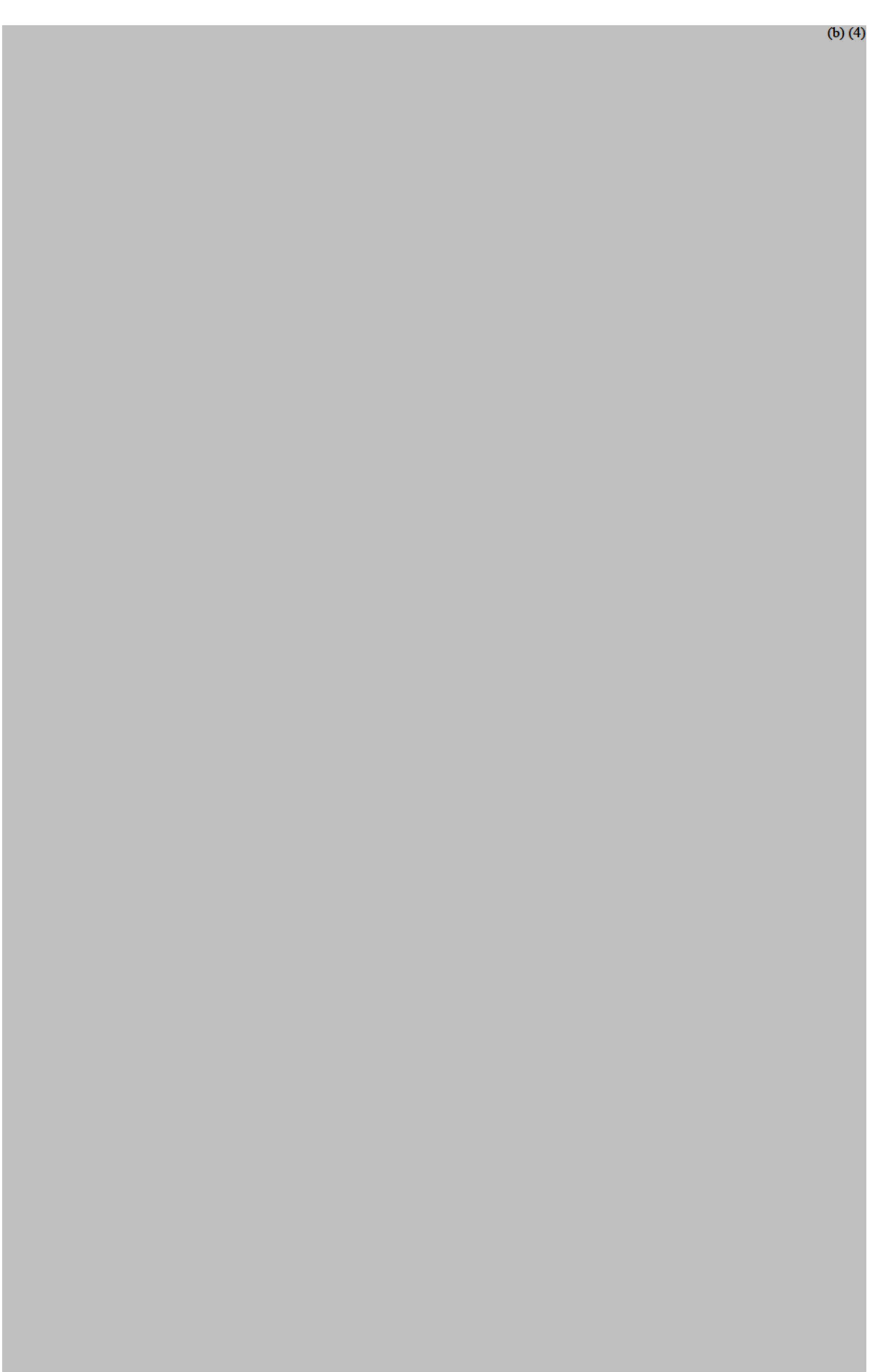
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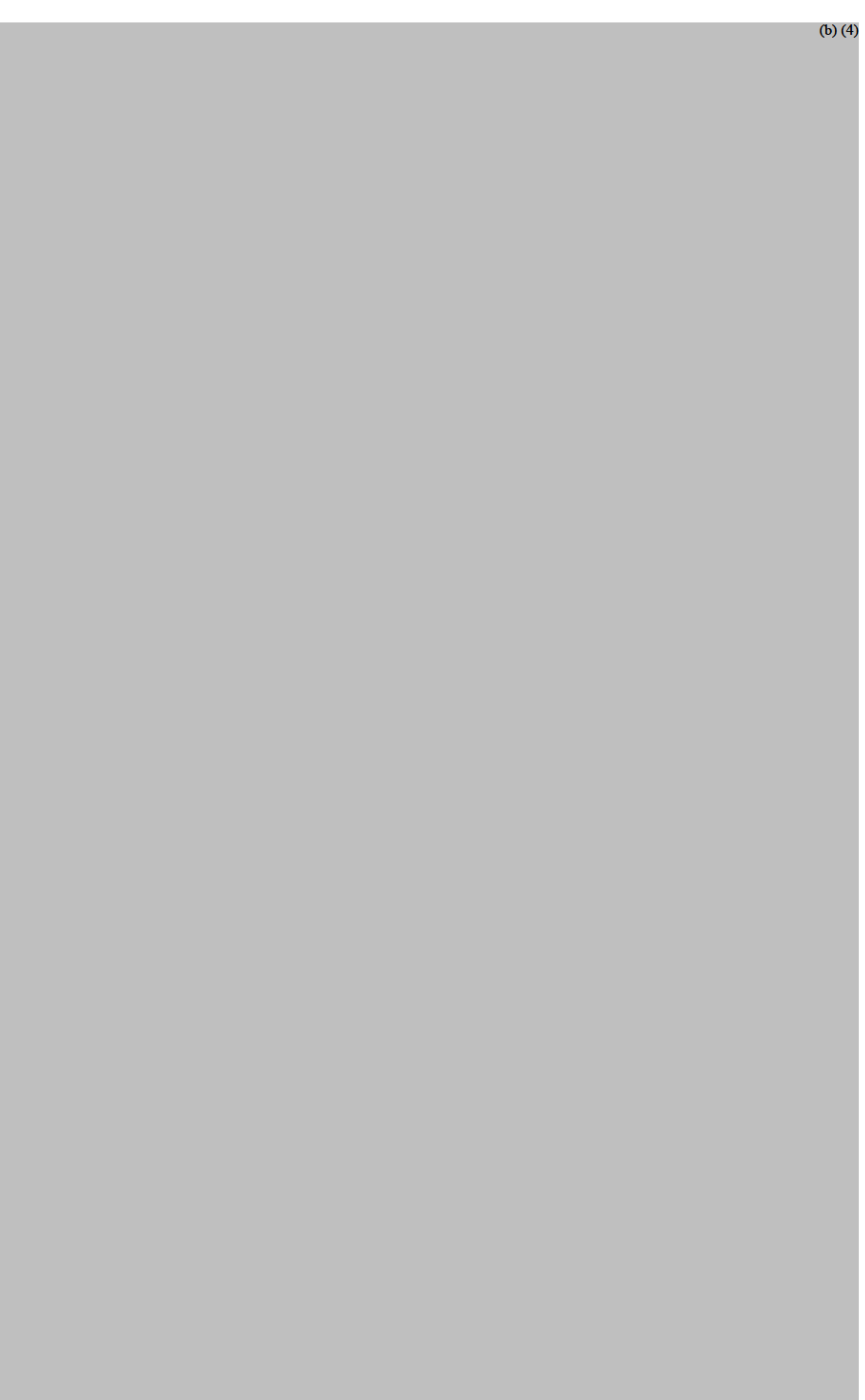
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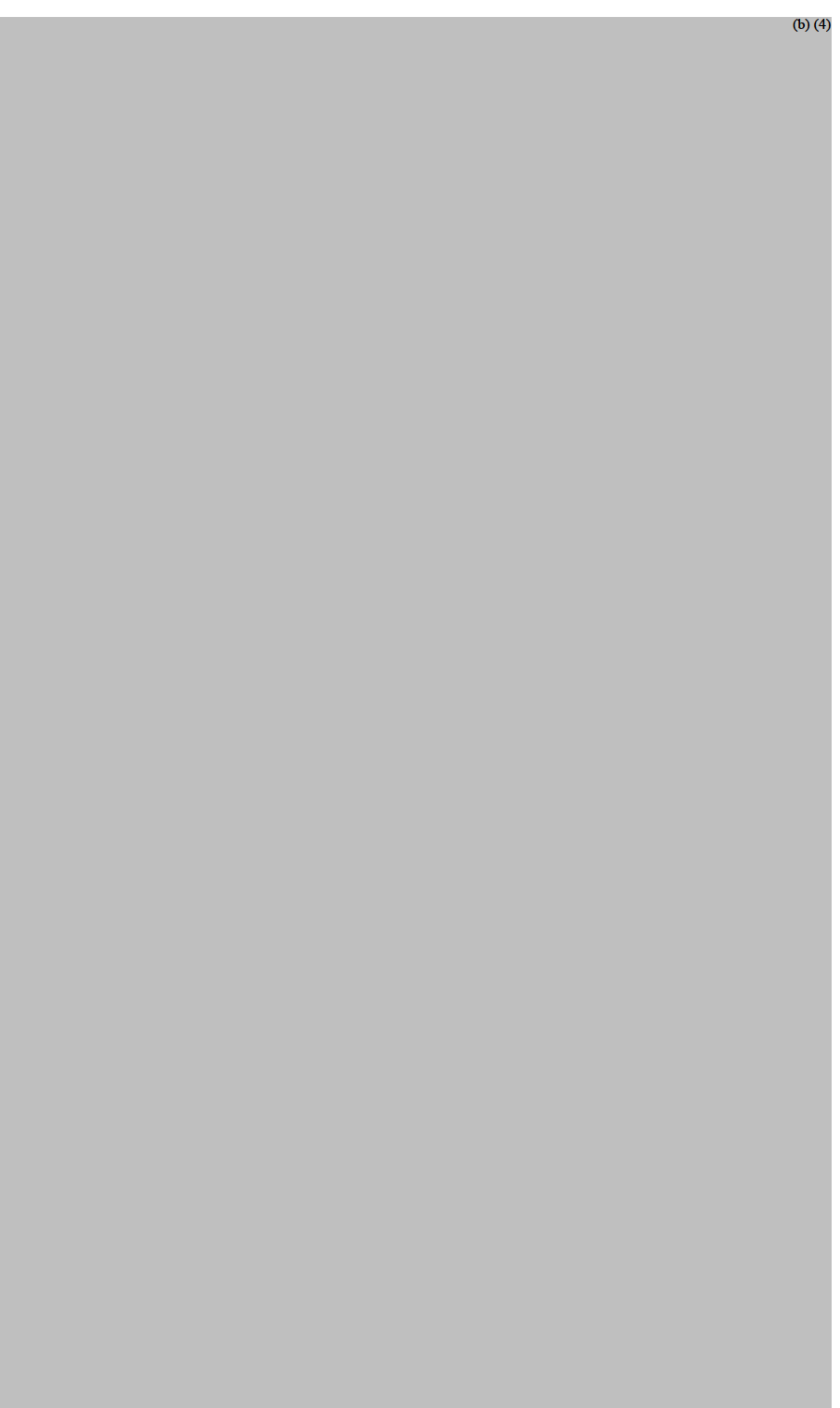
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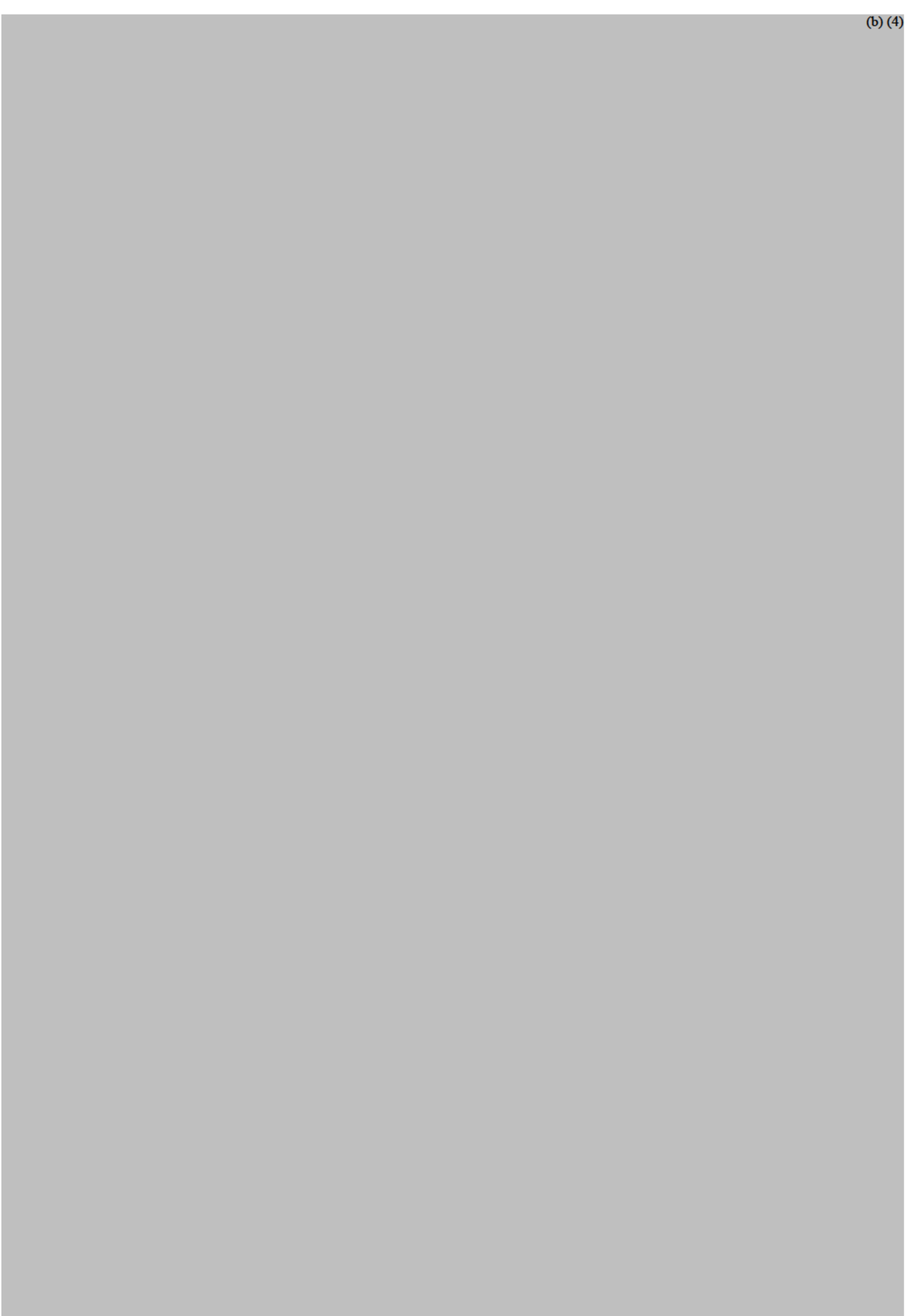

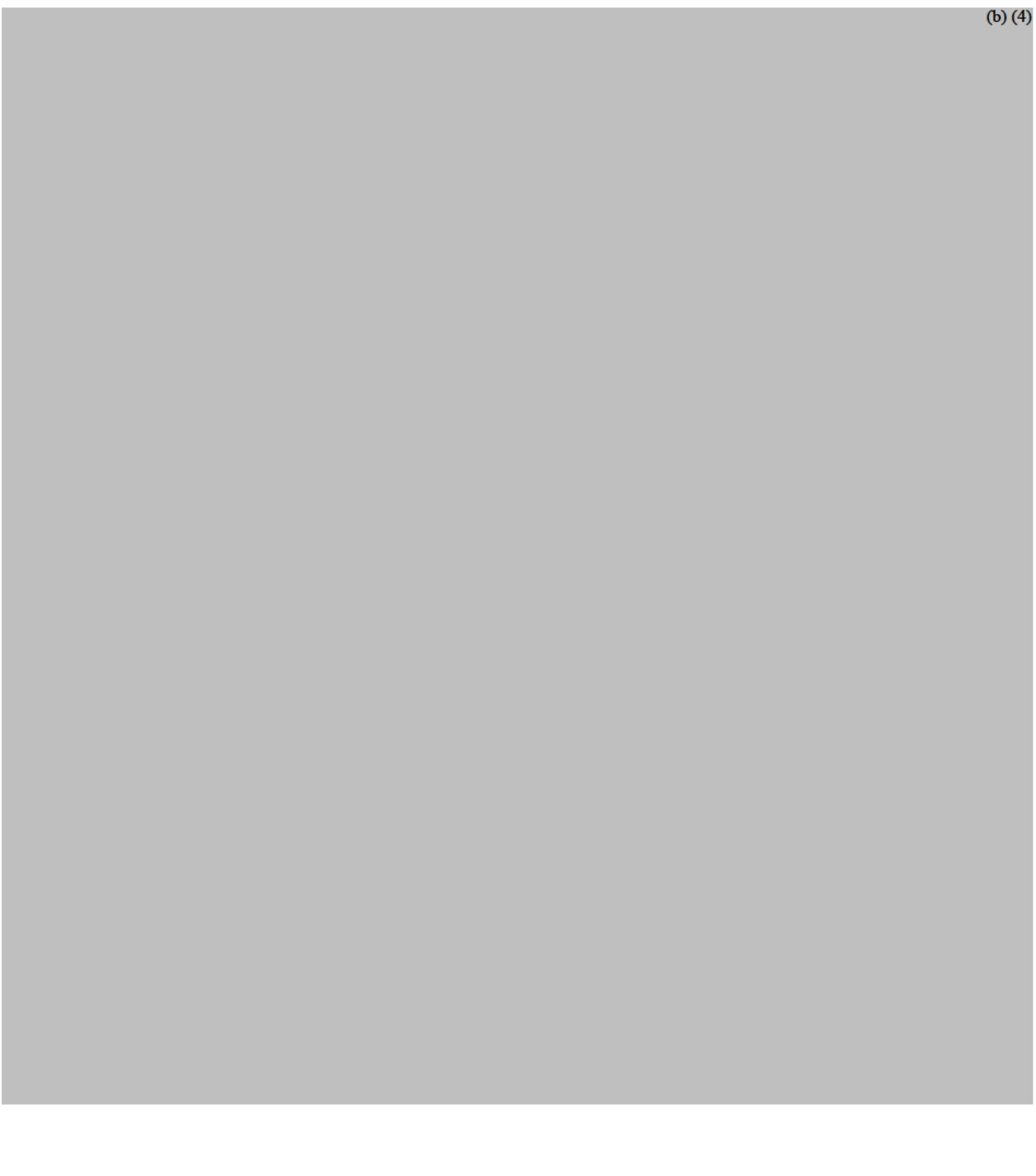
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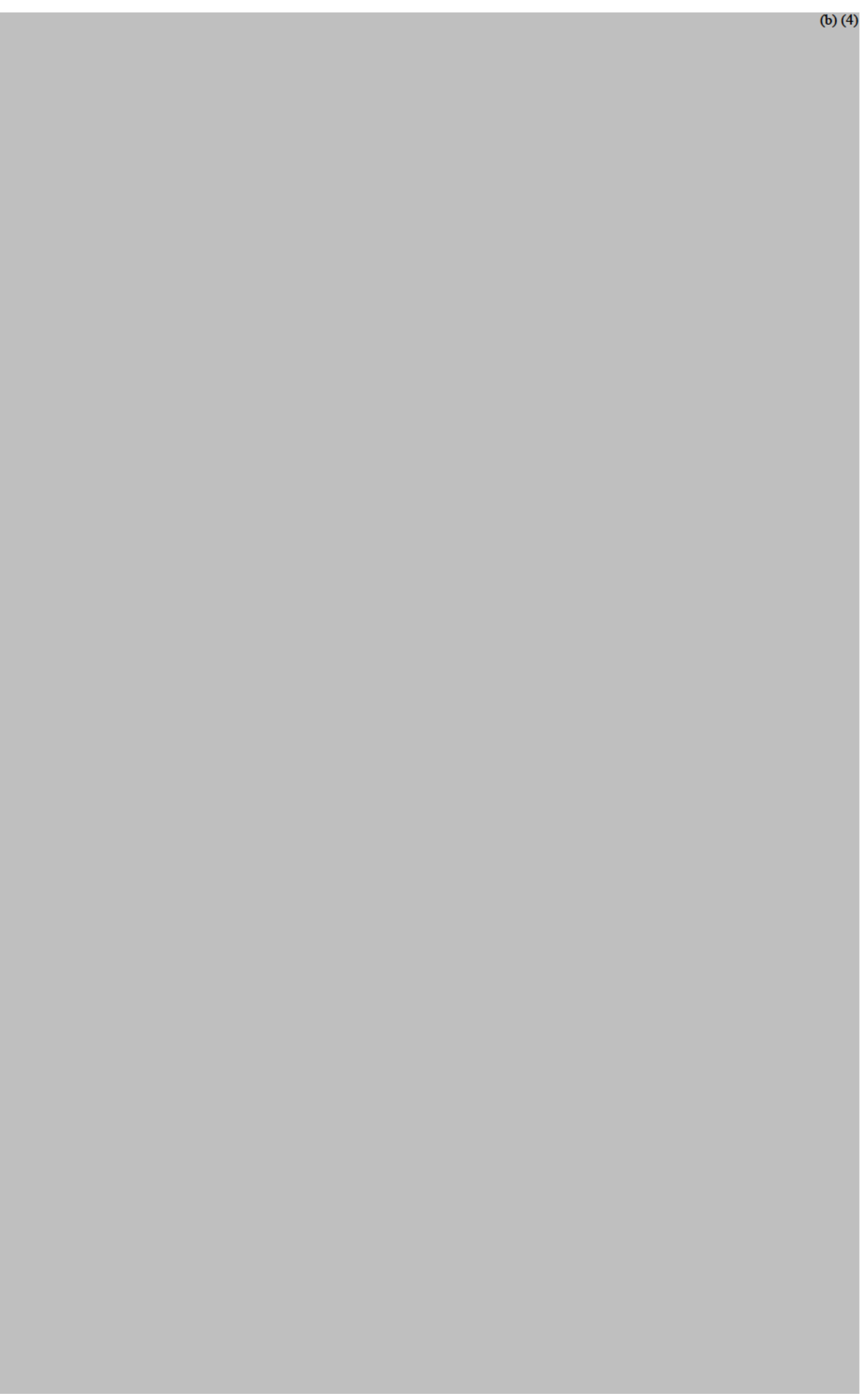
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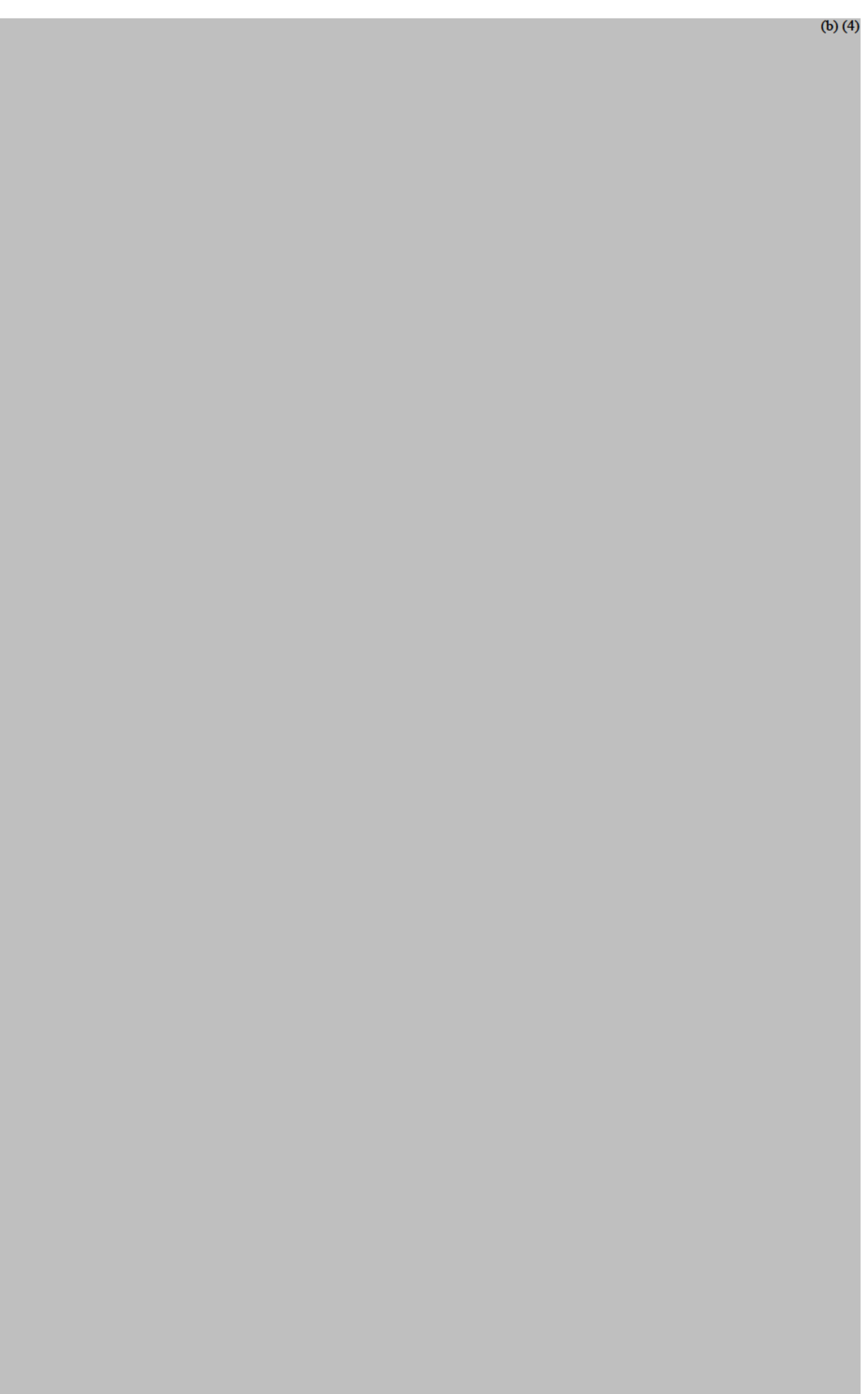
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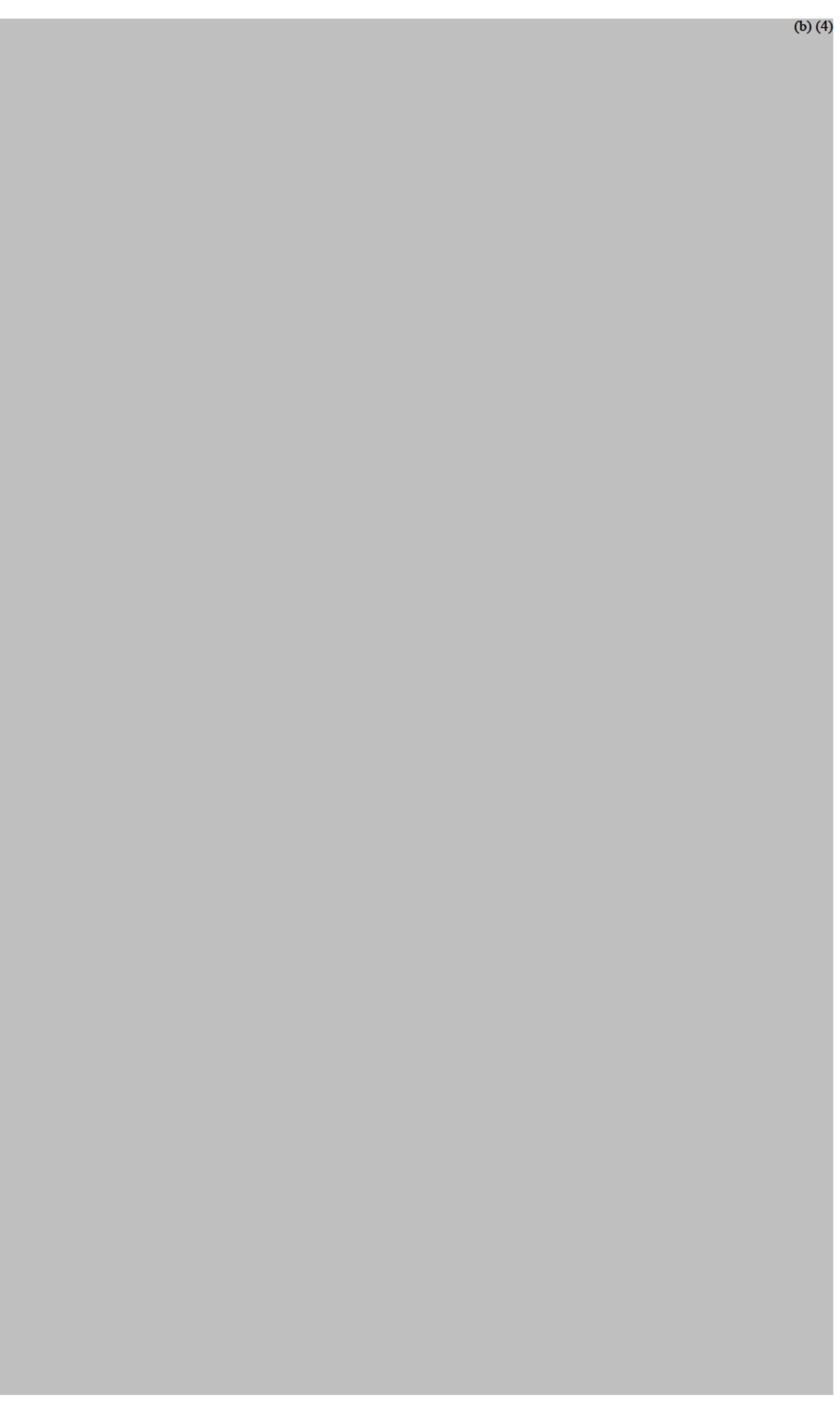
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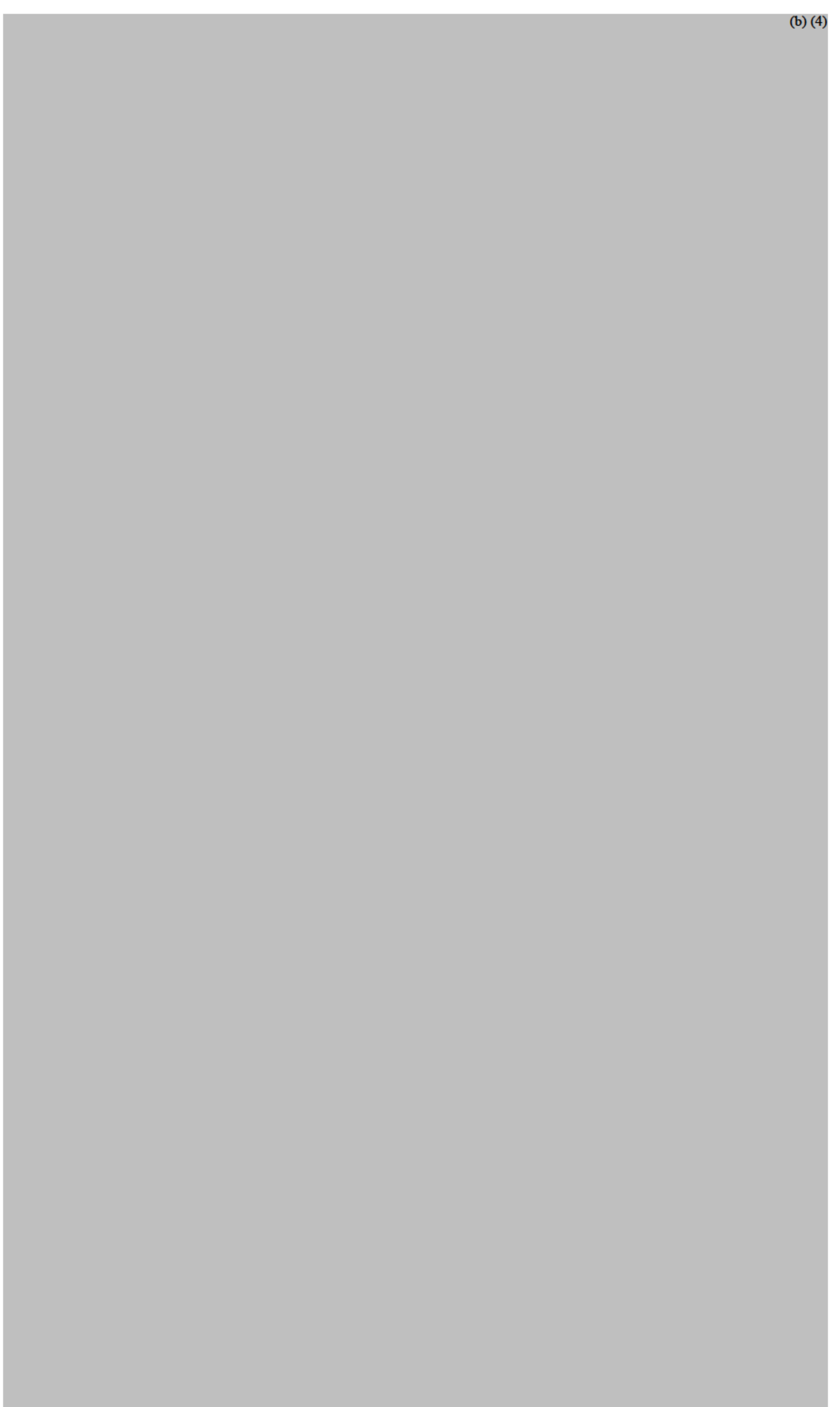
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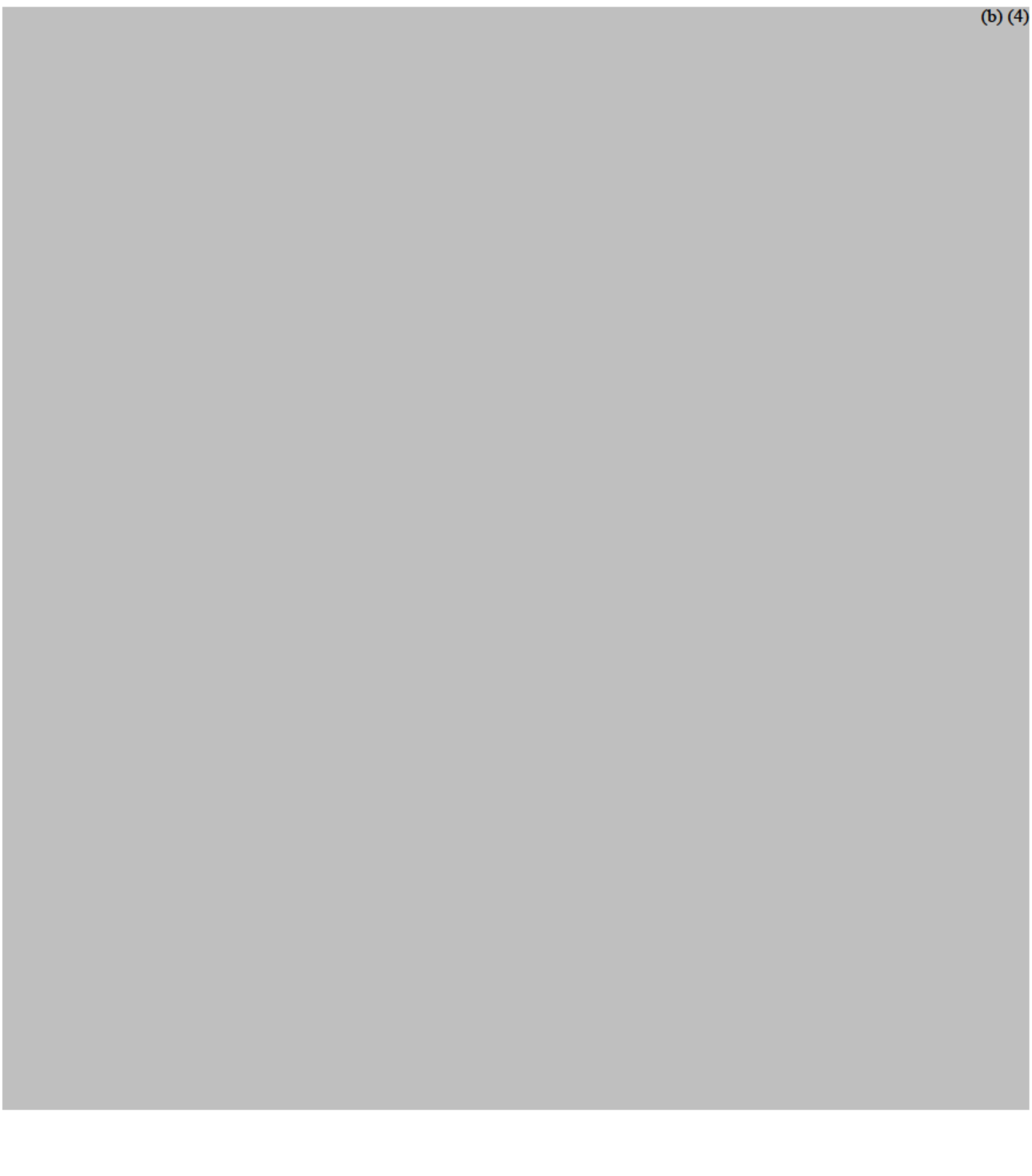
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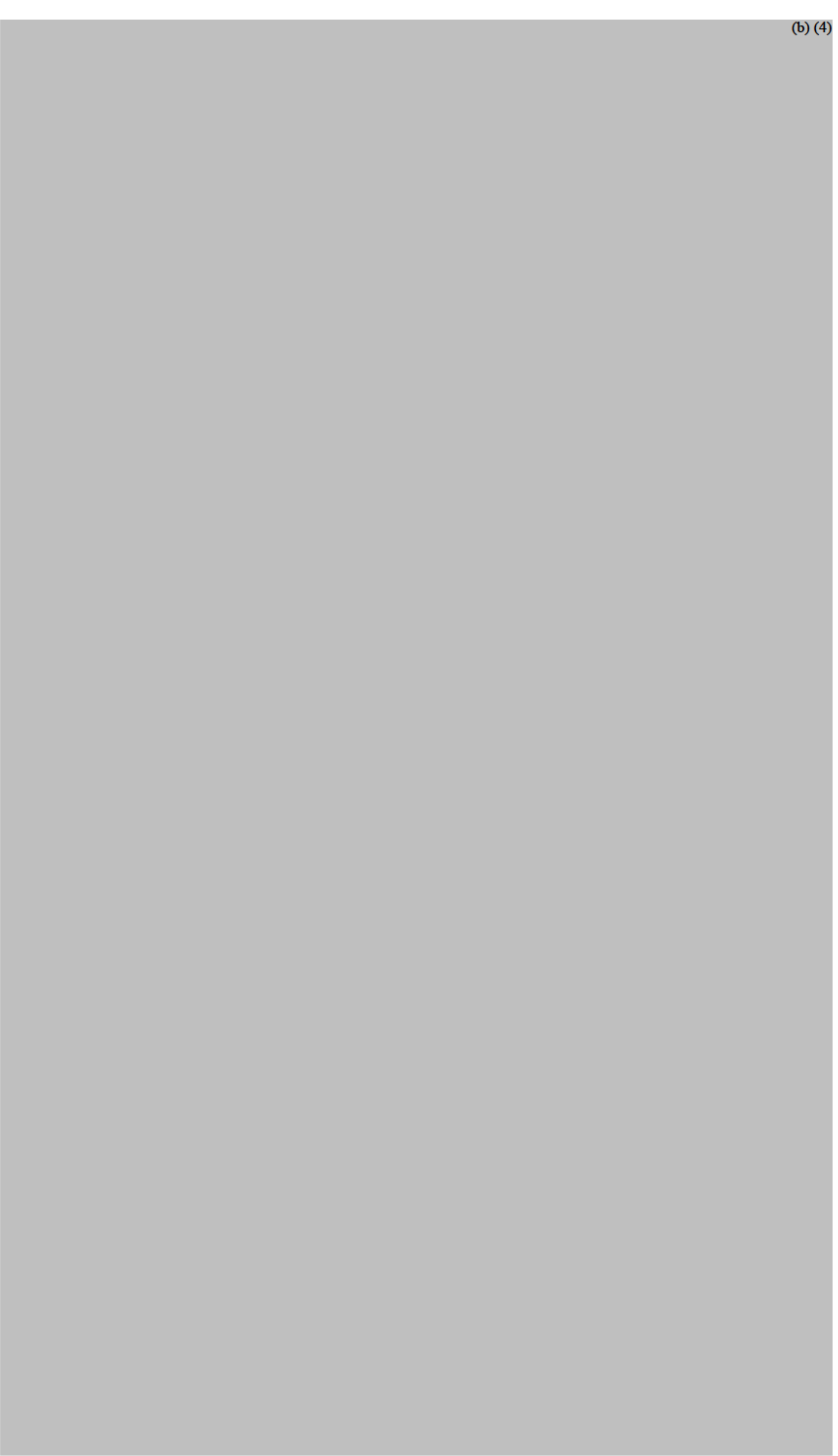
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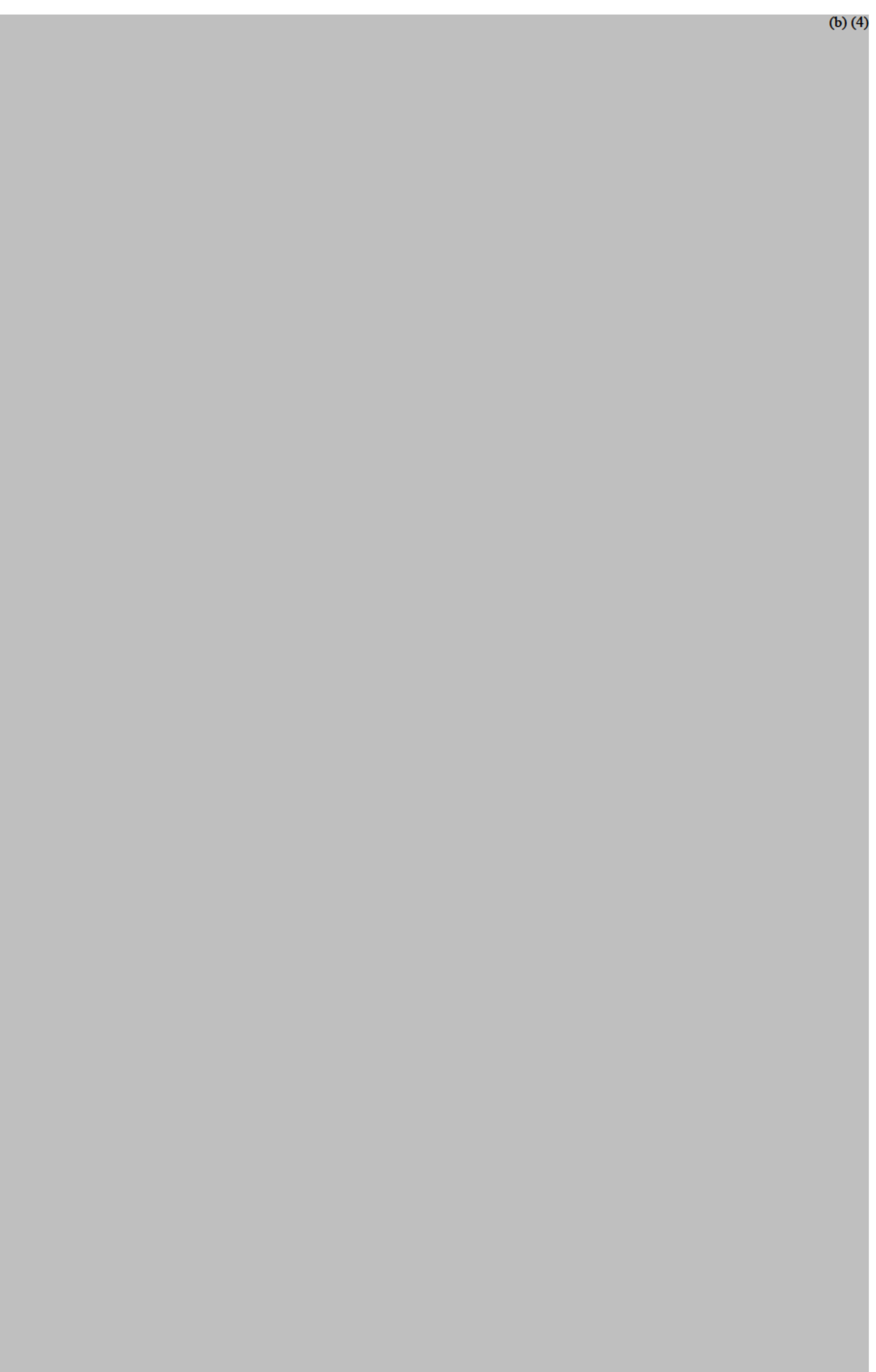
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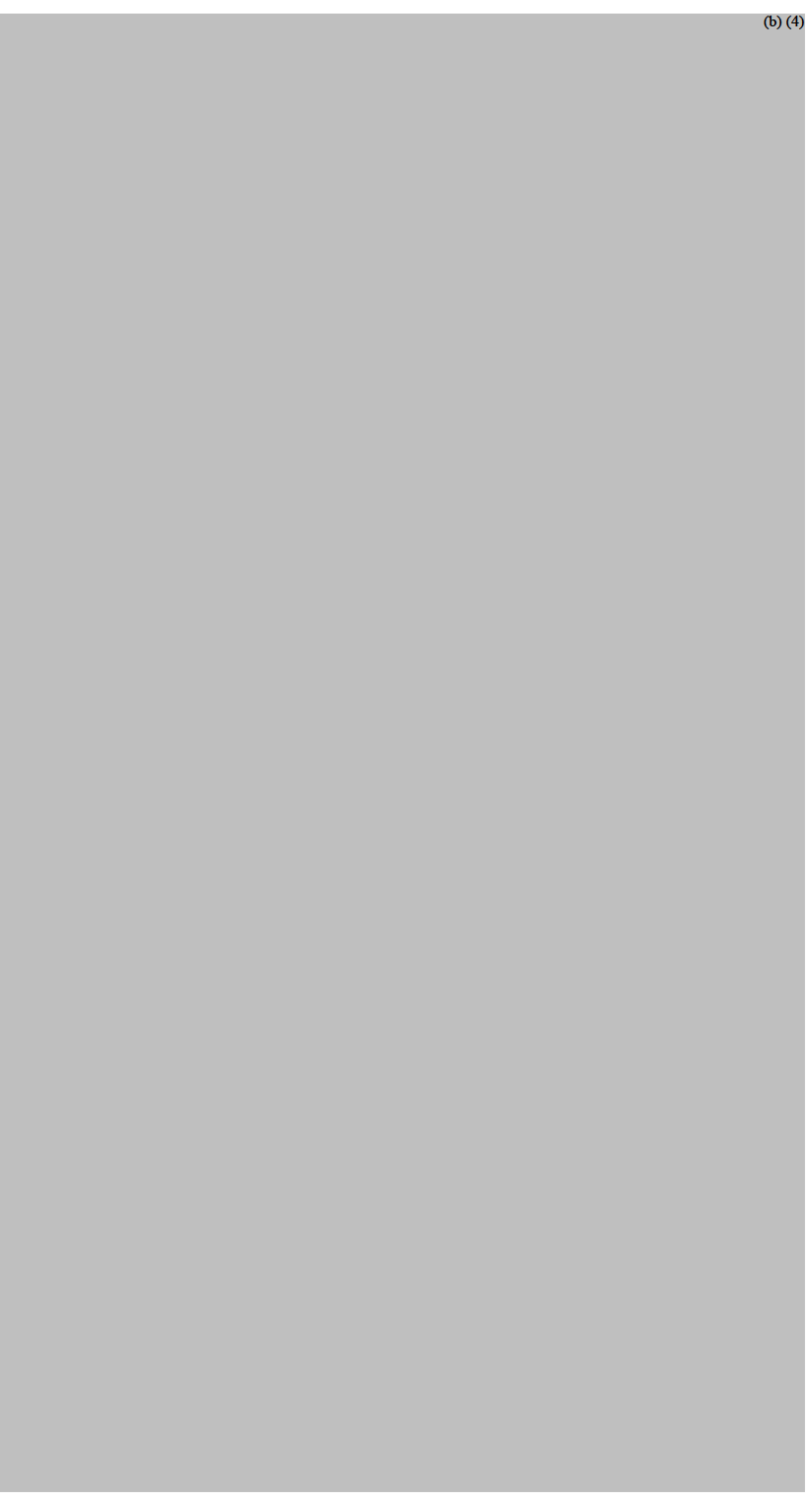
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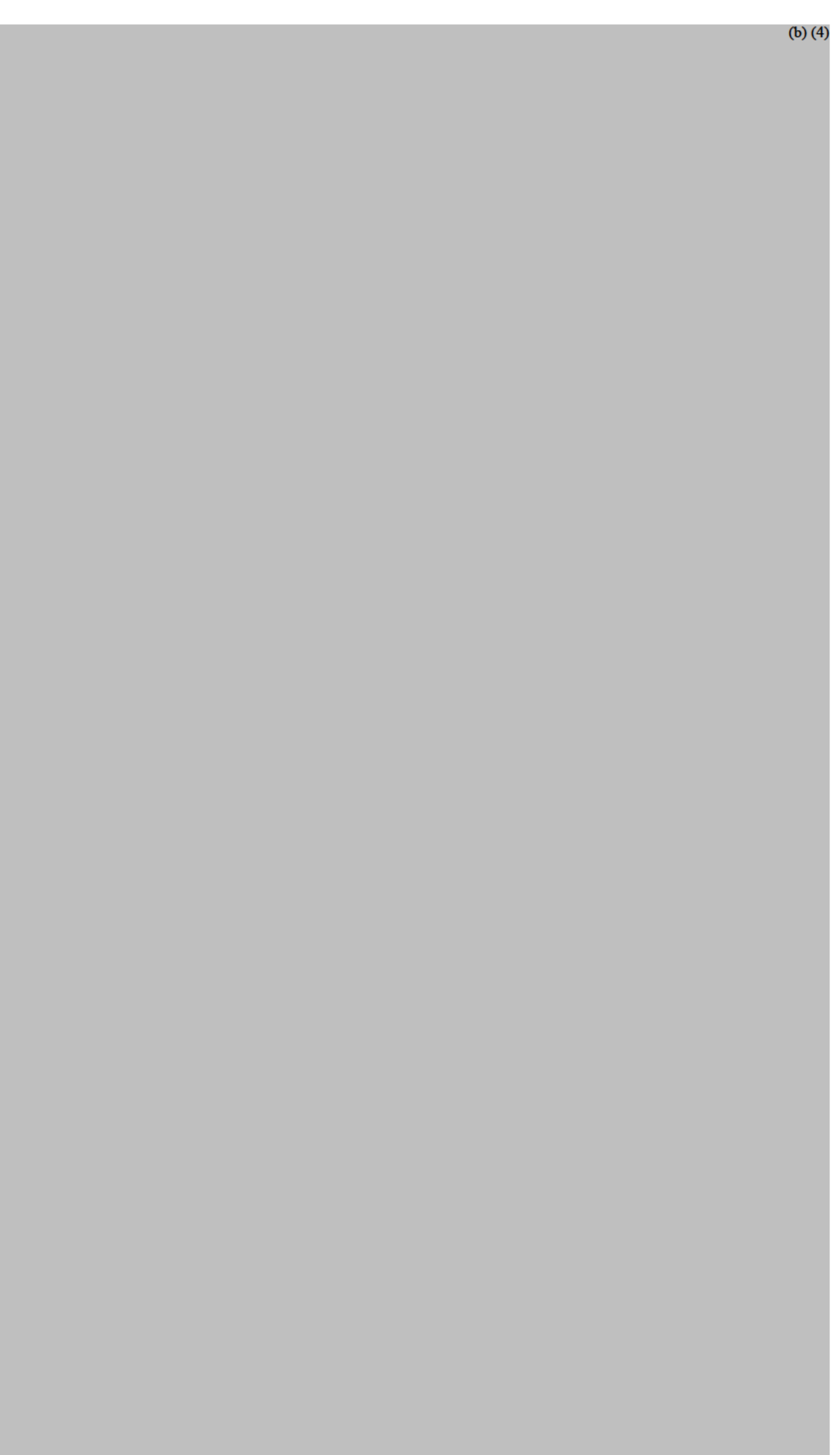
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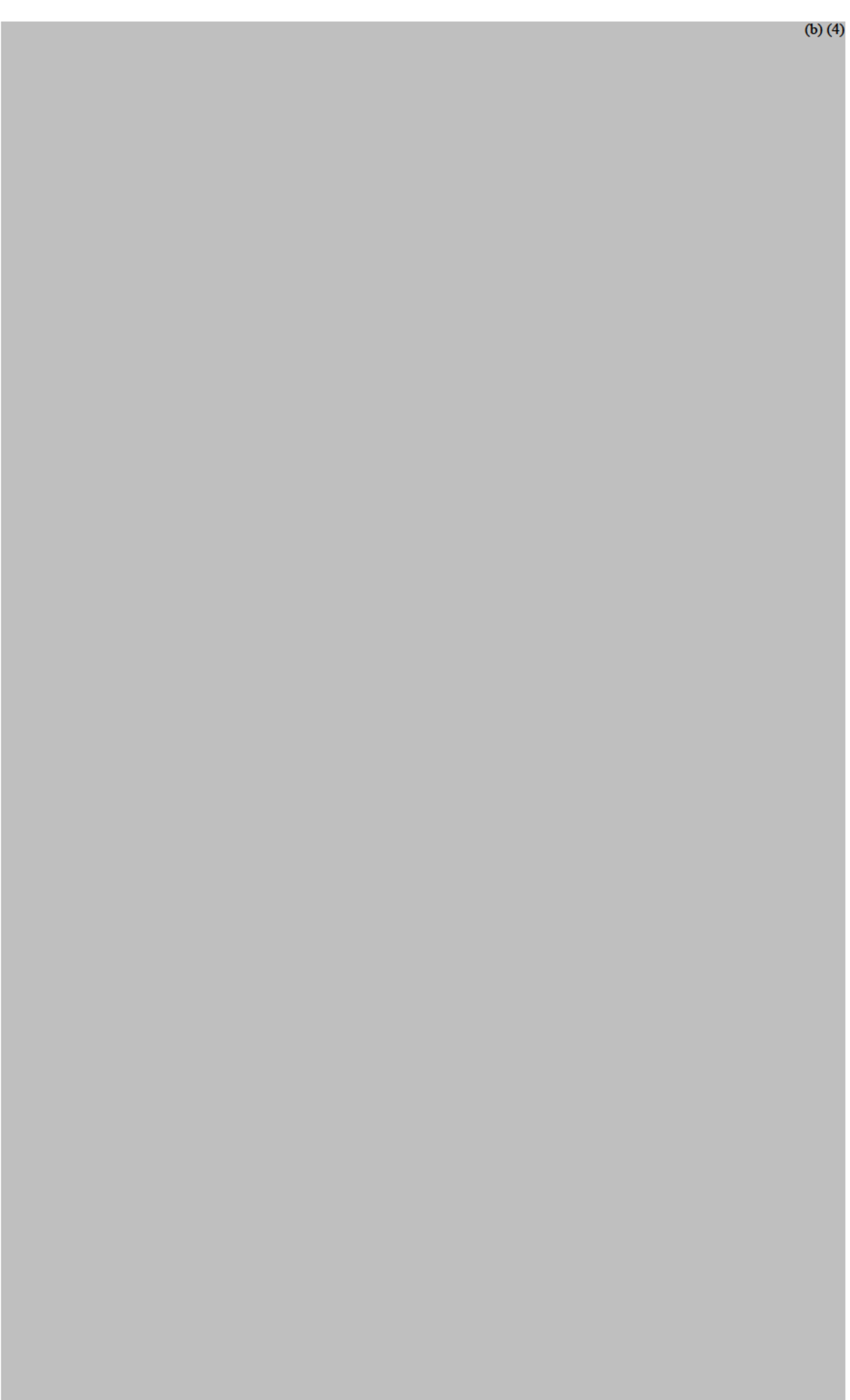

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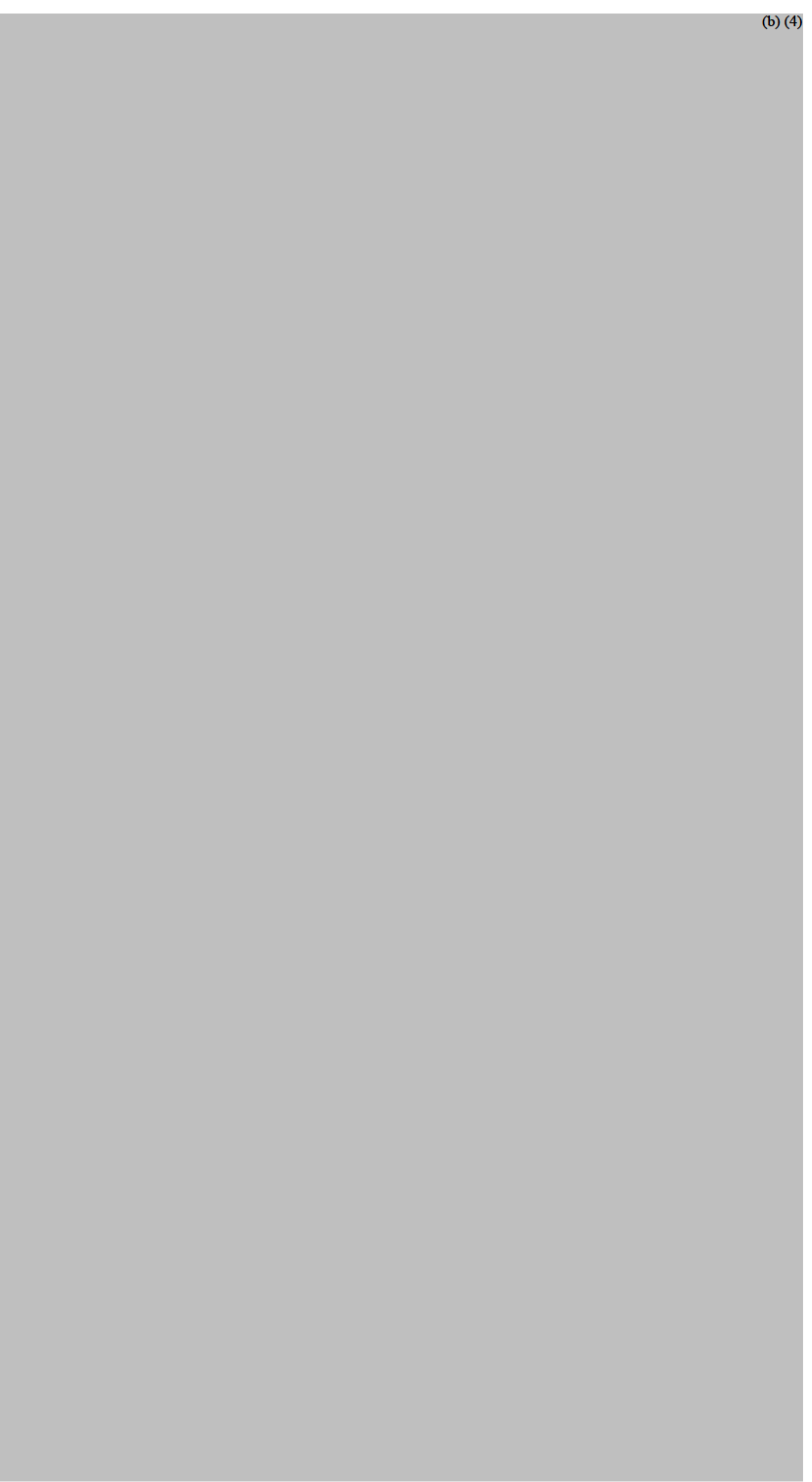
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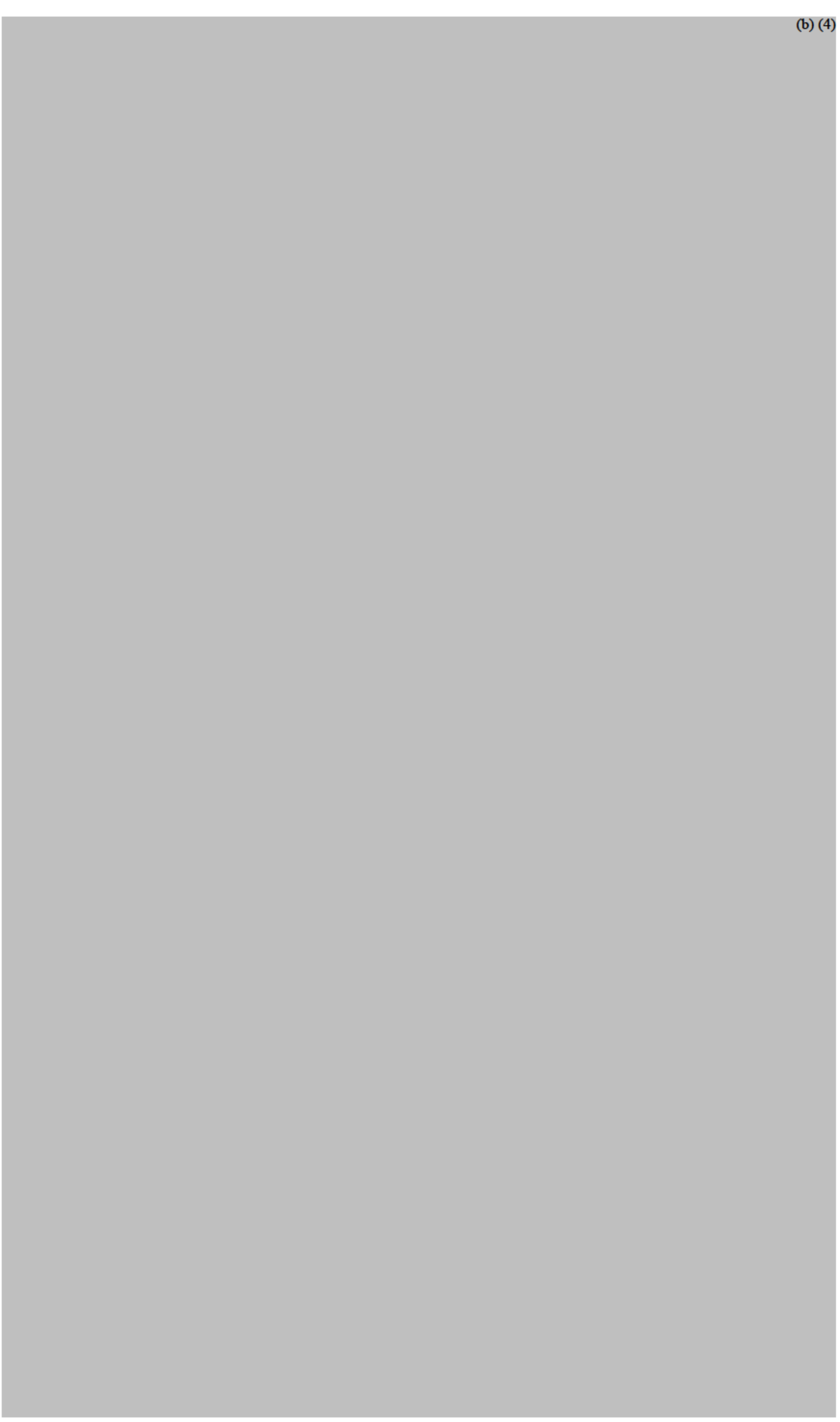
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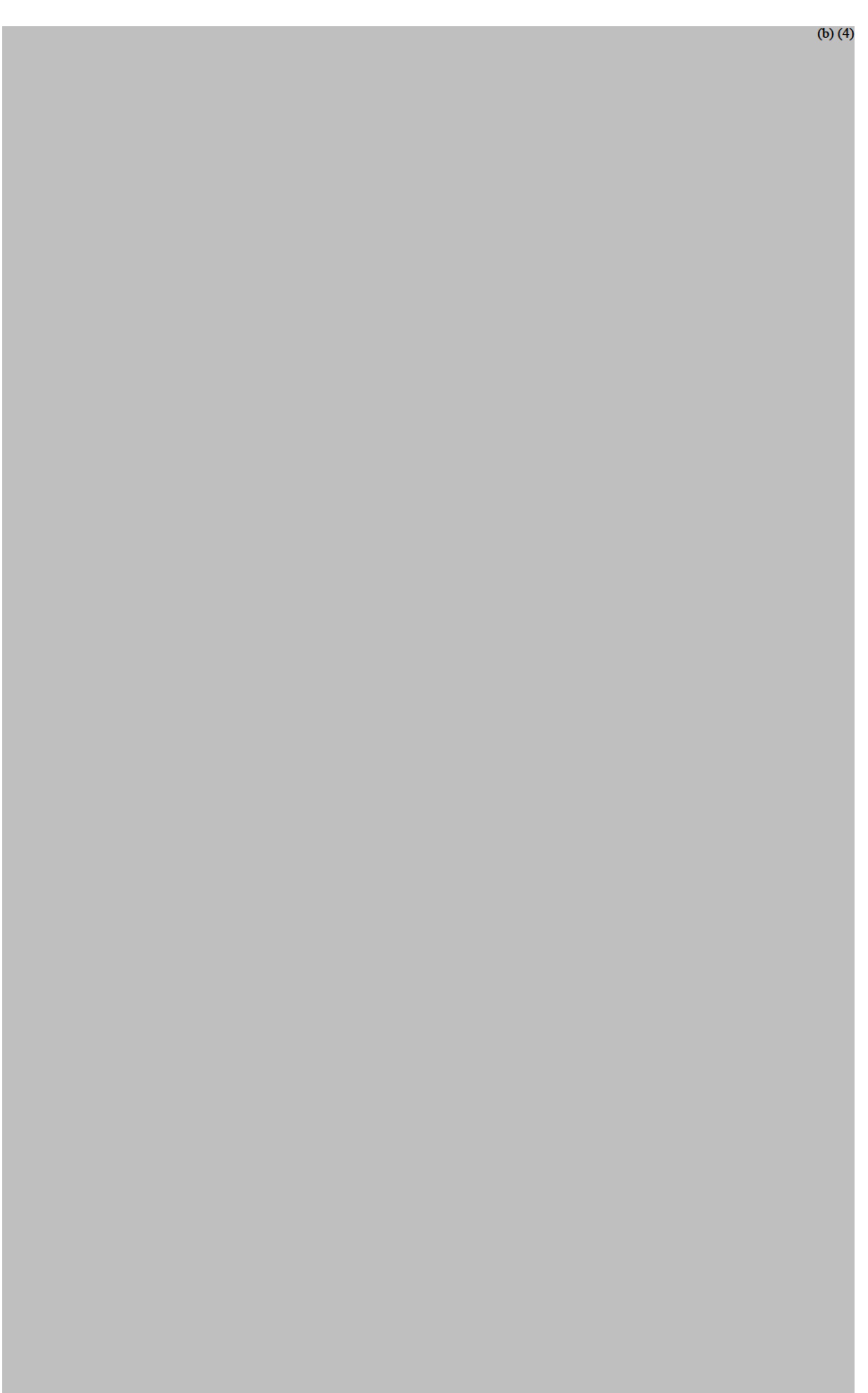
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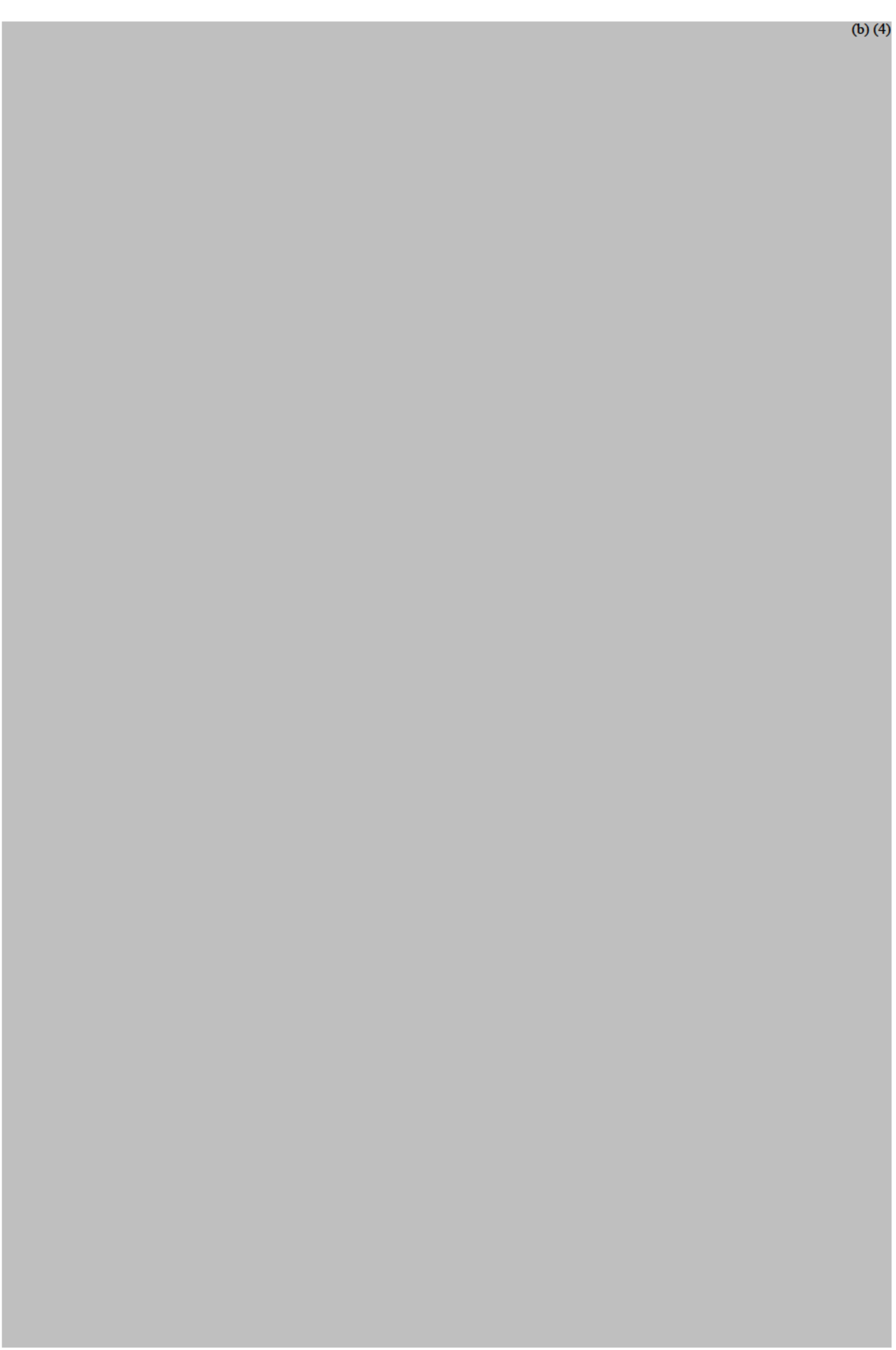

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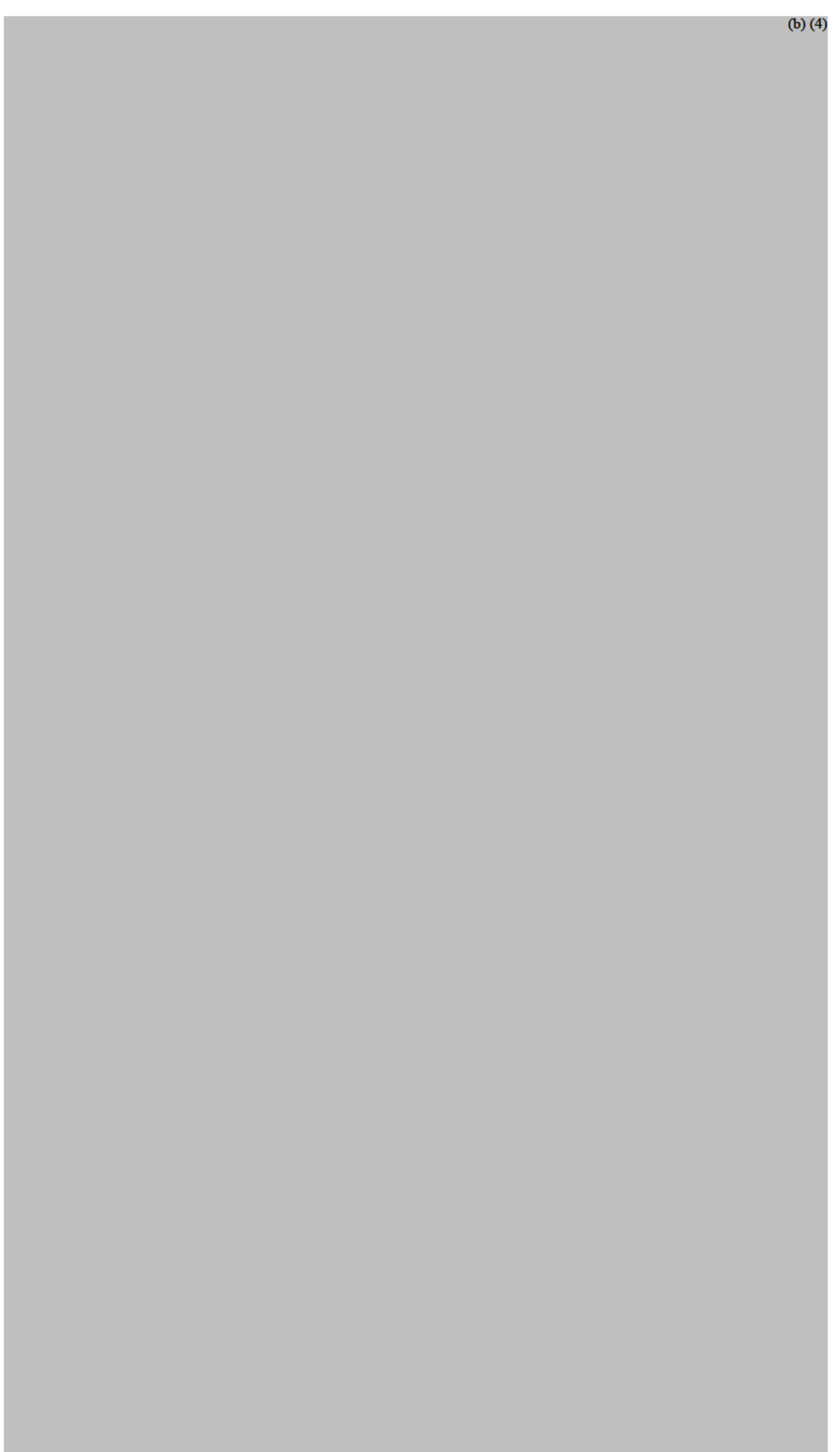

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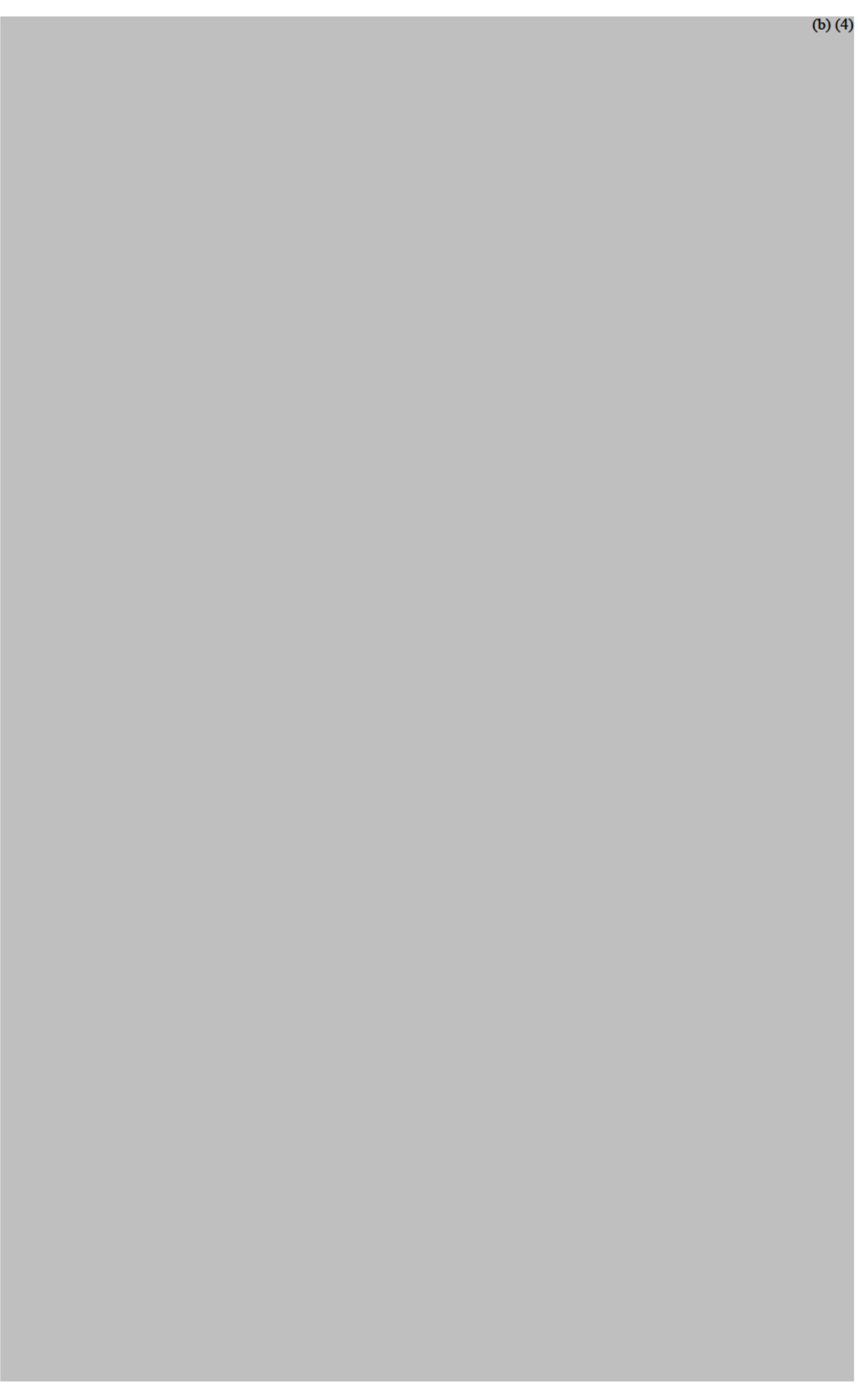
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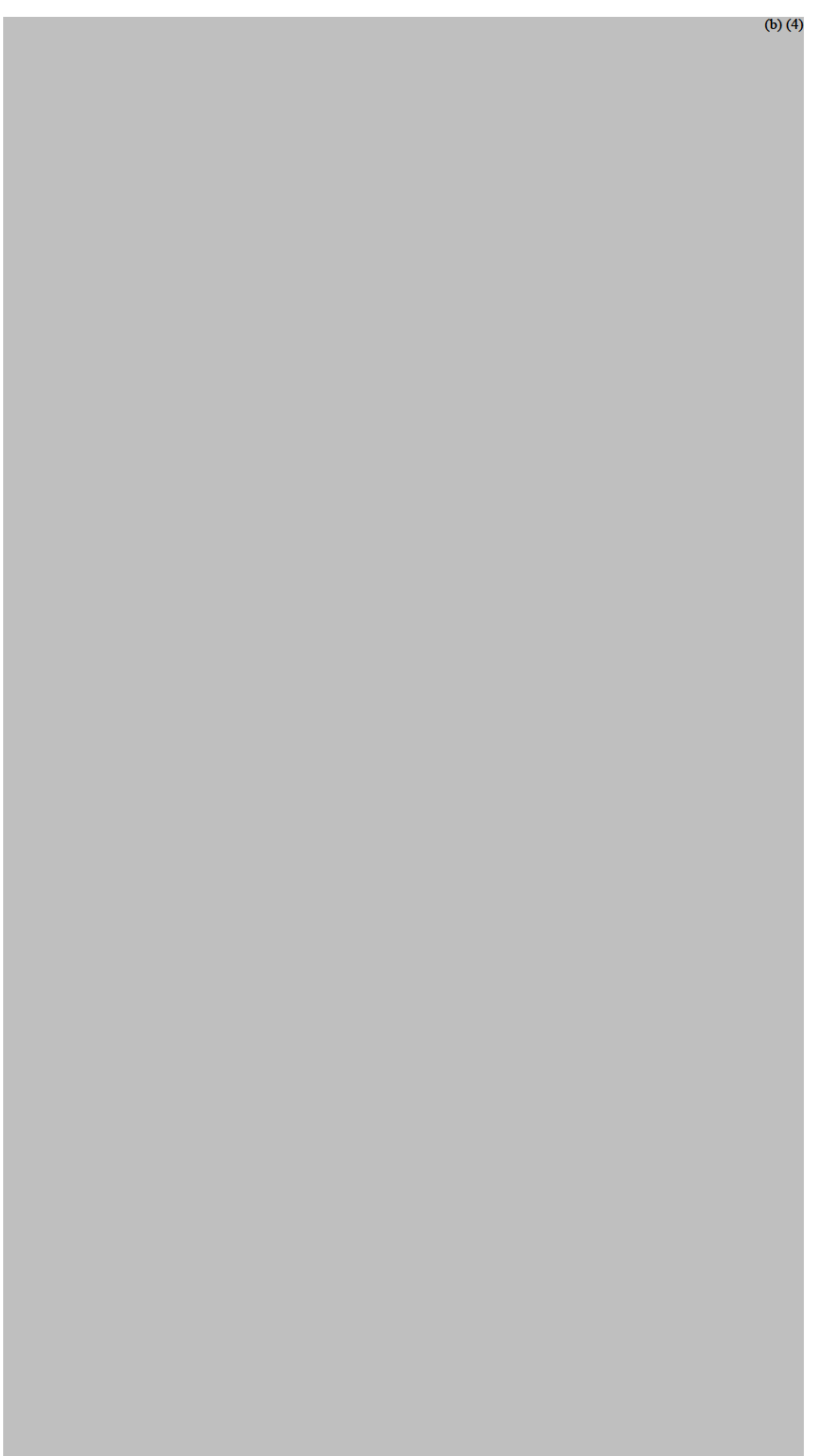

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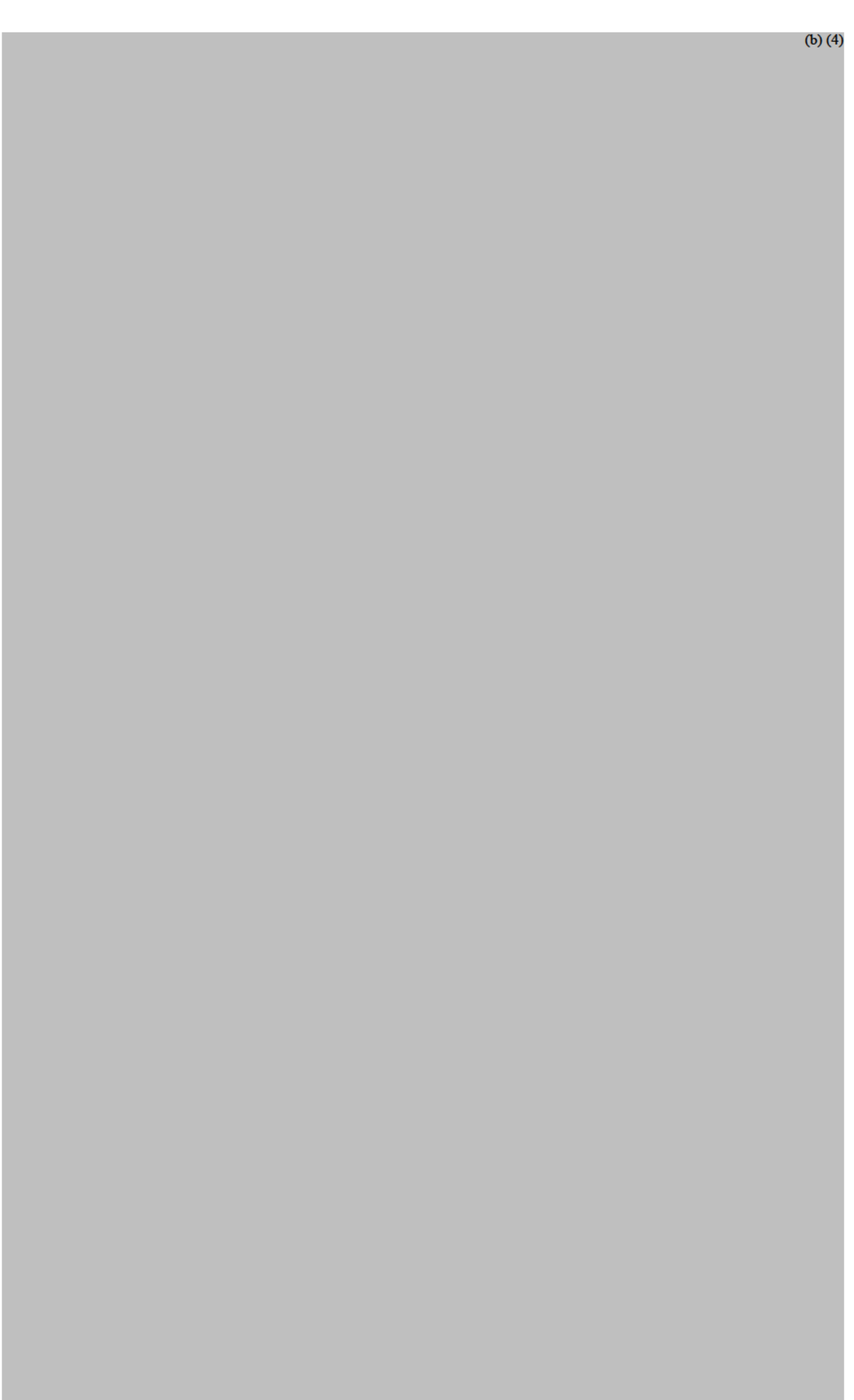
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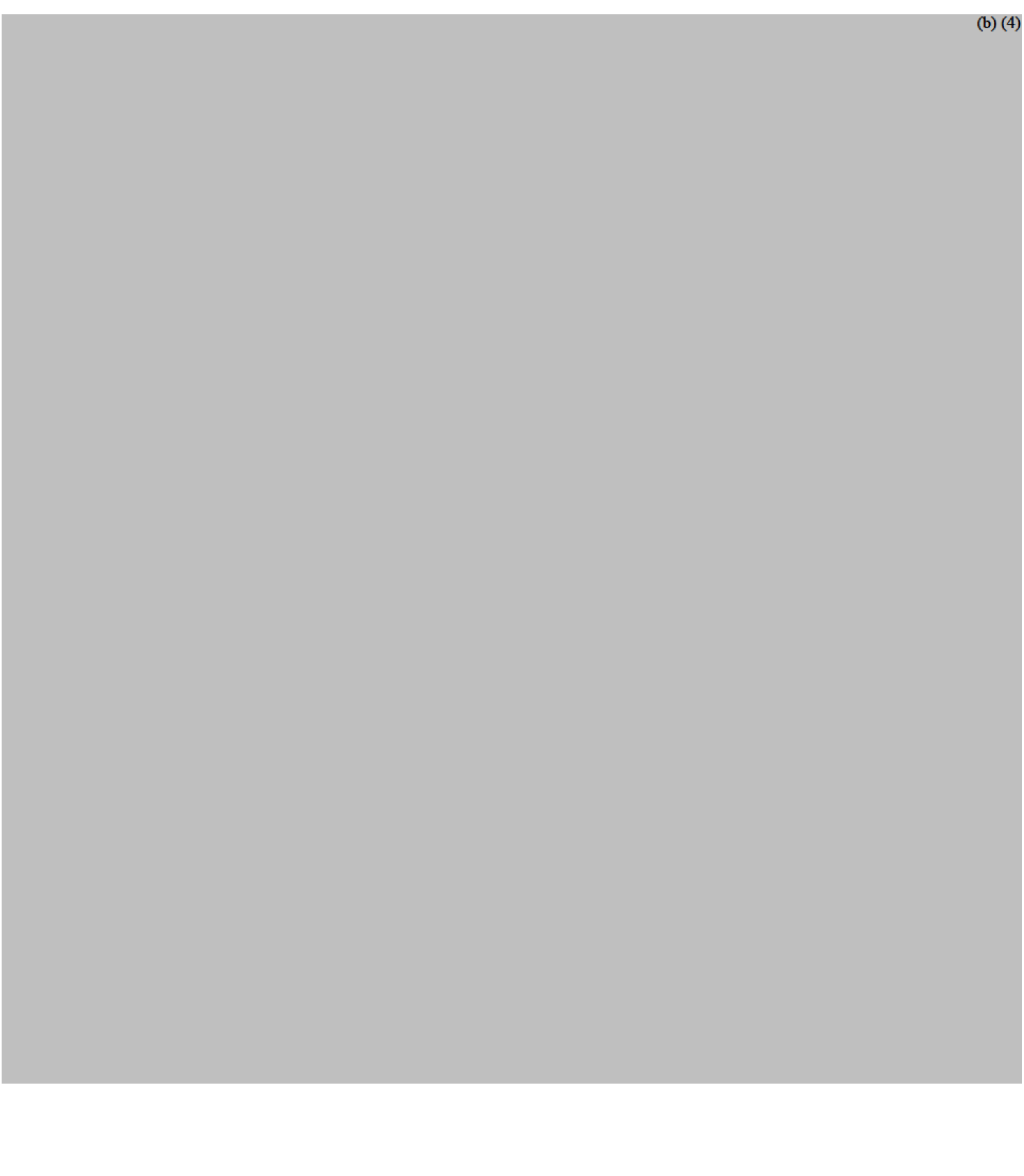
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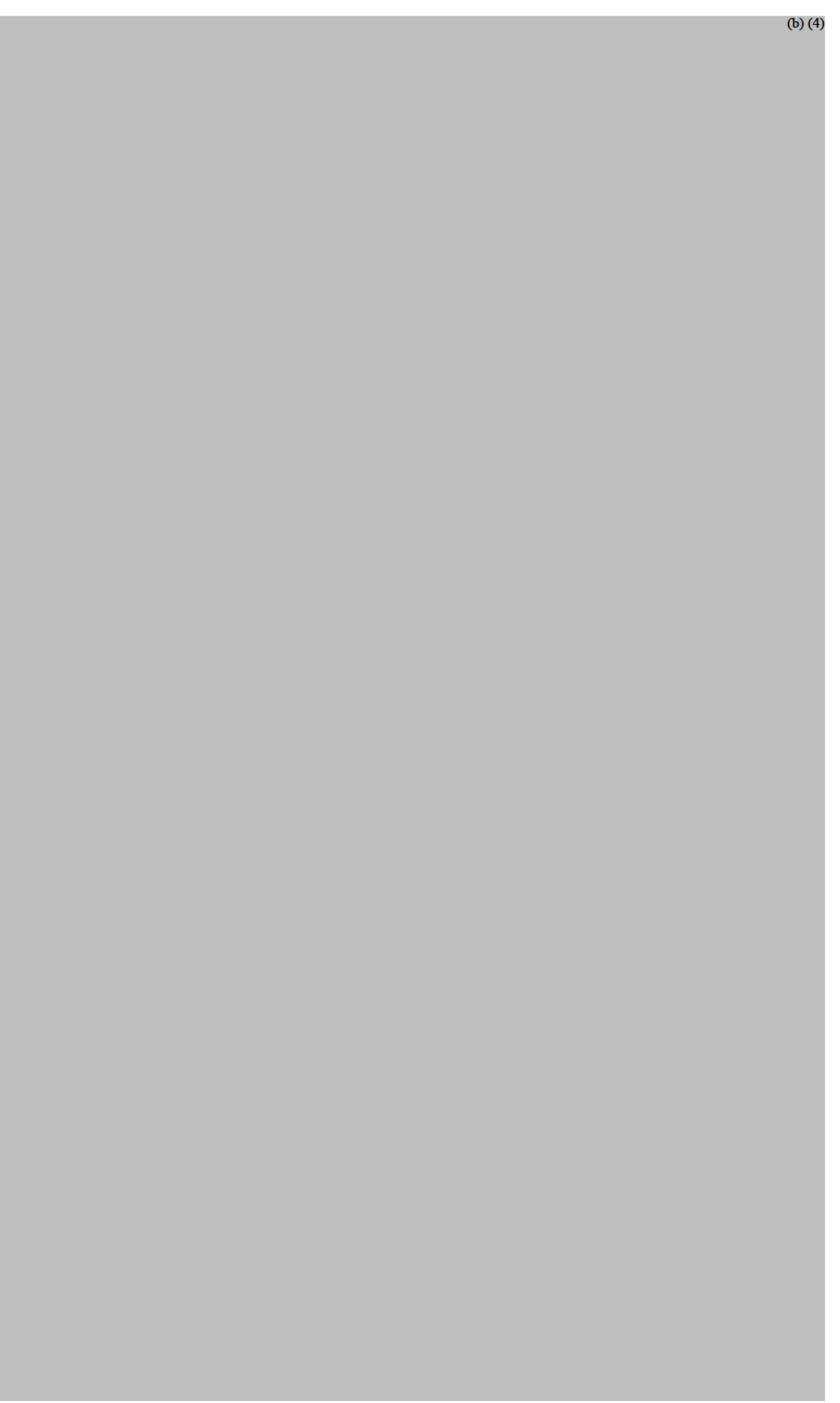
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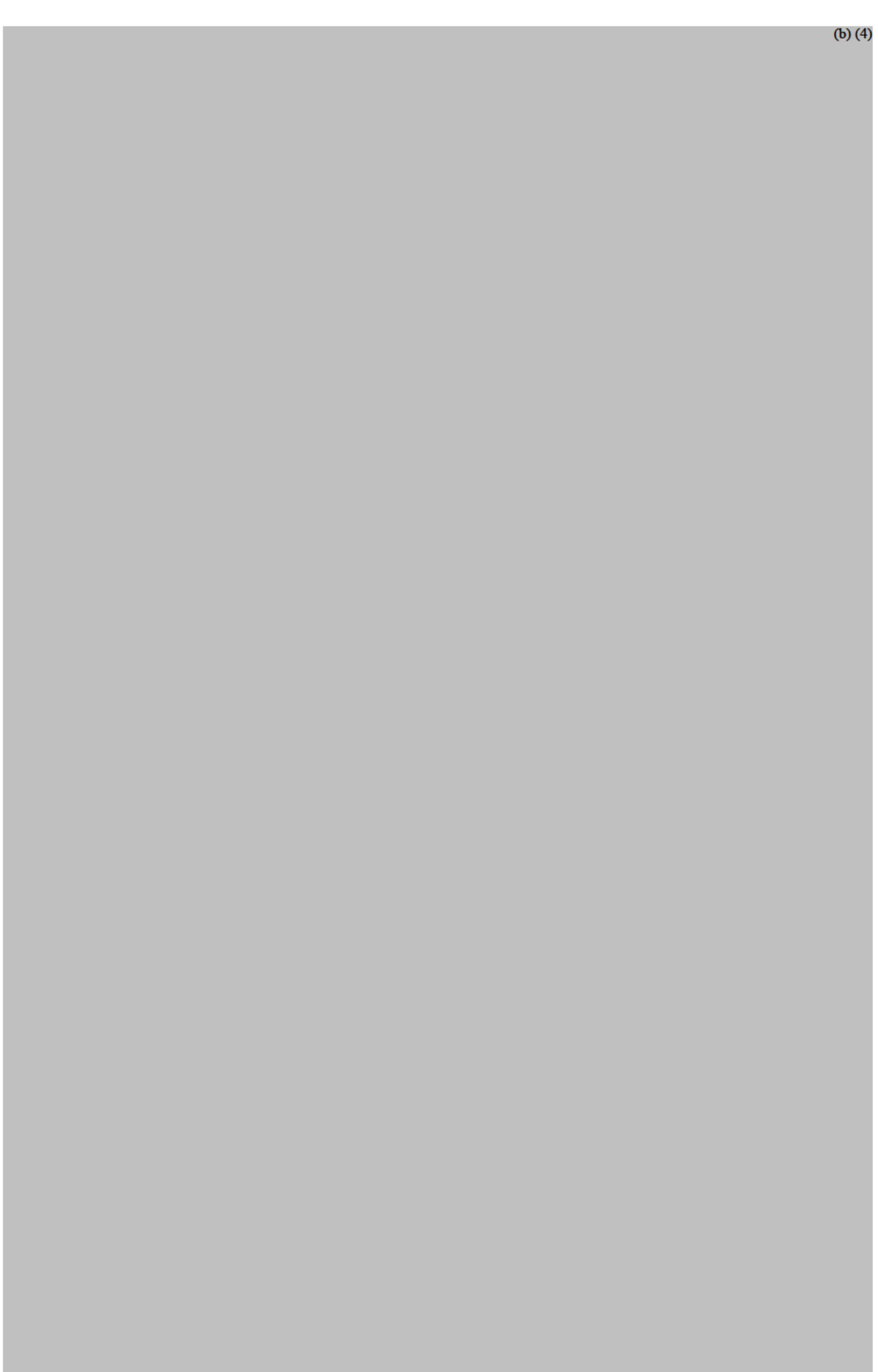
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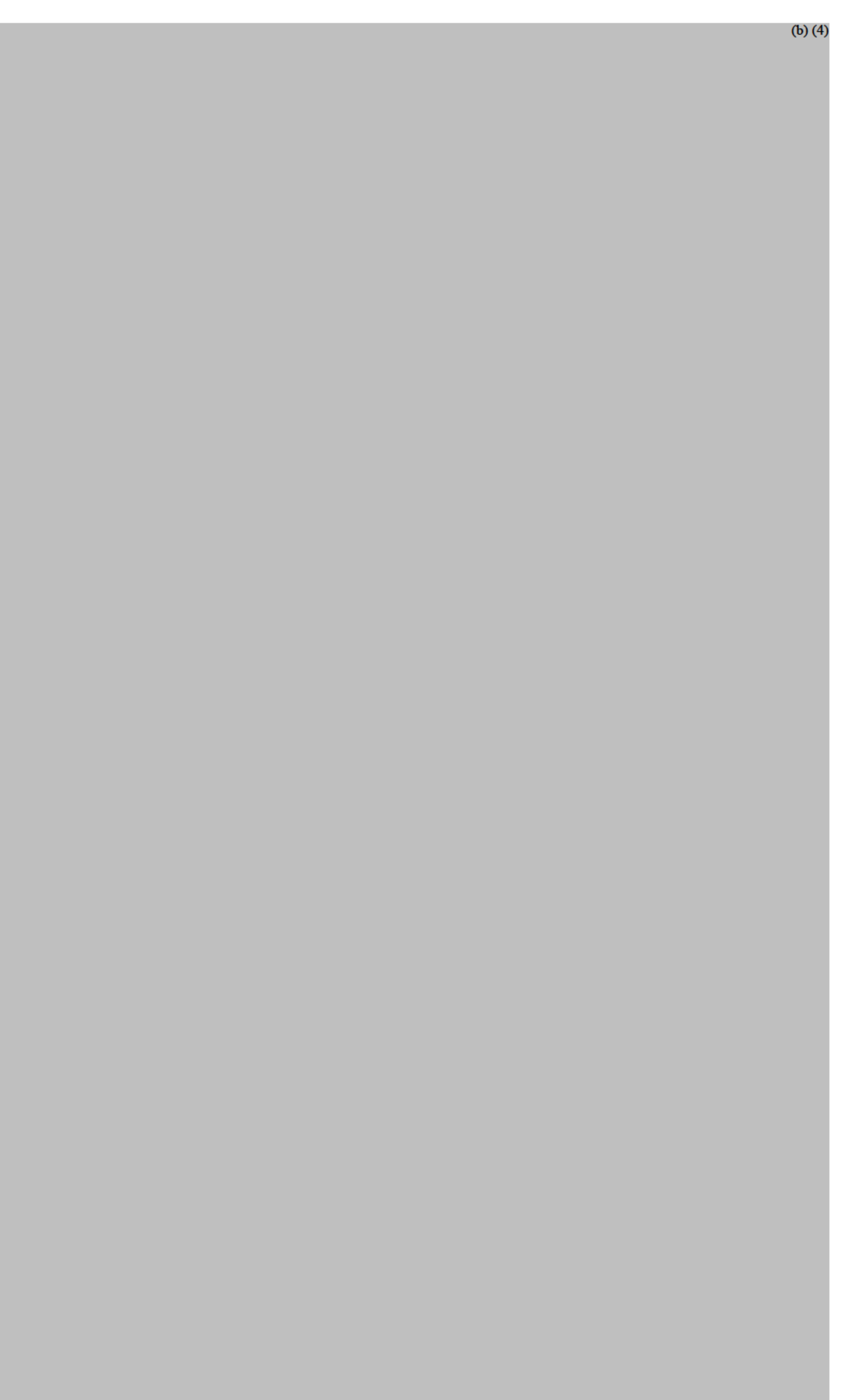

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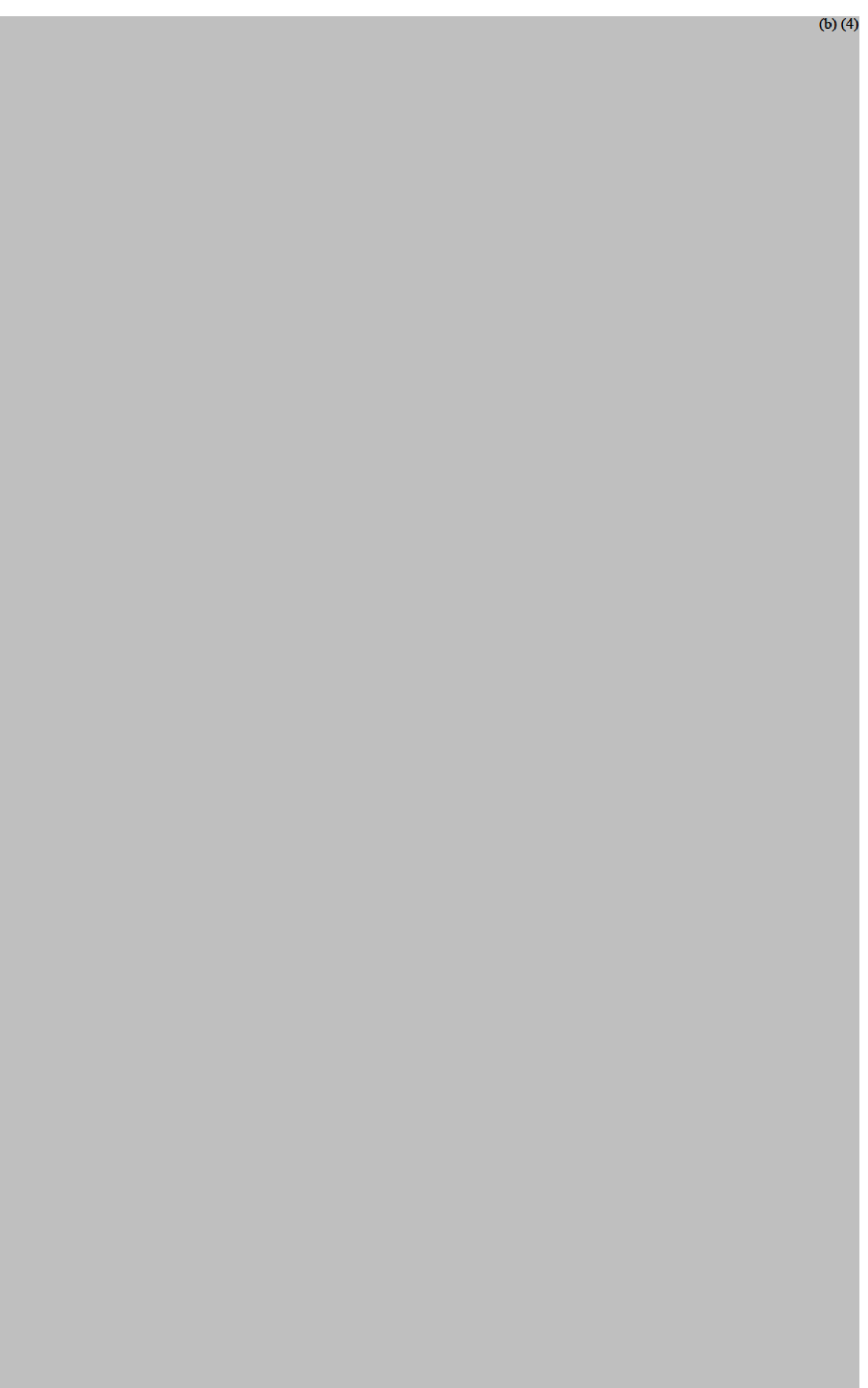
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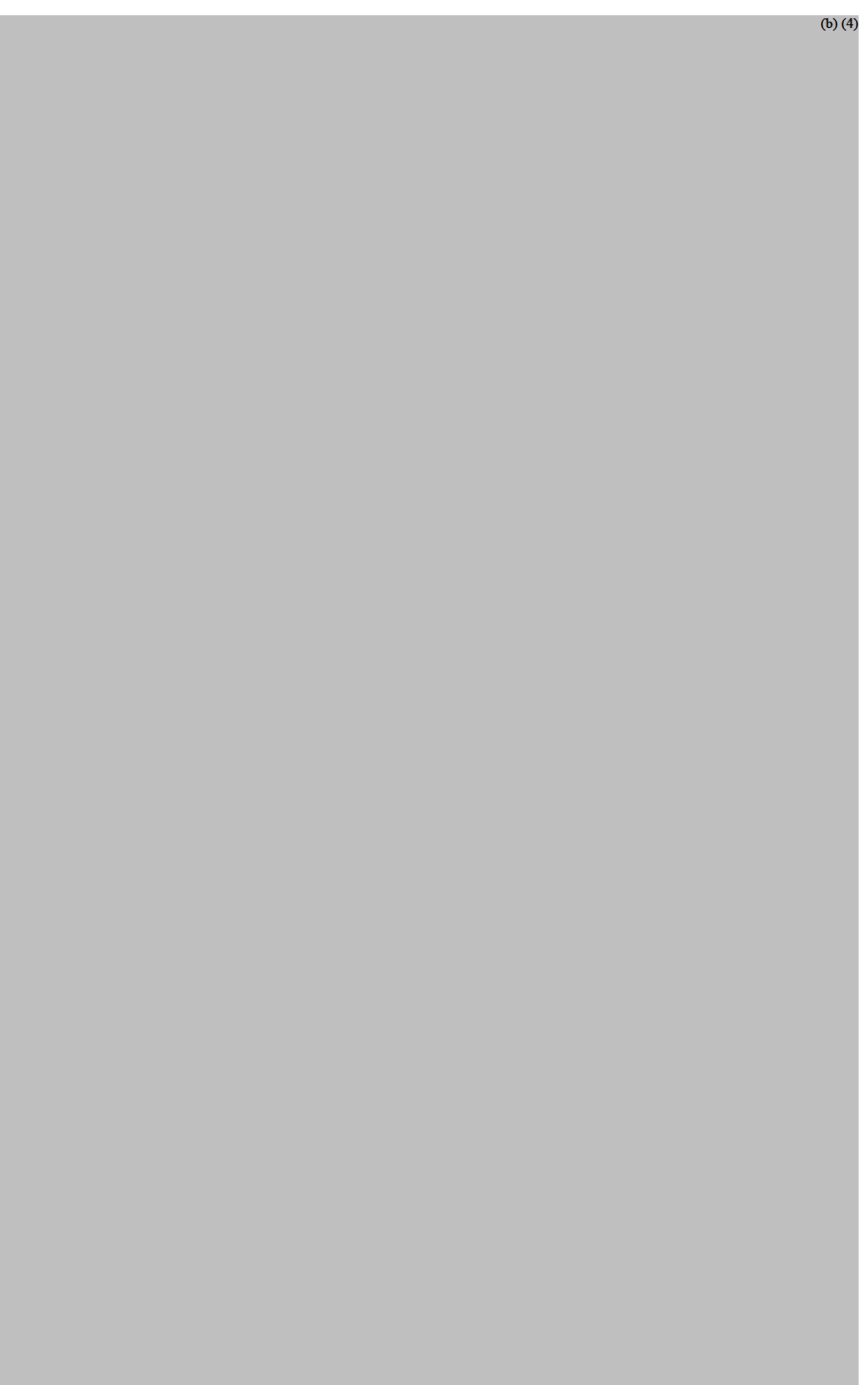
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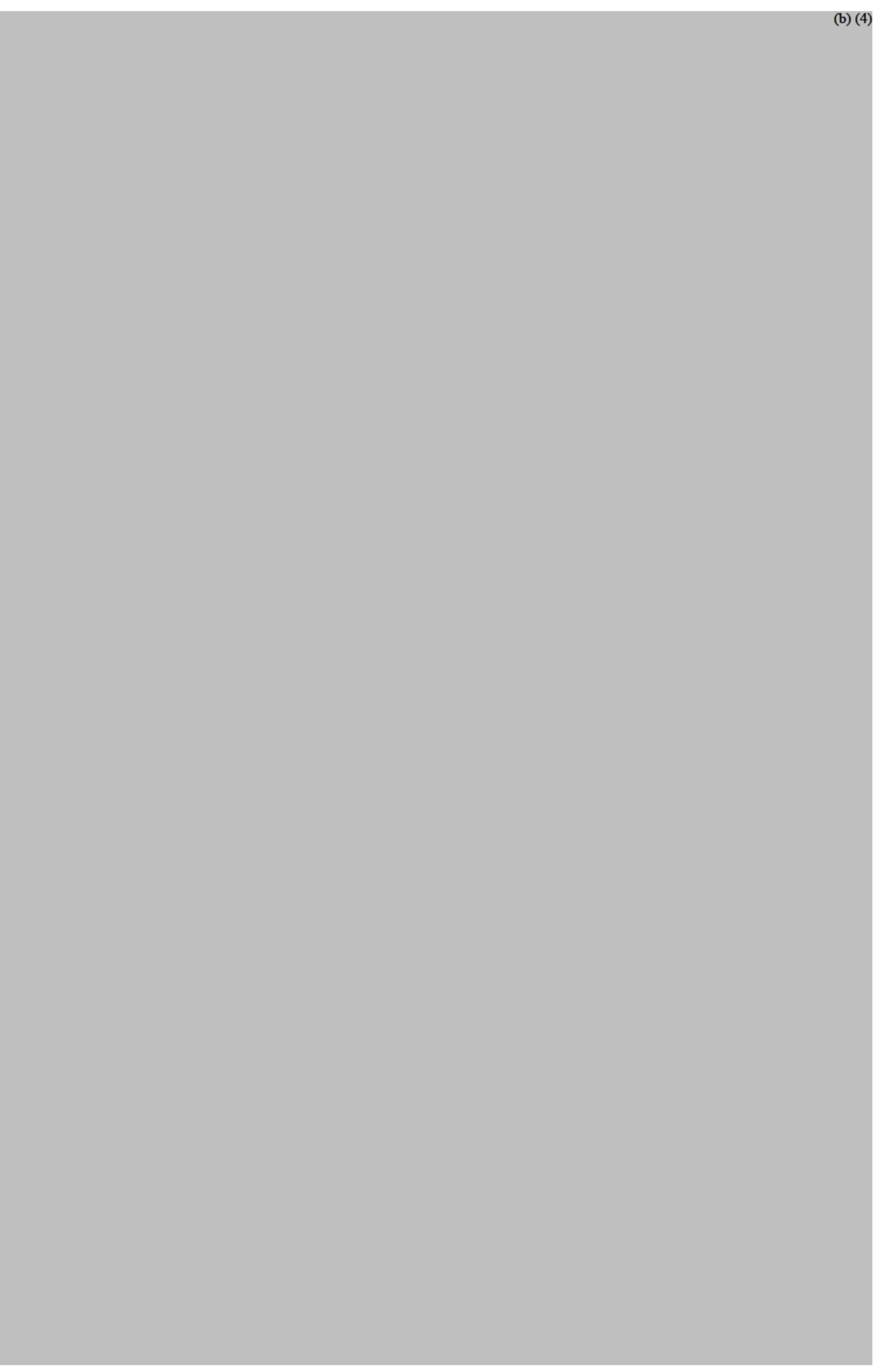
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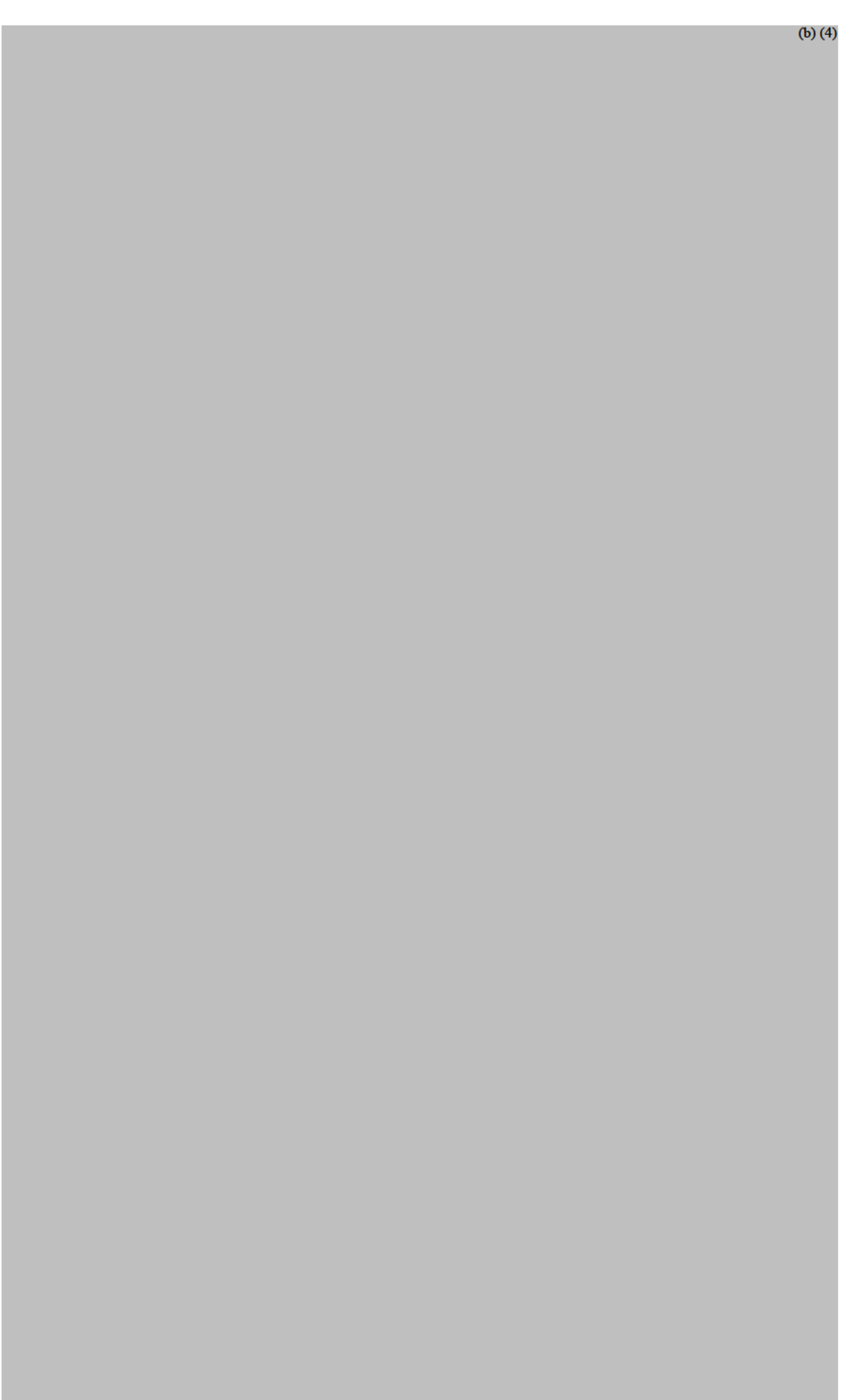
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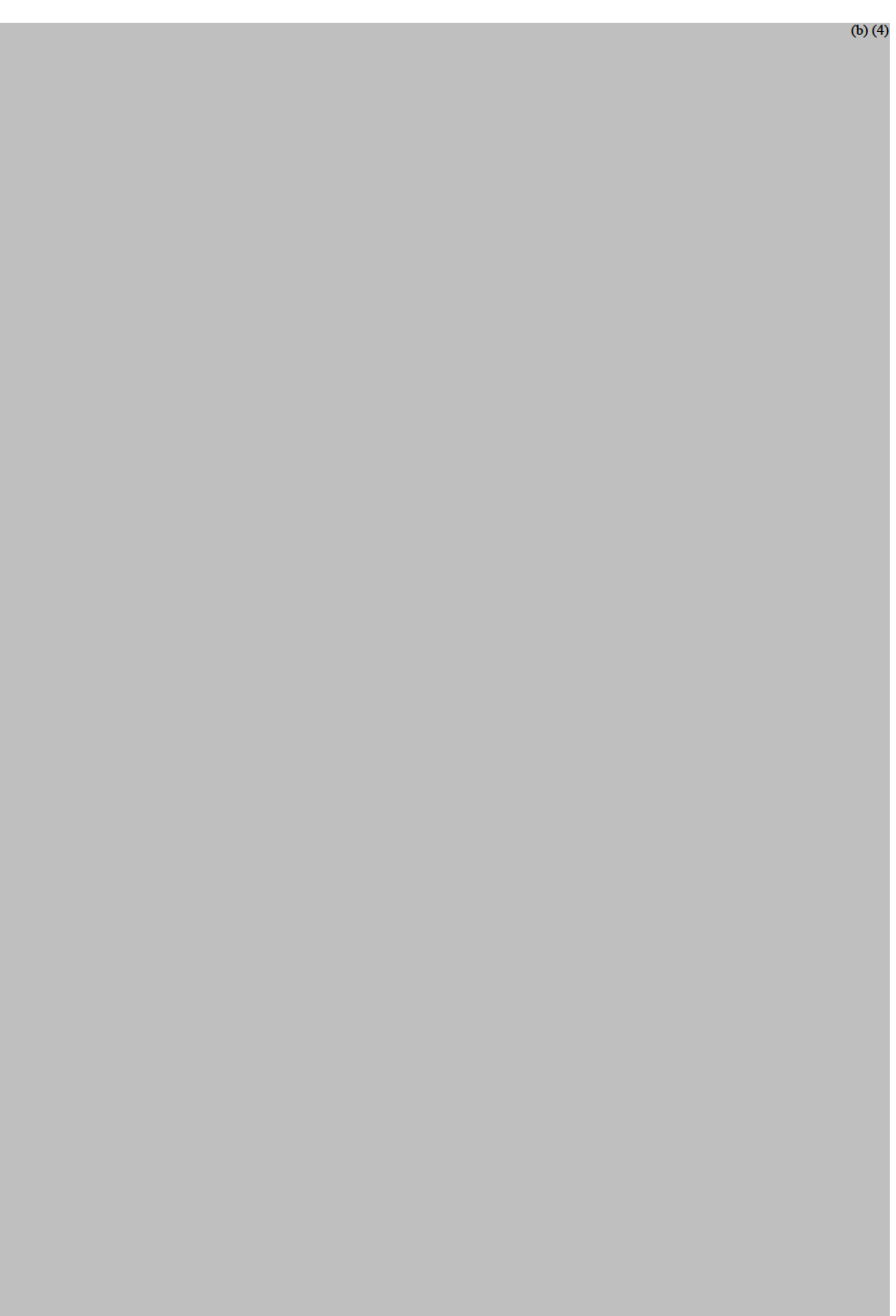

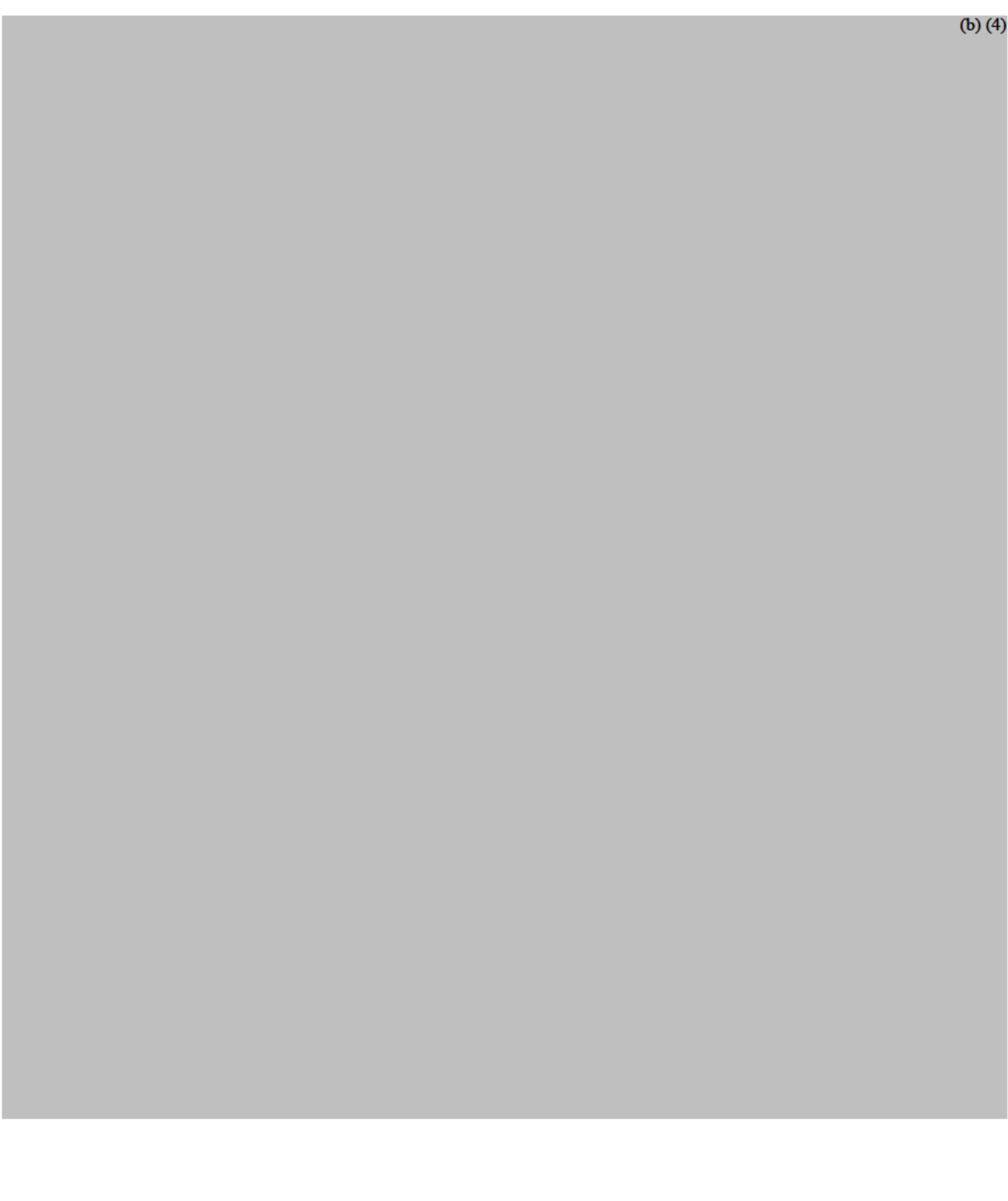
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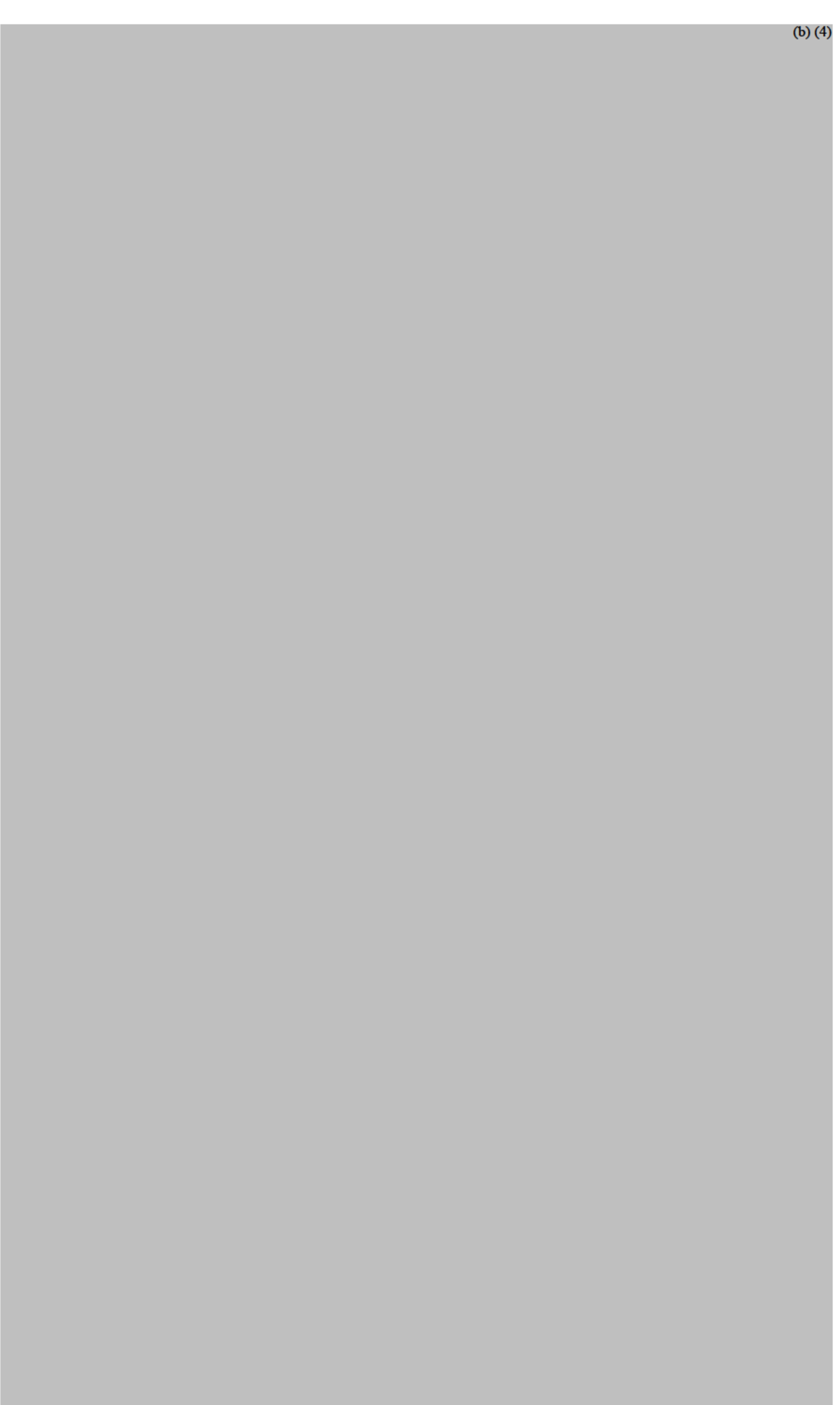
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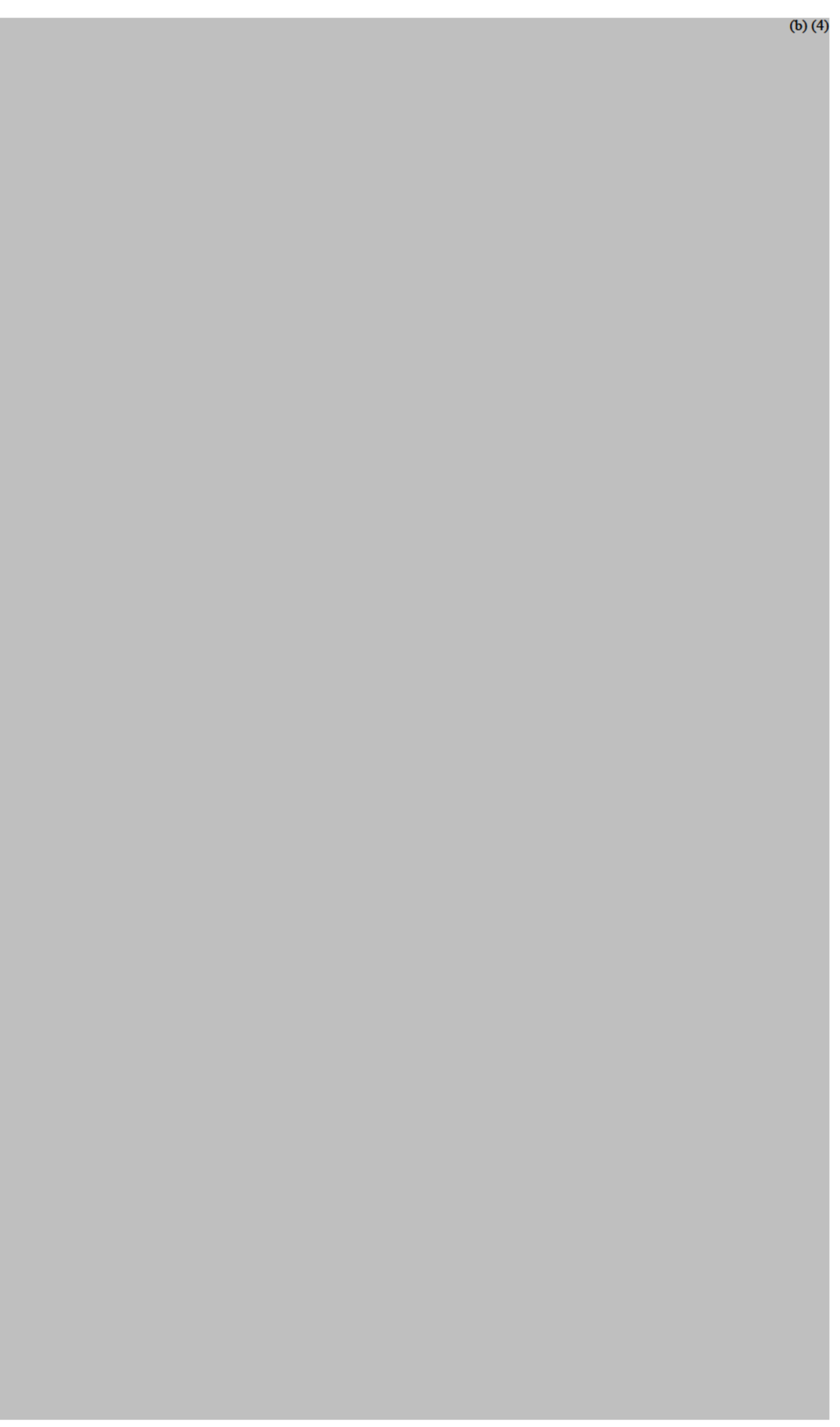
(b) (4)

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Gyeonggi-do, Korea. CJ Blossom Park
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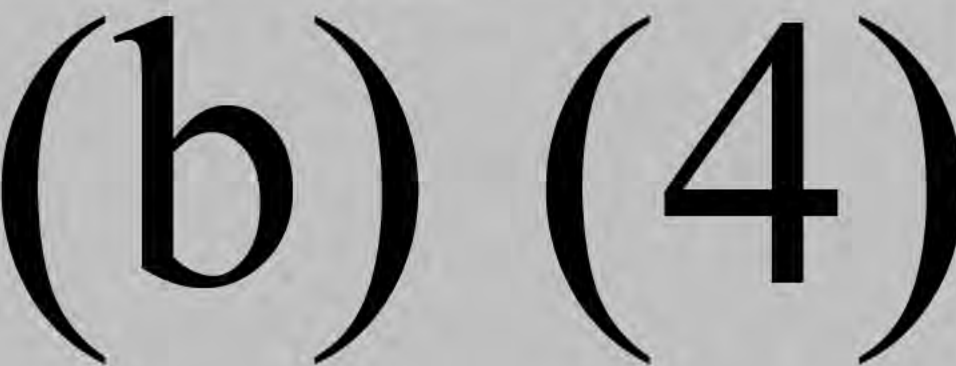
Certificate of analysis



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(b) (4)

Sep, 24, 2019

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Quantitation Report

Data File Name	002CALB.d
Acq/Data Batch	C:\Agilent\ICPMH1\DATA\190823.b
Acq Time	2019-08-28 15:08:09
Sample Name	BLANK
Sample Type	CalBlk
Comment	—
Prep Dilution	1.0000
Auto Dilution	1.0000
Total Dilution	1.0000
Operator Name	admin
Acq Mode	Spectrum
Cal Title	—
Cal Type	External Calibration
Last Calib	2019-09-24 11:25:5
Bkg File	—
Bkg Mode	Count Subtraction Re...
FQ BlankFile	—
WS Fit	Point to Point

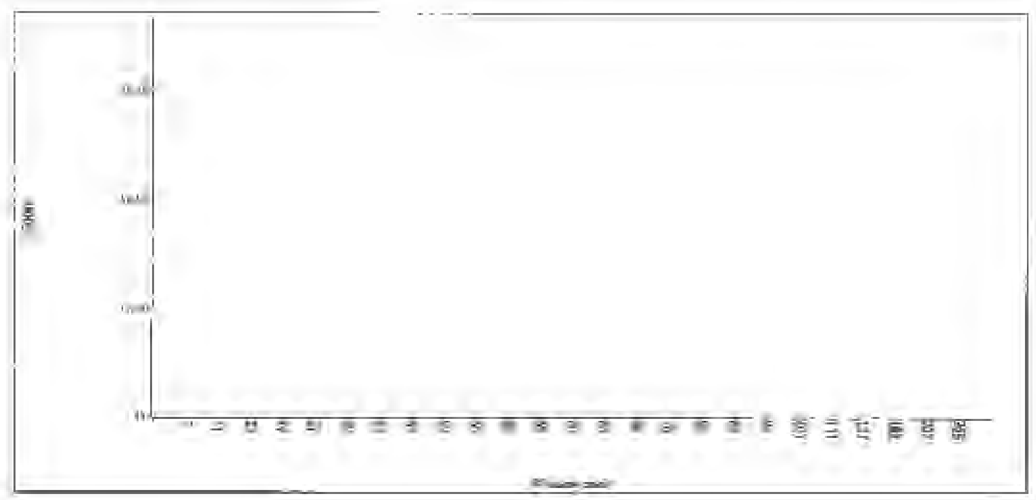
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time(sec)	Rep
As	75		He	<0.000	mg/kg	N/A	5.05		Pulse	0.3000	3
Pb	208		He	<0.000	mg/kg	N/A	166.67		Pulse	0.3000	3
Cd	111		H2	0.001	mg/kg	0.2	3.33		Pulse	0.1000	3
Hg	202		H2	0.000	mg/kg	0.3	26.67		Pulse	0.1000	3

ISTD Table:

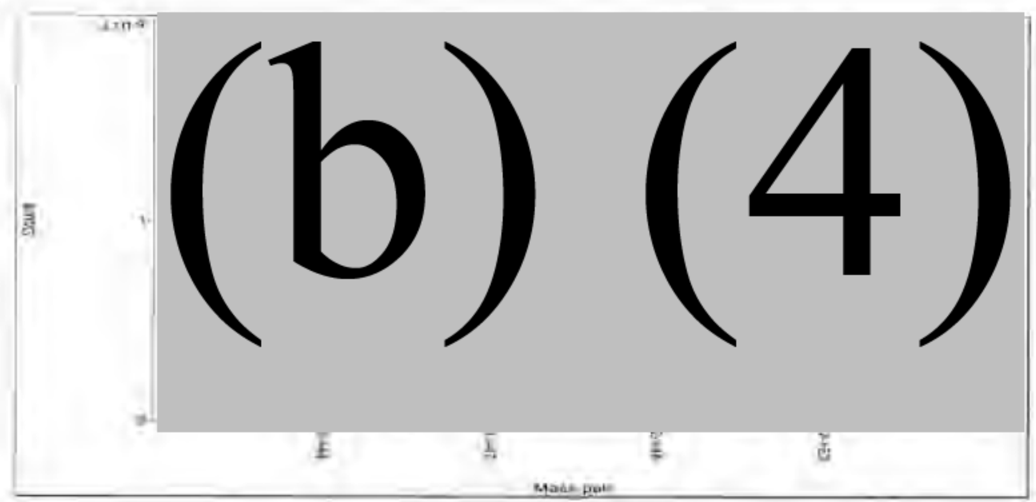
Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery %	Det.	Time(sec)	Rep

He

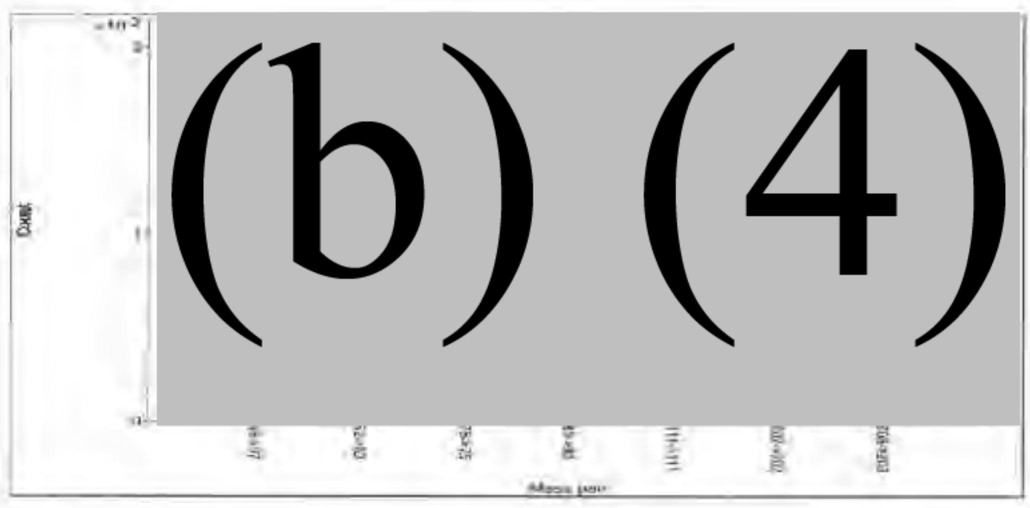


Quantitation Report

D2



H2



Quantitation Report

Data File Name 003CALB.d
Acq/Data Batch C:\Agilent\ICPMH\1\DATA\190928.b
Acq Time 2019-09-28 15:34:02
Sample Name STD-1
Sample Type CalStd
Comment —
Prep Dilution 1.0000
Auto Dilution 1.0000
Total Dilution 1.0000
Operator Name admin
Acq Mode Spectrum
Cal Title —
Cal Type External Calibration
Last Calib 2019-09-24 11:26:59
Bkg File —
Bkg Mode Count Subtraction for All
FQ Batch/File —
Wt Fit Point to Point

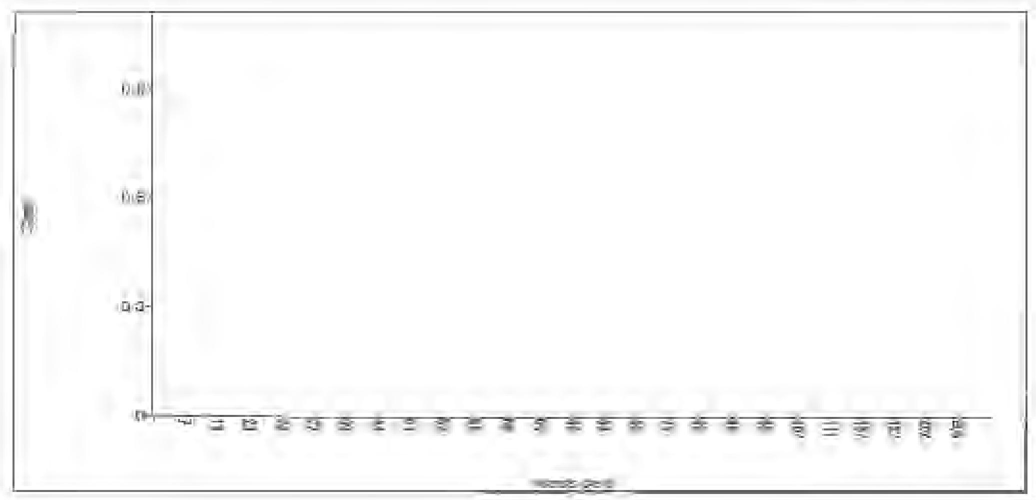
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det	Time(sec)	Rep
As	75		He	0.018	mg/kg	0.6	26735.20		Pulse	0.3000	3
Pb	208		He	0.041	mg/kg	1.3	680182.05		Pulse	0.3000	3
Cd	111		H2	0.044	mg/kg	2.7	188355.43		Pulse	0.1000	3
Hg	202		H2	0.000	mg/kg	5.7	2880.18		Pulse	0.1000	3

ISTD Table

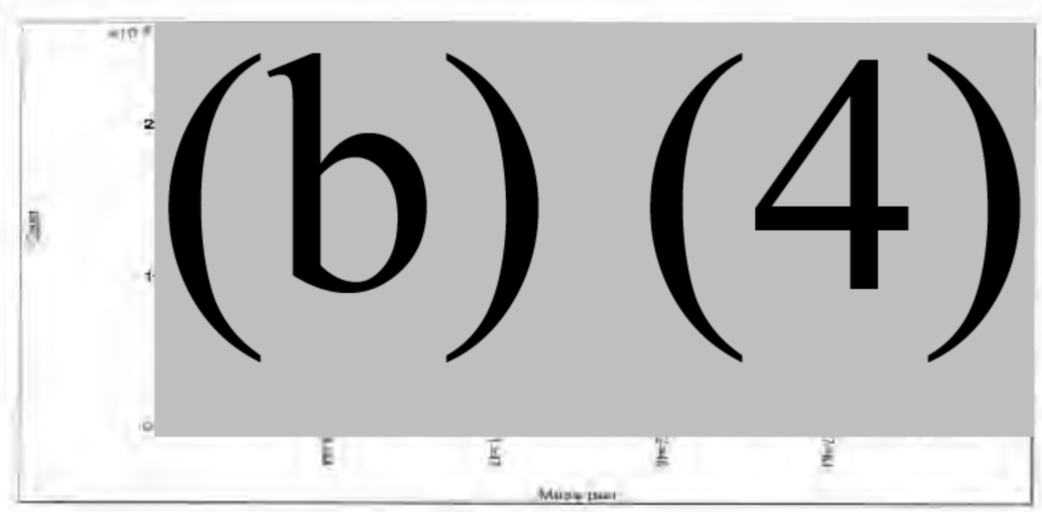
Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery%	Det	Time(sec)	Rep

He

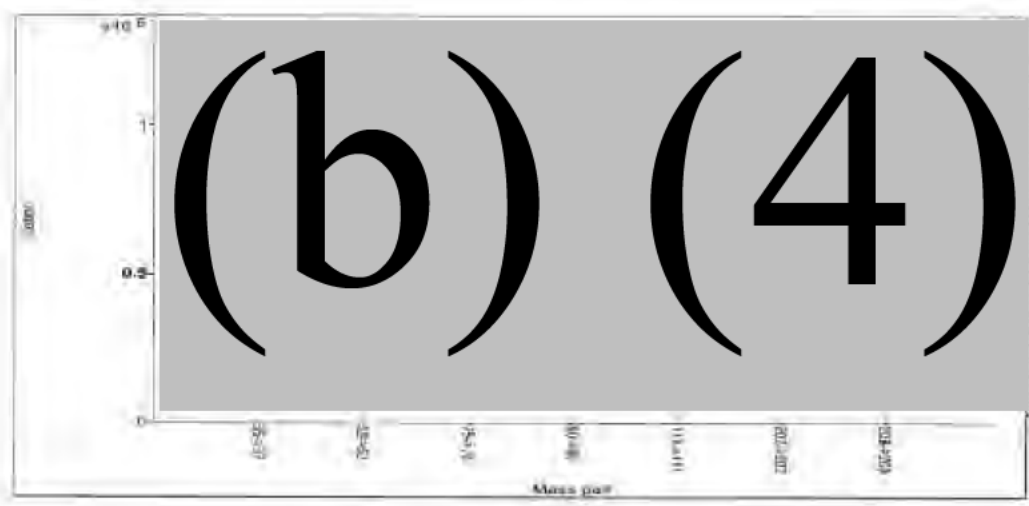


Quantitation Report

Q2



H2



Quantitation Report

Data File Name	004CAL5.d
Acq/Data Path	C:\Agilent\ICPMH\1\DATA\180628I.b
Acq Time	2019-06-26 15:18:59
Sample Name	STD-2
Sample Type	CalStd
Comment	
Prep Dilution	1.0000
Auto Dilution	1.0000
Total Dilution	1.0000
Operator Name	admin
Acq Mode	Spectrum
Cal Title	—
Cal Type	External Calibration
Last Calib	2019-09-24 11:28:59
Bkg File	—
Bkg Mode	Count Subtraction for All
FQ BlankFile	—
WS Fit	Point to Point

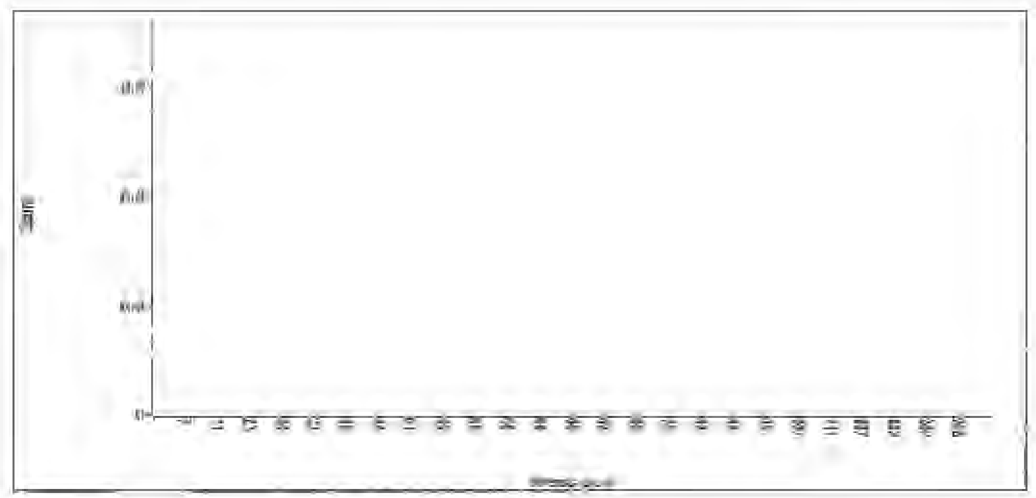
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time(sec)	Rep.
As	75		He	0.255	mg/kg	0.5	116245.27		Pulse	0.0800	3
Pb	208		He	0.208	mg/kg	1.4	2900301.40		Analog	0.0800	3
Cd	111		H2	0.252	mg/kg	0.5	682540.86		Pulse	0.1000	3
Hg	202		H2	0.080	mg/kg	2.9	19115.04		Pulse	0.1000	3

ISTD Table

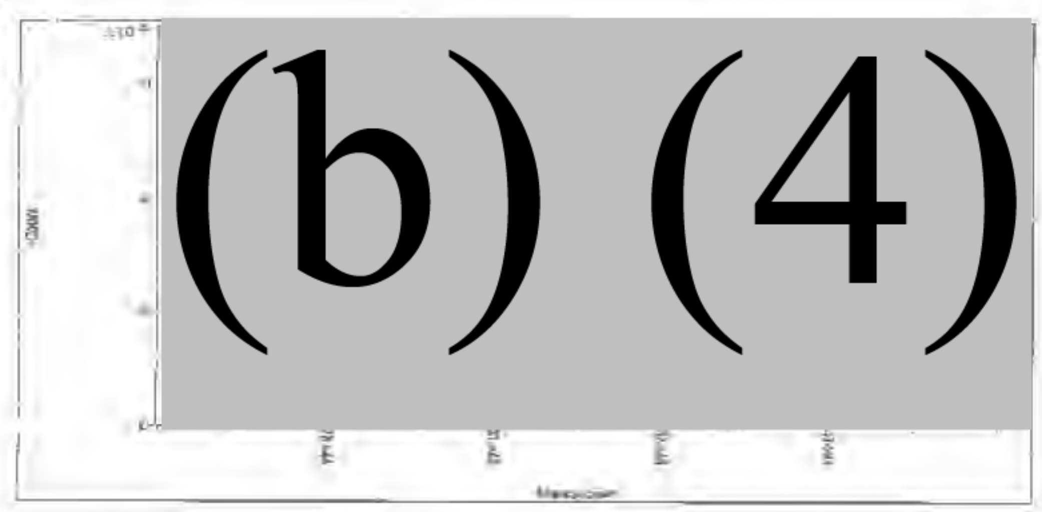
Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery%	Det.	Time(sec)	Rep.

He

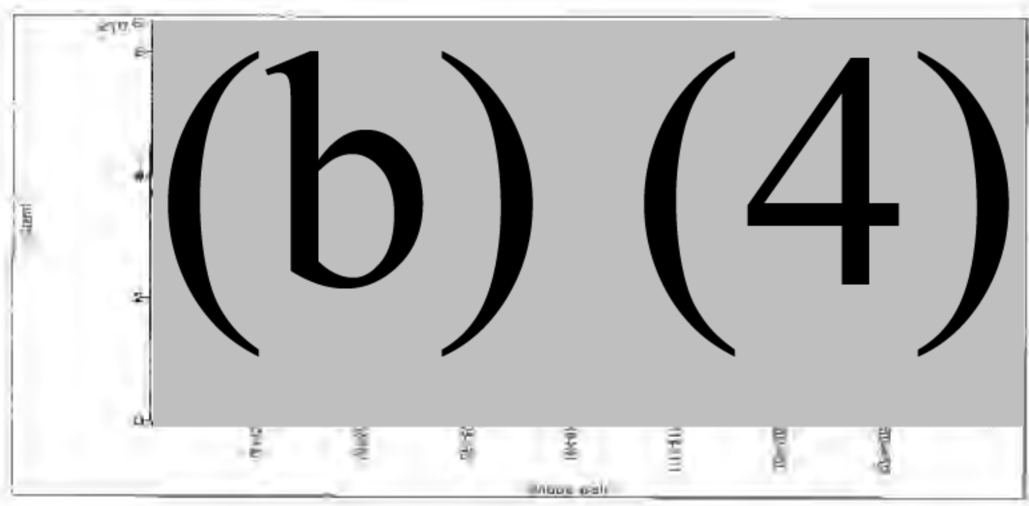


Quantitation Report

O2



H2



Quantitation Report

Data File Name	005CALS.d
Acq/Data Batch	C:\Agilent\ICPMH\1\DATA\190829.b
Acq Time	2019-08-29 15:23:51
Sample Name	STD-3
Sample Type	CalBlk
Comment	—
Prep Dilution	1.0000
Auto Dilution	1.0000
Total Dilution	1.0000
Operator Name	admin
Acq Mode	Spectrum
Cal Title	—
Cal Type	External Calibration
Last Calib	2019-09-24 11:25:51
Bkg File	—
Bkg Mode	Count Subtraction in W
FQ Batch File	—
MS Fit	Point to Point

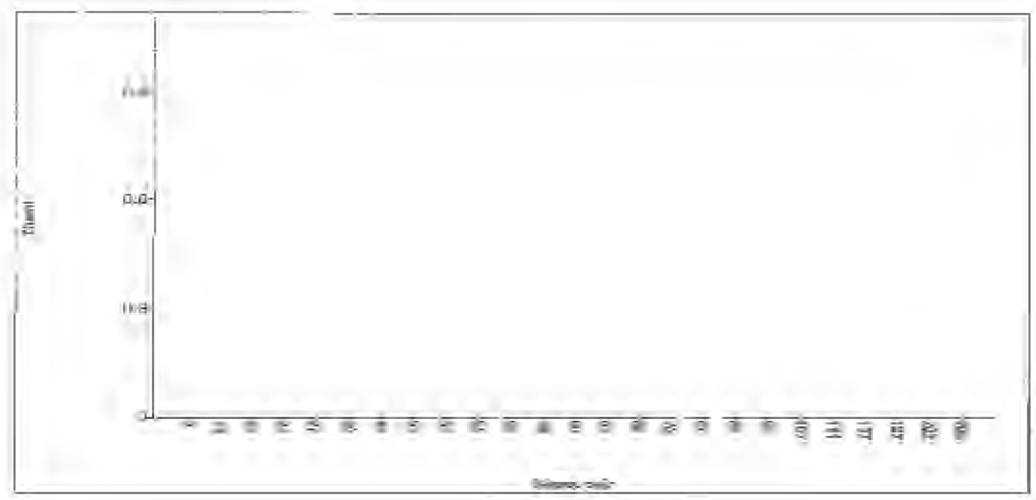
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time[sec]	Rep
As	75		He	0.882	mg/kg	1.2	402113.69		Pulse	0.3000	3
Pb	208		He	0.575	mg/kg	2.0	10579068.86		Analog	0.3000	3
Cd	111		H2	0.694	mg/kg	2.0	2003408.30		Analog	0.1000	3
Hg	202		H2	0.001	mg/kg	8.6	35005.92		Pulse	0.1000	3

ISTD Table

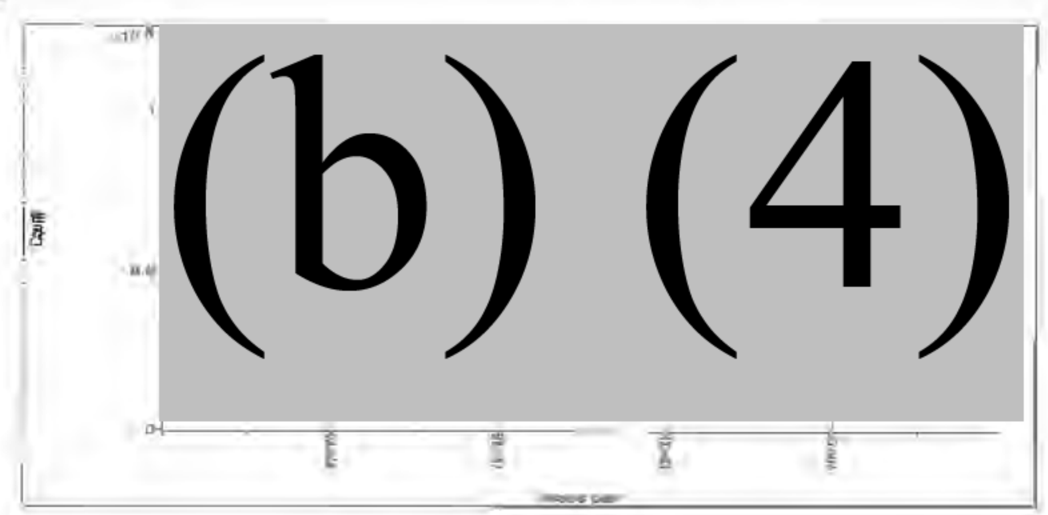
Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery %	Det.	Time[sec]	Rep

Hg

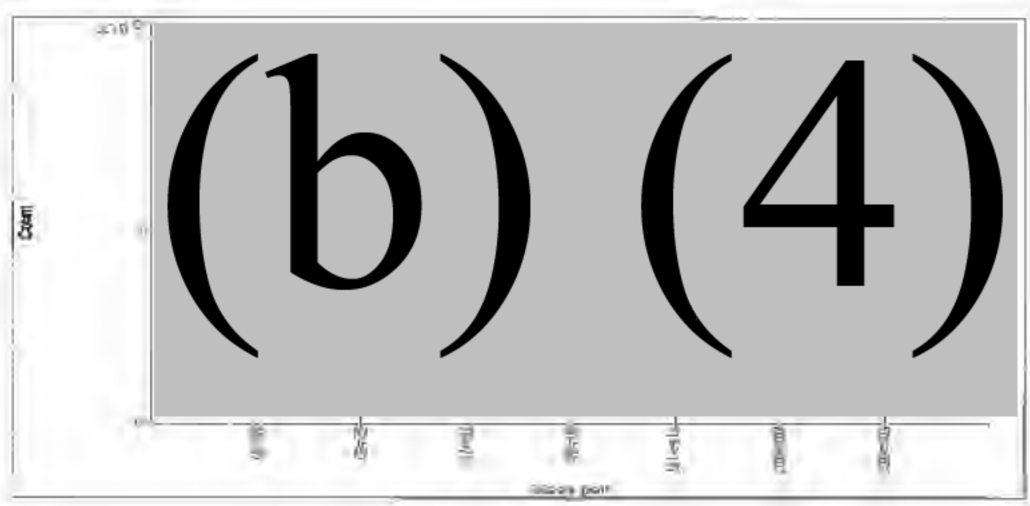


Quantitation Report

O2



N2



Quantitation Report

Data File Name	023SMPL.d
Acq/Data Batch	C:\Agilent\ICPMH\1\DATA\190628.b
Acq Time	2019-06-28 16:47:32
Sample Name	KIS_4
Sample Type	Sample
Comment	
Prep Dilution	100.0000
Auto Dilution	1.0000
Total Dilution	100.0000
Operator Name	admin
Acq Mode	Spectrum
Cal Title	
Cal Type	External Calibration
Last Calib	2019-09-24 11:28:53
Seq File	
Sbtr Mode	Count Subtraction for All
FQ BlankFile	007CBLK.d
VIS Fit	Point to Point

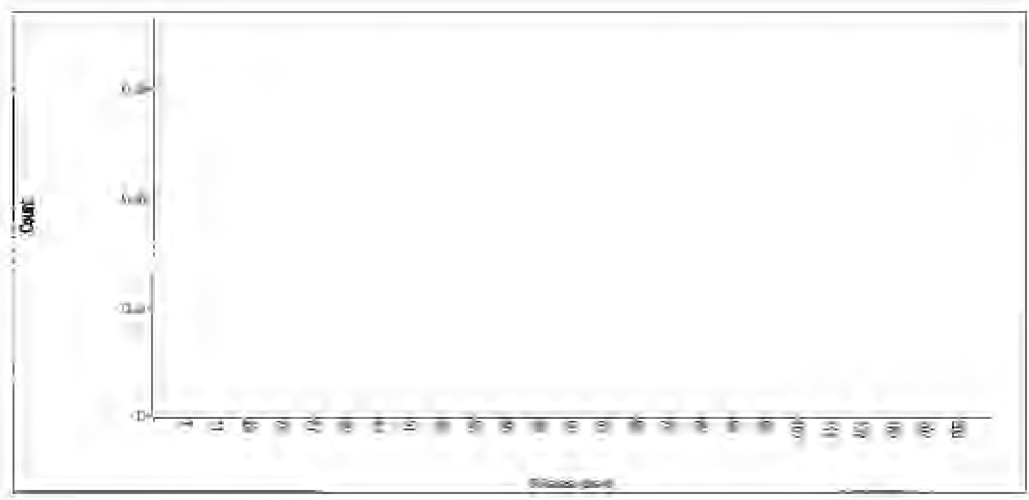
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time(sec)	Rep
As	75		He	<0.009	mg/kg	N/A	19.56		Pulse	0.0500	3
Pb	208		He	<0.002	mg/kg	N/A	112.22		Pulse	0.3000	3
Cd	111		H2	<0.001	mg/kg	N/A	6.67		Pulse	0.1000	3
Hg	202		H2	<0.020	mg/kg	N/A	46.57		Pulse	0.1000	3

ISTD Table

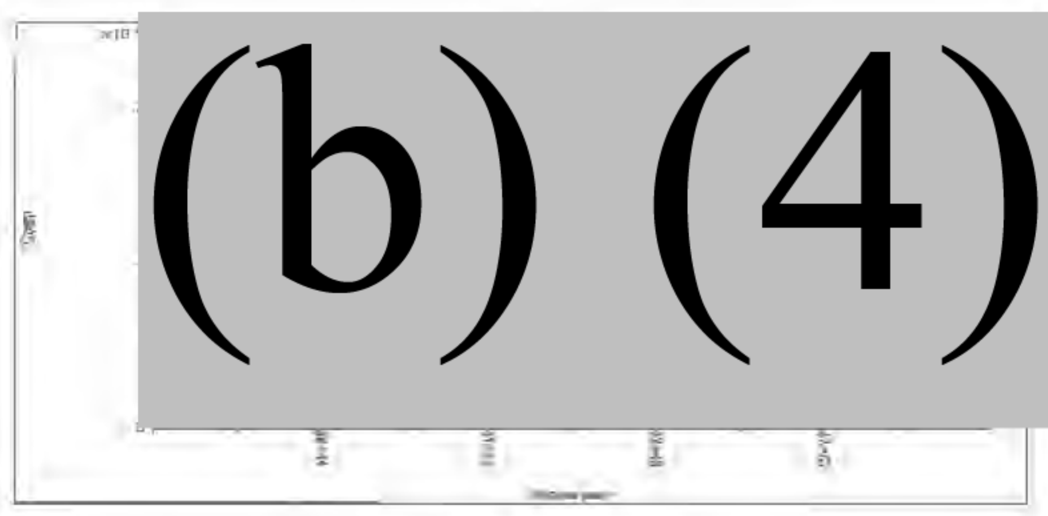
Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery %	Det.	Time(sec)	Rep

He



Quantitation Report

D2



H2

Quantitation Report

Data File Name	004SMPL.d
Acq/Data Batch	C:\Agilent\ICPMH\1\DATA\190829.b
Acq Time	2019-08-28 16:52:14
Sample Name	KIS_6
Sample Type	Sample
Comment	—
Prep Dilution	100.0000
Auto Dilution	1.0000
Total Dilution	100.0000
Operator Name	admin
Acq Mode	Spectrum
Cal File	—
Cal Type	External Calibration
Last Cal Update	2019-09-24 11:26:53
Blk File	—
Blk Mode	Count Subtraction for All
FQ Blank File	007QBLK.d
VIS Fit	Point to Point

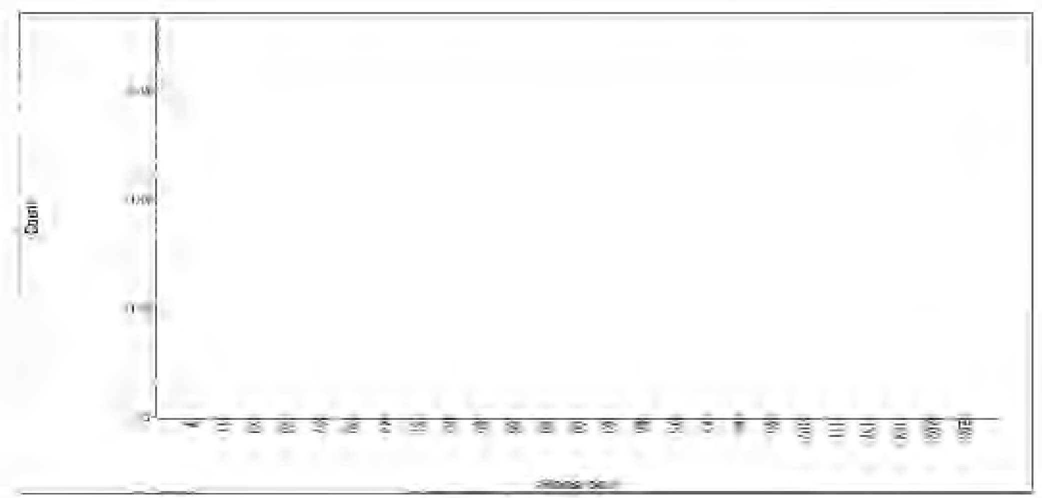
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time(Sec)	Rep
As	75		He	<0.002	mg/kg	N/A	14.41		Pulse	0.9000	3
Pb	208		He	<0.002	mg/kg	N/A	88.88		Pulse	0.3000	3
Cd	111		H2	<0.001	mg/kg	N/A	0.00		Pulse	0.1000	3
Hg	202		H2	<0.000	mg/kg	N/A	46.57		Pulse	0.1000	3

ISTD Table:

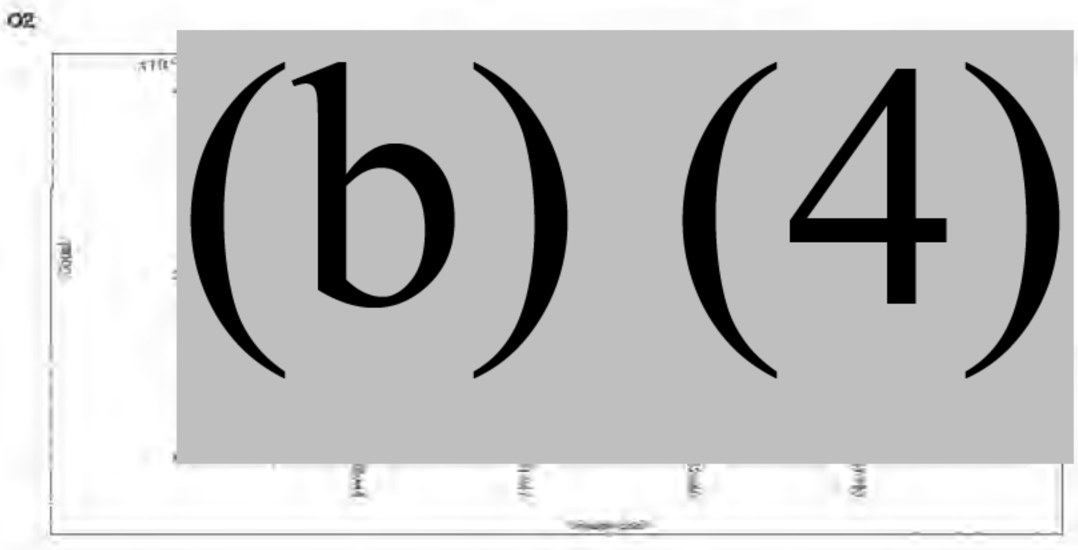
Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery %	Det.	Time(sec)	Rep

He

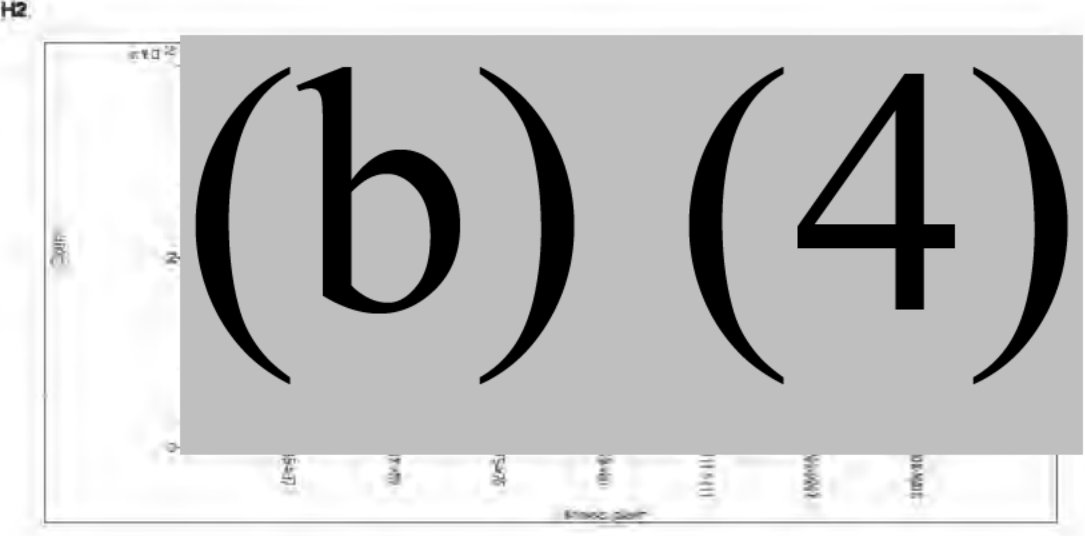


Quantitation Report

O2



H2



Quantitation Report

Data File Name	025SMPL.d
Acq/Data Batch	C:\Agilent\ICPMH\1\DATA\190828.b
Acq Time	2019-08-28 18:58:54
Sample Name	KIS_6
Sample Type	Sample
Comment	—
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Auto Dilution	1.0000
Total Dilution	100.0000
Operator Name	admin
Acq Mode	Spectrum
Cal Title	—
Cal Type	External Calibration
Last Calib	2019-08-24 11:26:53
Bkg File	—
Bkg Mode	Count Subtraction for All
FQ BlankFile	007QBLK.d
IS Fit	Point to Point

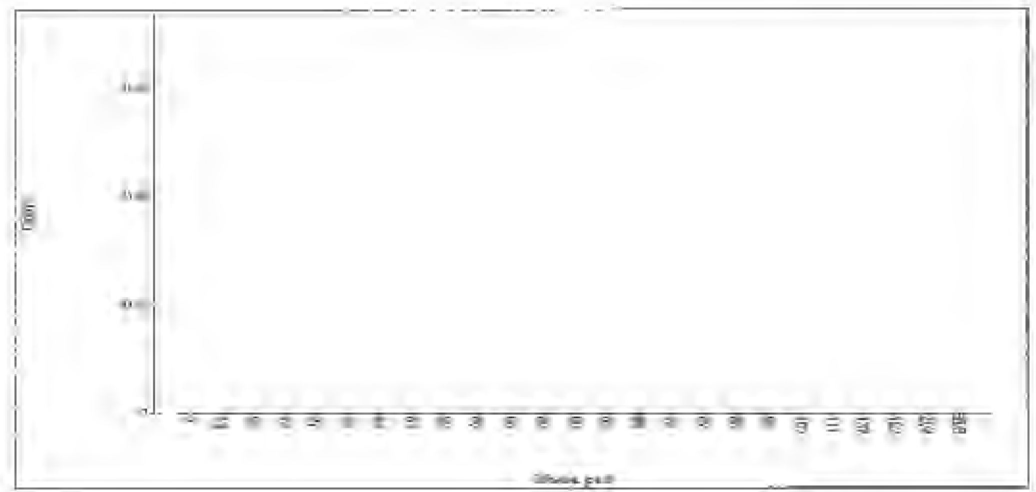
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time(sec)	Rep
As	75		He	<0.003	mg/kg	N/A	22.39		Pulse	0.3000	3
Pb	208		He	<0.003	mg/kg	N/A	152.22		Pulse	0.3000	3
Cd	111		H2	<0.001	mg/kg	N/A	0.00		Pulse	0.1000	3
Hg	202		H2	<0.000	mg/kg	N/A	60.00		Pulse	0.1000	3

ISTD Table

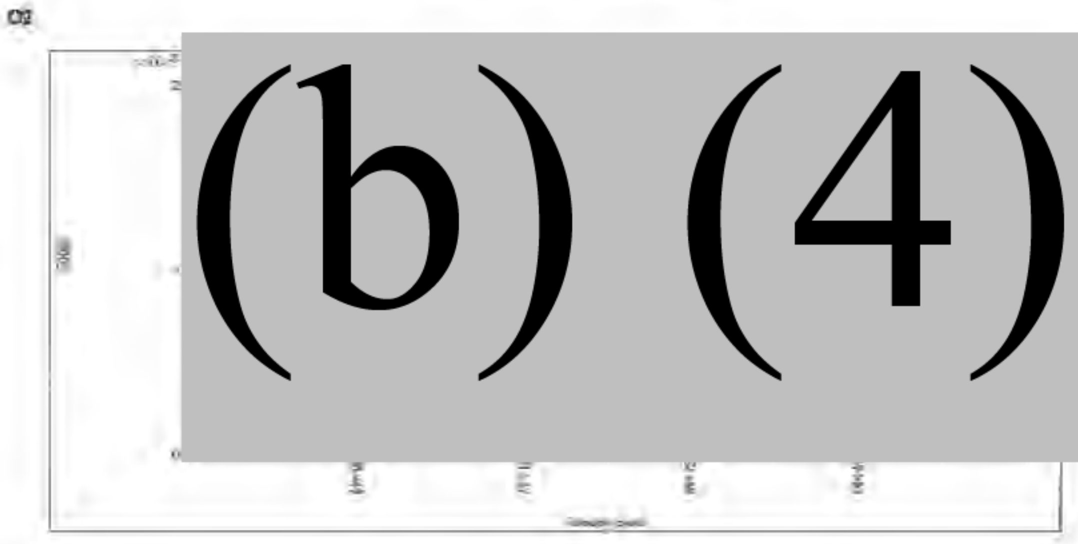
Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery %	Det.	Time(sec)	Rep

He



Quantitation Report

O2



H2

(b) (4)

AOAC Official Method 2015.01
Heavy Metals in Food

Inductively Coupled Plasma–Mass Spectrometry
First Action 2015

Note: The following is not intended to be used as a comprehensive training manual. Analytical procedures are written based on the assumption that they will be performed by technicians who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

{Applicable for the determination of heavy metals [arsenic (As), CAS No. 7440-38-2; cadmium (Cd), CAS No. 7440-43-9; lead (Pb), CAS No. 7439-92-1; and mercury (Hg), CAS No. 7439-97-6] at trace levels in food and beverage samples, including solid chocolate, fruit juice, fish, infant formula, and rice, using microwave digestion and inductively coupled plasma–mass spectrometry (ICP-MS).}

Caution: Nitric acid and hydrochloric acid are corrosive. When working with these acids, wear adequate protective gear, including eye protection, gloves with the appropriate resistance, and a laboratory coat. Use an adequate fume hood for all acids.

Hydrogen peroxide is a strong oxidizer and can react violently with organic material to give off oxygen gas and heat. Adequate protective gear should be worn.

Many of the chemicals have toxicities that are not well established and must be handled with care. For all known chemicals used, consult the Material Safety Data Sheet (MSDS) in advance.

The inductively coupled plasma–mass spectrometer emits UV light when the plasma is on. UV resistant goggles should be worn if working near the plasma.

The instrument generates high levels of radio frequency (RF) energy and is very hot when the plasma is on. In the case of an instrument failure, be aware of these potential dangers.

Safely store interference reduction technology (IRT) gases, such as oxygen, in a closed, ventilated cabinet. Use adequate caution with pressurized gases. Prior training or experience is necessary to change any gas cylinders. Oxygen gas can cause many materials to ignite easily.

Following microwave digestion, samples are hot to the touch. Allow the samples to cool to room temperature before opening the digestion vessels to avoid unexpected depressurization and potential release of toxic fumes.

A. Principle

Food samples are thoroughly homogenized and then prepared by microwave digestion and the addition of dilute solutions of gold (Au) and lutetium (Lu). The Au is used to stabilize the Hg in the preparation, and the Lu is used to assess the potential loss of analyte during the microwave digestion process.

A prepared, diluted, aqueous sample digestate is pumped through a nebulizer, where the liquid forms an aerosol as it enters a spray chamber. The aerosol separates into a fine aerosol mist and larger aerosol droplets. The larger droplets exit the spray chamber while the fine mist is transported into the ICP torch.

Inside the ICP torch, the aerosol mist is transported into a high-temperature plasma, where it becomes atomized and ionized as it passes through an RF load coil. The ion stream is then focused by a single ion

lens through a cylinder with a carefully controlled electrical field. For instruments equipped with dynamic reaction cell (DRC) or collision cell IRT, the focused ion stream is directed into the reaction/collision cell where, when operating with a pressurized cell, the ion beam will undergo chemical modifications and/or collisions to reduce elemental interferences. When not operating with a pressurized cell, the ion stream will remain focused as it passes through the cell with no chemical modification taking place.

The ion stream is then transported to the quadrupole mass filter, where only ions having a desired mass-to-charge ratio (*m/z*) are passed through at any moment in time. The ions exiting the mass filter are detected by a solid-state detector and the signal is processed by the data handling system.

B. Equipment

Perform routine preventative maintenance for the equipment used in this procedure.

An ultra-clean laboratory environment is critical for the successful production of quality data at ultra-low levels. All sample preparation must take place in a clean hood (Class 100). Metallic materials should be kept to a minimum in the laboratory and coated with an acrylic polymer gel where possible. Adhesive floor mats should be used at entrances to the laboratory and changed regularly to prevent the introduction of dust and dirt from the outside environment. Wear clean-room gloves and change whenever contact is made with anything non-ultra-clean. The laboratory floor should be wiped regularly to remove any particles without stirring up dust. *Note:* "Ultra-clean" (tested to be low in the analytes of interest) reagents, laboratory supplies, facilities, and sample handling techniques are required to minimize contamination in order to achieve the trace-level detection limits described herein.

(**a**) *Instrumentation*.--ICP-MS instrument, equipped with IRT with a free-running 40 MHz RF generator; and controllers for nebulizer, plasma, auxiliary, and reaction/collision flow control. The quadrupole mass spectrometer has a mass range of 5 to 270 atomic mass units (amu). The turbo molecular vacuum system achieves 10^{-6} torr or better. Recommended ICP-MS components include an RF coil, platinum skimmer and sampler cones, Peltier-cooled quartz cyclonic spray chamber, quartz or sapphire injector, micronebulizer, variable speed peristaltic pump, and various types of tubing (for gases, waste, and peristaltic pump). *Note*: The procedure is written specifically for use with a PerkinElmer ELAN DRC II ICP-MS (www.perkinelmer.com). Equivalent procedures may be performed on any type of ICP-MS instrument with equivalent IRT if the analyst is fully trained in the interpretation of spectral and matrix interferences and procedures for their correction, including the optimization of IRT. For example, collision cell IRT can be used for arsenic determination using helium gas.

(**b**) *Gases*.--High-purity grade liquid argon (>99.996%). Additional gases are required for IRT (such as ultra-x grade, 99.9999% minimum purity oxygen, used for determination of As in DRC mode with some PerkinElmer ICP-MS instruments).

(**c**) *Analytical balance*.--Standard laboratory balance suitable for sample preparation and capable of measuring to 0.1 mg.

(**d**) *Clean-room gloves*.--Tested and certified to be low in the metals of interest.

(**e**) *Microwave digestion system*.--Laboratory microwave digestion system with temperature control and an adequate supply of chemically inert digestion vessels. The microwave should be appropriately vented and corrosion resistant.

(*1*) The microwave digestion system must sense the temperature to within ±2.5°C and automatically adjust the microwave field output power within 2 s of sensing. Temperature sensors should be accurate

to ±2°C (including the final reaction temperature of 190°C). Temperature feedback control provides the primary control performance mechanism for the method.

(*2*) The use of microwave equipment with temperature feedback control is required to control the unfamiliar reactions of unique or untested food or beverage samples. These tests may require additional vessel requirements, such as increased pressure capabilities.

(**f**) *Autosampler cups.*--15 and 50 mL; vials are precleaned by soaking in 2-5% (v/v) HNO_3 overnight, rinsed three times with reagent water/deionized water (DIW), and dried in a laminar flow clean hood. For the 50 mL vials, as these are used to prepare standards and bring sample preparations to final volume, the bias and precision of the vials must be assessed and documented prior to use. The recommended procedure for this is as follows:

(1) For every case of vials from the same lot, remove 10 vials.

(2) Tare each vial on an analytical balance, and then add reagent water up to the 20 mL mark. Repeat procedure by adding reagent water up to the 50 mL mark.

(3) Measure and record the mass of reagent water added, and then calculate the mean and RSD of the 10 replicates at each volume.

(4) To evaluate bias, the mean of the measurements must be with ±3% of the nominal volume. To evaluate precision, the RSD of the measurements must be ≤3% using the stated value (20 or 50 mL) in place of the mean.

(**g**) *Spatulas*.--To weigh out samples; should be acid-cleaned plastic (ideally Teflon) and cleaned by soaking in 2% (v/v) HNO_3 prior to use.

C. Reagents and Standards

Reagents may contain elemental impurities that could negatively affect data quality. High-purity reagents should always be used. Each reagent lot should be tested and certified to be low in the elements of interest before use.

(**a**) *DIW*.--ASTM Type I; demonstrated to be free from the metals of interest and potentially interfering substances.

(**b**) *Nitric acid (HNO_3).*—Concentrated; tested and certified to be low in the metals of interest.

(c) *Hydrogen peroxide (H_2O_2).*–Optima grade or equivalent, 30-32% assay.

(**d**) *Stock standard solutions.*--Obtained from a reputable and professional commercial source.

(*1*) *Single-element standards*.--Obtained for each determined metal, as well as for any metals used as internal standards and interference checks.

(*2*) *Second source standard*.--Independent from the single-element standard; obtained for each determined metal.

(*3*) *Multi-element stock standard solution*.--Elements must be compatible and stable in solutions together. Stability is determined by the vendor; concentrations are then verified before use of the standard.

(**e**) *Internal standard solution*.--For analysis of As, Cd, Pb, and Hg in food matrices, an internal standard solution of 40 µg/L rhodium (Rh), indium (In), and thulium (Tm) is recommended. Rh is analyzed in DRC

mode for correction of the As signal. In addition, the presence of high levels of elements, such as carbon and chlorine, in samples can increase the effective ionization of the plasma and cause a higher response factor for arsenic in specific samples. This potential interference is addressed by the on-line addition of acetic acid (or another carbon source, such as methanol), which greatly increases the effective ionization of incompletely ionized analytes, and decreases the potential increase caused by sample characteristics. The internal standard solution should be prepared in 20% acetic acid.

(**f**) *Calibration standards.*--Fresh calibration standards should be prepared every day, or as needed.

(*1*) Dilute the multi-element stock standard solutions into 50 mL precleaned autosampler vials with 5% HNO_3 in such a manner as to create a calibration curve. The lowest calibration standard (STD 1) should be equal to or less than the limit of quantitation (LOQ) when recalculated in units specific to the reported sample results.

(*2*) *See* Table **2015.01A** for recommended concentrations for the calibration curve.

Table 2015.01A. Recommended concentrations for the calibration curve

Standard	As, µg/L	Cd, µg/L	Pb, µg/L	Hg, µg/L
0	0.00	0.00	0.000	0.00
1	0.01	0.01	0.005	0.01
2	0.02	0.02	0.010	0.05
3	0.10	0.10	0.050	0.10
4	0.50	0.50	0.250	0.50
5	5.00	5.00	2.500	2.00
6	20.00	20.00	10.000	5.00

(**g**) *Initial calibration verification (ICV) solution.*--Made up from second source standards in order to verify the validity of the calibration curve.

(**h**) *Calibration solutions*.--Daily optimization, tuning, and dual detector calibration solutions, as needed, should be prepared and analyzed per the instrument manufacturer’s suggestions.

(**i**) *Certified Reference Materials (CRMs)*.--CRMs should preferably match the food matrix type being analyzed and contain the elements of interest at certified concentrations above the LOQ. Recommended reference materials include NIST SRM 1568a (Rice Flour), NIST SRM 1548a (Typical Diet), NRCC CRM DORM-3 (Dogfish Muscle), and NIST SRM 2976 (Mussel Tissue).

(**j**) *Spiking solution*.--50 mg/L Au and Lu in 5% (v/v) HNO_3. Prepared from single-element standards.

D. Contamination and Interferences

(**a**) Well-homogenized samples and small reproducible aliquots help minimize interferences.

(**b**) *Contamination.*—(*1*) Contamination of the samples during sample handling is a great risk. Extreme care should be taken to avoid this. Potential sources of contamination during sample handling include using metallic or metal-containing homogenization equipment, laboratory ware, containers, and sampling equipment.

(*2*) Contamination of samples by airborne particulate matter is a concern. Sample containers must remain closed as much as possible. Container lids should only be removed briefly and in a clean environment during sample preservation and processing, so that exposure to an uncontrolled environment is minimized.

(**c**) *Laboratory.*--(*1*) All laboratory ware (including pipet tips, ICP-MS autosampler vials, sample containers, extraction apparatus, and reagent bottles) should be tested for the presence of the metals of interest. If necessary, the laboratory ware should be acid-cleaned, rinsed with DIW, and dried in a Class 100 laminar flow clean hood.

(*2*) All autosampler vials should be cleaned by storing them in 2% (v/v) HNO_3 overnight and then rinsed three times with DIW. Then dry vials in a clean hood before use. Glass volumetric flasks should be soaked in about 5% HNO_3 overnight prior to use.

(*3*) All reagents used for analysis and sample preparation should be tested for the presence of the metals of interest prior to use in the laboratory. Due to the ultra-low detection limits of the method, it is imperative that all the reagents and gases be as low as possible in the metals of interest. It is often required to test several different sources of reagents until an acceptable source has been found. Metals contamination can vary greatly from lot to lot, even when ordering from the same manufacturer.

(*4*) Keep the facility free from all sources of contamination for the metals of interest. Replace laminar flow clean hood HEPA filters with new filters on a regular basis, typically once a year, to reduce airborne contaminants. Metal corrosion of any part of the facility should be addressed and replaced. Every piece of apparatus that is directly or indirectly used in the processing of samples should be free from contamination for the metals of interest.

(**d**) *Elemental interferences*.--Interference sources that may inhibit the accurate collection of ICP-MS data for trace elements are addressed below.

(*1*) *Isobaric elemental interferences*.--Isotopes of different elements that form singly or doubly charged ions of the same *m/z* and cannot be resolved by the mass spectrometer. Data obtained with isobaric overlap must be corrected for that interference.

(*2*) *Abundance sensitivity*.--Occurs when part of an elemental peak overlaps an adjacent peak. This often occurs when measuring a small *m/z* peak next to a large *m/z* peak. The abundance sensitivity is affected by ion energy and quadrupole operating pressure. Proper optimization of the resolution during tuning will minimize the potential for abundance sensitivity interferences.

(*3*) *Isobaric polyatomic interferences.*--Caused by ions, composed of multiple atoms, which have the same *m/z* as the isotope of interest, and which cannot be resolved by the mass spectrometer. These ions are commonly formed in the plasma or the interface system from the support gases or sample components. The objective of IRT is to remove these interferences, making the use of correction factors unnecessary when analyzing an element in DRC mode. Elements not determined in DRC mode can be corrected by using correction equations in the ICP-MS software.

(**e**) *Physical interferences.*--(*1*) Physical interferences occur when there are differences in the response of the instrument from the calibration standards and the samples. Physical interferences are associated with the physical processes that govern the transport of sample into the plasma, sample conversion processes in the plasma, and the transmission of ions through the plasma-mass spectrometer interface.

(*2*) Physical interferences can be associated with the transfer of solution to the nebulizer at the point of nebulization, transport of aerosol to the plasma, or during excitation and ionization processes in the plasma. High levels of dissolved solids in a sample can result in physical interferences. Proper internal

standardization (choosing internal standards that have analytical behavior similar to the associating elements) can compensate for many physical interferences.

(**f**) *Resolution of interferences.*—(*1*) For elements that are subject to isobaric or polyatomic interferences (such as As), it is advantageous to use the DRC mode of the instrument. This section specifically describes a method of using IRT for interference removal for As using a PerkinElmer DRC II and oxygen as the reaction gas. Other forms of IRT may also be appropriate.

(*a*) Arsenic, which is monoisotopic, has an *m/z* of 75 and is prone to interferences from many sources, most notably from chloride (Cl), which is common in many foods (e.g., salt). Argon (Ar), used in the ICP-MS plasma, forms a polyatomic interference with Cl at *m/z* 75 [$^{35}Cl + ^{40}Ar = ^{75}(ArCl)$].

(*b*) When arsenic reacts with the oxygen in the DRC cell, $^{75}As^{16}O$ is formed and measured at *m/z* 91, which is free of most interferences. The potential ^{91}Zr interference is monitored for in the following ways: ^{90}Zr and ^{94}Zr are monitored for in each analytical run, and if a significant Zr presence is detected, then $^{75}As^{16}O$ measured at *m/z* 91 is evaluated against the ^{75}As result. If a significant discrepancy is present, then samples may require analysis using alternative IRT, such as collision cell technology (helium mode).

(*c*) Instrument settings used (for PerkinElmer DRC II): DRC settings for $^{91}(AsO)$ and ^{103}Rh include an RPq value of 0.7 and a cell gas flow rate of 0.6 L/min. Cell conditions, especially cell gas flow rates, may be optimized for specific analyte/matrix combinations, as needed. In such cases, the optimized methods will often have slightly different RPq and cell gas flow values.

(*2*) For multi-isotopic elements, more than one isotope should be measured to monitor for potential interferences. For reporting purposes, the most appropriate isotope should be selected based on review of data for matrix interferences and based on the sensitivity (or relative abundance) of each isotope. The table below lists the recommended isotopes to measure. Low abundance isotopes are not recommended for this method as it is specifically applicable for ultra-low level concentrations (8-10 ppb LOQs). *See* Table **2015.01B**.

Table 2015.01B. Recommended isotopes for analysis

Element	Isotope, amu	Isotopic abundance, %	Potential interferences
Cd	111	13	MoO^+
	114	29	MoO^+, Sn^+
Hg	200	23	WO^+
	202	30	WO^+
Pb[a]	Sum of 206, 207, and 208	99	OsO^+

[a] Allowance for isotopic variability of lead isotopes.

(**g**) *Memory effects*.—Minimize carryover of elements in a previous sample in the sample tubing, cones, torch, spray chamber, connections, and autosampler probe by rinsing the instrument with a reagent blank after samples high in metals concentrations are analyzed. Memory effects for Hg can be minimized through the addition of Au to all standard, samples, and quality control (QC) samples.

E. Sample Handling and Storage

(**a**) Food and beverage samples should be stored in their typical commercial storage conditions (either frozen, refrigerated, or at room temperature) until analysis. Samples should be analyzed within 6 months of preparation.

(**b**) If food or beverage samples are subsampled from their original storage containers, ensure that containers are free from contamination for the elements of concern.

F. Sample Preparation

(**a**) Weigh out sample aliquots (typically 0.25 g of as-received or wet sample) into microwave digestion vessels.

(**b**) Add 4 mL of concentrated HNO_3 and 1 mL of 30% hydrogen peroxide (H_2O_2) to each digestion vessel.

(**c**) Add 0.1 mL of the 50 mg/L Au + Lu solution to each digestion vessel.

(**d**) Cap the vessels securely (and insert into pressure jackets, if applicable). Place the vessels into the microwave system according to the manufacturer's instructions, and connect the appropriate temperature and/or pressure sensors.

(**e**) Samples are digested at a minimum temperature of 190°C for a minimum time of 10 min. Appropriate ramp times and cool down times should be included in the microwave program, depending on the sample type and model of microwave digestion system. Microwave digestion is achieved using temperature feedback control. Microwave digestion programs will vary depending on the type of microwave digestion system used. When using this mechanism for achieving performance-based digestion targets, the number of samples that may be simultaneously digested may vary. The number will depend on the power of the unit, the number of vessels, and the heat loss characteristics of the vessels. It is essential to ensure that all vessels reach at least 190°C and be held at this temperature for at least 10 min. The monitoring of one vessel as a control for the batch/carousel may not accurately reflect the temperature in the other vessels, especially if the samples vary in composition and/or sample mass. Temperature measurement and control will depend on the particular microwave digestion system.

(1) Note: a predigestion scheme for samples that react vigorously to the addition of the acid may be required.

(2) The method performance data presented in this method was produced using a Berghof Speedwave 4 microwave digestion system, with the program listed in Table **2015.01C** (steps 1 and 2 are a predigestion step).

Table 2015.01C. Digestion program for Berghof Speedwave 4 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	145	1	1
2	50	1	1
3	145	1	1
4	170	1	10
5	190	1	10

(3) Equivalent results were achieved using the program listed in Table **2015.01D** on a CEM MARS 6 microwave digestion system using the 40-position carousel and 55 mL Xpress digestion vessels.

Table 2015.01D. Digestion program for CEM MARS 6 microwave			
Step	Temp., °C	Ramp, min	Hold, min
1	190	20	10
2	Cool down	NA	10

(4) For infant formula samples, the program described in Table **2015.01E** has been shown to work effectively.

Table 2015.01E. Digestion program for infant formula			
Step	Temp., °C	Ramp, min	Hold, min
1	180	20	20
2	Cool down	NA	20
3	200	20	20
4	Cool down	NA	20

(**f**) Allow vessels to cool to room temperature and slowly open. Open the vessels carefully, as residual pressure may remain and digestate spray is possible. Pour the contents of each vessel into an acid-cleaned 50 mL HDPE centrifuge tube and dilute with DIW to a final volume of 20 mL.

(**g**) Digestates are diluted at least 4x prior to analysis with the 1% (v/v) HNO_3 diluent. When the metals concentration of a sample is unknown, the samples may be further diluted or analyzed using a total quantification method prior to being analyzed with a comprehensive quantitative method. This protects the instrument and the sample introduction system from potential contamination and damage.

(**h**) Food samples high in calcium carbonate ($CaCO_3$) will not fully digest. In such cases, the CRM can be used as a gauge for an appropriate digestion time.

(**i**) QC samples to be prepared with the batch (a group of samples and QC samples that are prepared together) include a minimum of three method blanks, duplicate for every 10 samples, matrix spike/matrix spike duplicate (MS/MSD) for every 10 samples, blank spike, and any matrix-relevant CRMs that are available.

G. Procedure

(**a**) *Instrument startup*.--(*1*) Instrument startup routine and initial checks should be performed per manufacturer recommendations.

(*2*) Ignite the plasma and start the peristaltic pump. Allow plasma and system to stabilize for at least 30 min.

(**b**) *Optimizations.*--(*1*) Perform an optimization of the sample introduction system (e.g., *X-Y* and *Z* optimizations) to ensure maximum sensitivity.

(*2*) Perform an instrument tuning or mass calibration routine whenever there is a need to modify the resolution for elements, or monthly (at a minimum), to ensure the instrument's quadrupole mass filtering performance is adequate. Measured masses should be ±0.1 amu of the actual mass value, and the resolution (measured peak width) should conform to manufacturer specifications.

(*3*) Optimize the nebulizer gas flow for best sensitivity while maintaining acceptable oxide and double-charged element formation ratios.

(*4*) Perform a daily check for instrument sensitivity, oxide formation ratios, double-charged element formation ratios, and background. If the performance check is not satisfactory, additional optimizations (a "full optimization") may be necessary.

(**c**) *Internal standardization and calibration*.--(*1*) Following precalibration optimizations, prepare and analyze the calibration standards prepared as described in **C**(**e**).

(*2*) Use internal standardization in all analyses to correct for instrument drift and physical interferences. Refer to **D**(**e**)(*2*). Internal standards must be present in all samples, standards, and blanks at identical concentrations. Internal standards can be added using a second channel of the peristaltic pump to produce a responses that is clear of the pulse-to-analog detector interface.

(*3*) Multiple isotopes for some analytes may be measured, with only the most appropriate isotope (as determined by the analyst) being reported.

(*4*) Use IRT for the quantification of As using the Rh internal standard.

(**d**) *Sample analysis*.--(*1*) Create a method file for the ICP-MS.

(*2*) Enter sample and calibration curve information into the ICP-MS software.

(*3*) Calibrate the instrument and ensure the resulting standard recoveries and correlation coefficients meet specifications (**H**).

(*4*) Start the analysis of the samples.

(*5*) Immediately following the calibration, an initial calibration blank (ICB) should be analyzed. This demonstrates that there is no carryover of the analytes of interest and that the analytical system is free from contamination.

(*6*) Immediately following the ICB, an ICV should be analyzed. This standard must be prepared from a different source than the calibration standards.

(*7*) A minimum of three reagent/instrument blanks should be analyzed following the ICV. These instrument blanks can be used to assess the background and variability of the system.

(*8*) A continuing calibration verification (CCV) standard should be analyzed after every 10 injections and at the end of the run. The CCV standard should be a mid-range calibration standard.

(*9*) An instrument blank should be analyzed after each CCV (called a continuing calibration blank, or CCB) to demonstrate that there is no carryover and that the analytical system is free from contamination.

(*10*) Method of Standard Additions (MSA) calibration curves may be used any time matrix interferences are suspected.

(*11*) Post-preparation spikes (PS) should be prepared and analyzed whenever there is an issue with the MS recoveries.

(**e**) Export and process instrument data.

H. Quality Control

(**a**) The correlation coefficients of the weighted-linear calibration curves for each element must be ≥0.995 to proceed with sample analysis.

(**b**) The percent recovery of the ICV standard should be 90-110% for each element being determined.

(**c**) Perform instrument rinses after any samples suspected to be high in metals, and before any method blanks, to ensure baseline sensitivity has been achieved. Run these rinses between all samples in the batch to ensure a consistent sampling method.

(**d**) Each analytical or digestion batch must have at least three preparation (or method) blanks associated with it if method blank correction is to be performed. The blanks are treated the same as the samples and must go through all of the preparative steps. If method blank correction is being used, all of the samples in the batch should be corrected using the mean concentration of these blanks. The estimated method detection limit (EMDL) for the batch is equal to 3 times the standard deviation (SD) of these blanks.

(**e**) For every 10 samples (not including quality control samples), a matrix duplicate (MD) sample should be analyzed. This is a duplicate of a sample that is subject to all of the same preparation and analysis steps as the original sample. Generally, the relative percent difference (RPD) for the replicate should be ≤30% for all food samples if the sample concentrations are greater than 5 times the LOQ. RPD is calculated as shown below. An MSD may be substituted for the MD, with the same control limits.

$$RPD = 200\ x\ \frac{|S1 - S2|}{S1 + S2}$$

where S1 = concentration in the first sample and S2 = concentration in the duplicate.

(**f**) For every 10 samples (not including quality control samples), an MS and MSD should be performed. The percent recovery of the spikes should be 70-130% with an RPD ≤30% for all food samples.

(*1*) If the spike recovery is outside of the control limits, an MSA curve that has been prepared and analyzed may be used to correct for the matrix effect. Samples may be corrected by the slope of the MSA curve if the correlation coefficient of the MSA curve is ≥0.995.

(*a*) The MSA technique involves adding known amounts of standard to one or more aliquots of the processed sample solution. This technique attempts to compensate for a sample constituent that enhances or depresses the analyte signal, thus producing a different slope from that of the calibration standards. It will not correct for additive interferences which cause a baseline shift.

(*b*) The best MSA results can be obtained by using a series of standard additions. To equal volumes of the sample are added a series of standard solutions containing different known quantities of the analyte(s), and all solutions are diluted to the same final volume. For example, addition 1 should be prepared so that the resulting concentration is approximately 50% of the expected concentration of the native sample. Additions 2 and 3 should be prepared so that the concentrations are approximately 100% and 150%, respectively, of the expected native sample concentration. Determine the concentration of each solution and then plot on the vertical axis of a graph, with the concentrations of the known standards plotted on the horizontal axis. When the resulting line is extrapolated to zero absorbance, the point of interception of the abscissa is calculated MSA-corrected concentration of the analyte in the sample. A linear regression program may be used to obtain the intercept concentration.

(*c*) For results of the MSA technique to be valid, take into consideration the following limitations:

(*i*) The apparent concentrations from the calibration curve must be linear (0.995 or greater) over the concentration range of concern.

(*ii*) The effect of the interference should not vary as the ratio of analyte concentration to sample matrix changes, and the MSA curve should respond in a similar manner as the analyte.

(*2*) If the sample concentration levels are sufficiently high, the sample may be diluted to reduce the matrix effect. Samples should be diluted with the 1% (v/v) HNO_3 diluent. For example, to dilute a sample by a 10x dilution factor, pipette 1 mL of the digested sample into an autosampler vial, and add 9 mL of the 1% (v/v) HNO_3 diluent. MS/MSD sets should be performed at the same dilution factor as the native sample.

(*3*) Spike at 1-10 times the level of a historical sample of the same matrix type, or, if unknown, spike at 1-5 times a typical value for the matrix. Spiking levels should be no lower than 10 times the LOQ.

(**g**) Percent recoveries of the CRMs should be 75-125% of their certified value.

(**h**) Percent recoveries of the CCV standards should be within 85-115%. Sample results may be CCV-corrected using the mean recovery of the bracketing CCVs. This should only be done after careful evaluation of the data. The instrument should show a trending drift of CCV recoveries and not just a few anomalous outliers.

(**i**) CCBs should be monitored for the effects of carryover and for possible system contamination. If carryover of the analyte at levels greater than 10 times the MDL is observed, the sample results may not be reportable.

(**j**) Absolute response of any one internal standard should not vary from the original response in the calibration blank by more than 60-125%. Some analytical samples, such as those containing concentrations of the internal standard and tissue digestates, can have a serious effect on the internal standard intensities, but this does not necessarily mean that the analytical system is out of control. In some situations, it is appropriate to reprocess the samples using a different internal standard monitored in the analysis. The data should be carefully evaluated before doing this.

(**k**) The recovery of the Lu that was spiked into the sample preparation prior to digestion should be evaluated to assess any potential loss of analyte during the process. The concentration of Lu in the sample preparation is 0.25 mg/L, and for samples diluted 4x at the instrument, this is equivalent to 62.5 µg/L at the instrument (if samples are diluted more than 4x, this must be taken into account). The Lu recovery should be no less than 75% of the original spiked concentration.

(**l**) Refer to Table **2015.01F** for a summary of all recommended quality control samples, minimum frequency at which they are to be analyzed, acceptance criteria for each, and appropriate corrective action if the acceptance criteria are not met.

Table 2015.01F. Summary of quality control samples

QC sample	Measure	Minimum frequency	Acceptance criteria	Corrective action
Calibration standards	Linearity of the calibration curve	Analyzed once per analytical day	Correlation coefficient ≥0.995, 1st standard ≤MRL, low standard recovery = 75-125%, all other standard recoveries = 80-120%	Reanalyze suspect calibration standard. If criteria still not met, then re-prepare standards and recalibrate the instrument.
Internal standards	Variation in sample properties between samples and standards	Each standard, blank, and sample is spiked with internal standard	60-125% recovery compared to calibration blank	If the responses of the internal standards in the following CCB are within the limit, rerun the sample at an additional 2x dilution. If not, then samples must be reanalyzed with a new calibration.
Lu digestion check spike	Assessment of potential loss during digestion	Added to every digested samples	Recovery ≥75%	Re-prepare the sample
Initial calibration verification (ICV)	Independent check of system performance	One following instrument calibration	Recovery = 90-110%	Correct problem prior to continuing analysis. Recalibrate if necessary.
Continuing calibration verification (CCV)	Accuracy	At beginning and end of analysis and one per 10 injections	Recovery = 85-115%	Halt analysis, correct problem, recalibrate, and reanalyze affected samples
Method blanks (MB)	Contamination from reagents, lab ware, etc.	Minimum of three per batch	Mean ≤ MRL; SD ≤ MDL or MBs <1/10th sample result	Determine and eliminate cause of contamination. Affected samples must be re-prepared and reanalyzed.
Method duplicates (MD)	Method precision within a given matrix	Minimum of one per 10 samples	RPD ≤ 30% or ±2x LOQ if results ≤5x LOQ	If RPD criteria not met, then sample may be re-prepared and reanalyzed, but this is not required. Sample matrix may be inhomogeneous. A post-digestion duplicate (PDD) can be analyzed to evaluate instrument precision.

Matrix spikes/matrix spike duplicates (MS/MSD)	Method accuracy and precision within a given matrix	Minimum of one per 10 samples	Recovery = 70-130% and RPD ≤ 30%	If RPD > 30%, results must be qualified
Post-preparation spike (PS)	Check for matrix interference	When required (samples spiked too low/high, dilution test fails, etc.)	Recovery = 75-125%	Analyze samples using MSA or results flagged accordingly
Laboratory fortified blank (LFB) or blank spike (BS)	Method accuracy	Minimum of one per batch	Recovery = 75-125%	If LFB recovery is outside of the control limit, then batch must be re-prepared and reanalyzed
Certified Reference Material (CRM)	Method accuracy	Must be matrix-matched to samples; minimum of one per batch	Recovery = 75-125% unless limits set by CRM manufacturer are greater or element/CRM specific limits have been established	If CRM true value is ≥5x the LOQ and recovery is outside of the control limit, then batch must be re-prepared and reanalyzed

I. Method Performance

(**a**) Limit of detection (LOD) and LOQ were determined through the analysis of 23 method blanks (*see* Table **2015.01G**). LOD was calculated as 3 times the SD of the results of the blanks, and LOQ was calculated as 2 times the value of the LOD, except where the resulting LOQ would be less than the lowest calibration point, in which case LOQ was elevated and set at the lowest calibration point and LOD was calculated as 1/3 of the LOQ. All LOQs achieved are ≤10 µg/kg for all food matrices and ≤8 µg/kg for liquid matrices, such as infant formula.

Table 2015.01G. Method blank results and LOD/LOQ, µg/kg

Method blanks	^{91}(AsO)	^{111}Cd	^{114}Cd	Pb	^{200}Hg	^{202}Hg
MB-01	2.83	0.229	0.270	1.90	1.61	0.95
MB-02	1.48	-0.088	0.270	0.14	1.48	1.13
MB-03	1.80	0.007	0.115	0.13	0.76	0.25
MB-04	1.03	0.154	0.288	0.12	1.46	0.33
MB-05	1.43	0.010	0.259	1.84	1.28	0.27
MB-06	1.07	0.105	0.096	3.02	0.87	0.76
MB-07	2.31	-0.002	0.297	2.67	0.89	0.44
MB-08	1.20	0.285	0.200	4.24	0.55	0.28
MB-09	1.05	0.002	0.182	0.09	0.96	0.25
MB-10	2.12	0.047	0.150	0.19	0.71	0.02
MB-11	2.09	-0.145	0.226	0.12	0.64	0.57
MB-12	1.44	0.037	0.165	0.18	0.45	0.50
MB-13	0.70	-0.122	0.160	0.17	0.81	0.19
MB-14	1.12	-0.001	0.074	0.14	0.85	0.21
MB-15	2.33	0.097	0.207	0.11	0.18	0.17
MB-16	1.53	-0.117	0.146	0.16	1.33	1.09
MB-17	1.79	-0.070	0.180	0.03	3.46	2.19
MB-18	1.90	0.049	0.115	0.06	3.30	2.36
MB-19	1.18	0.043	0.224	0.39	4.01	2.78
MB-20	1.24	-0.060	0.199	0.07	0.99	0.56
MB-21	0.92	0.165	0.120	0.03	0.73	0.33
MB-22	1.69	0.005	0.186	0.09	0.60	0.25
MB-23	2.13	0.171	0.152	0.08	0.41	-0.23
SD	0.54	0.113	0.063	1.18	1.01	0.77
LOD	1.6	0.50[a]	0.50[a]	3.5	3.0	2.3
LOQ	3.3	1.60[a]	1.60[a]	7.1	6.0	4.6

[a] Adjusted to conform to lowest calibration point.

(**b**) Sample-specific LOQs for several matrices, based on LOQs determined by the default method, and adjusted for changes in sample mass for particular samples, are shown in Table **2015.01H**. Values have been rounded up to the nearest part-per-billion.

Table 2015.01H. Sample-specific LOQs

Sample	LOQ, µg/kg (as received)			
	As	Cd	Pb	Hg
Infant formula	2	1	4	3
Chocolate	4	2	8	6
Rice flour	4	2	8	6
Fruit juice	1	1	2	2

(c) Numerous relevant CRMs were analyzed to establish method accuracy. Example percent recoveries are provided in Table **2015.01I** (recoveries have been omitted for CRMs that do not provide a certified value or if the certified value is less than the LOQ).

Table 2015.01I. Recoveries for numerous relevant CRMs

Certified Reference Material	As, %	Cd, %	Pb, %	Hg, %
DOLT-4 Dogfish Liver	104	97	87	114
DORM-3 Fish Protein	105	109	94	114
DORM-4 Fish Protein	105	91	91	81
NIST 1548a Typical Diet	103	95	113	NA
NIST 1568a Rice Flour	98	99	NA	NA
NIST 1946 Lake Superior Fish Tissue	119	NA	NA	101
TORT-2 Lobster Hepatopancreas	109	104	95	116
TORT-3 Lobster Hepatopancreas	113	89	86	86

(d) *Standard Method Performance Requirements*SM (AOAC SMPR 2012.007) for repeatability, reproducibility, and recovery for the method are shown in the Table **2015.01J**. *See* Appendix A (*J. AOAC Int.*, future issue) for detailed method performance information supporting acceptance of the method.

Table 2015.01J. AOAC SMPR 2012.007

Concentration range, µg/kg	Repeatability, %	Reproducibility, %	Recovery, %
LOQ–100	15	32	60-115
100–1000	11	16	80-115
>1000	7.3	8	80-115

(e) Detailed method performance information supporting acceptance of the method is on file with AOAC and the method author and is available upon request. Method validation samples were prepared and analyzed for all applicable matrices. In general, all SMPR criteria were met for As, Cd, Hg, and Pb in the matrices apple juice, infant formula, cocoa powder, and rice flour.

J. AOAC Int. (future issue)

AOAC SMPR 2012.007
J. AOAC Int. **96**, 704(2013)
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Genetic Stability of Dried L-Valine Fermentation Product Producing Strain, *Corynebacterium glutamicum* KCCM80058

< Confidential >

ORIGINAL FINAL REPORT DATE: November 08, 2018

CJ Blossom Park

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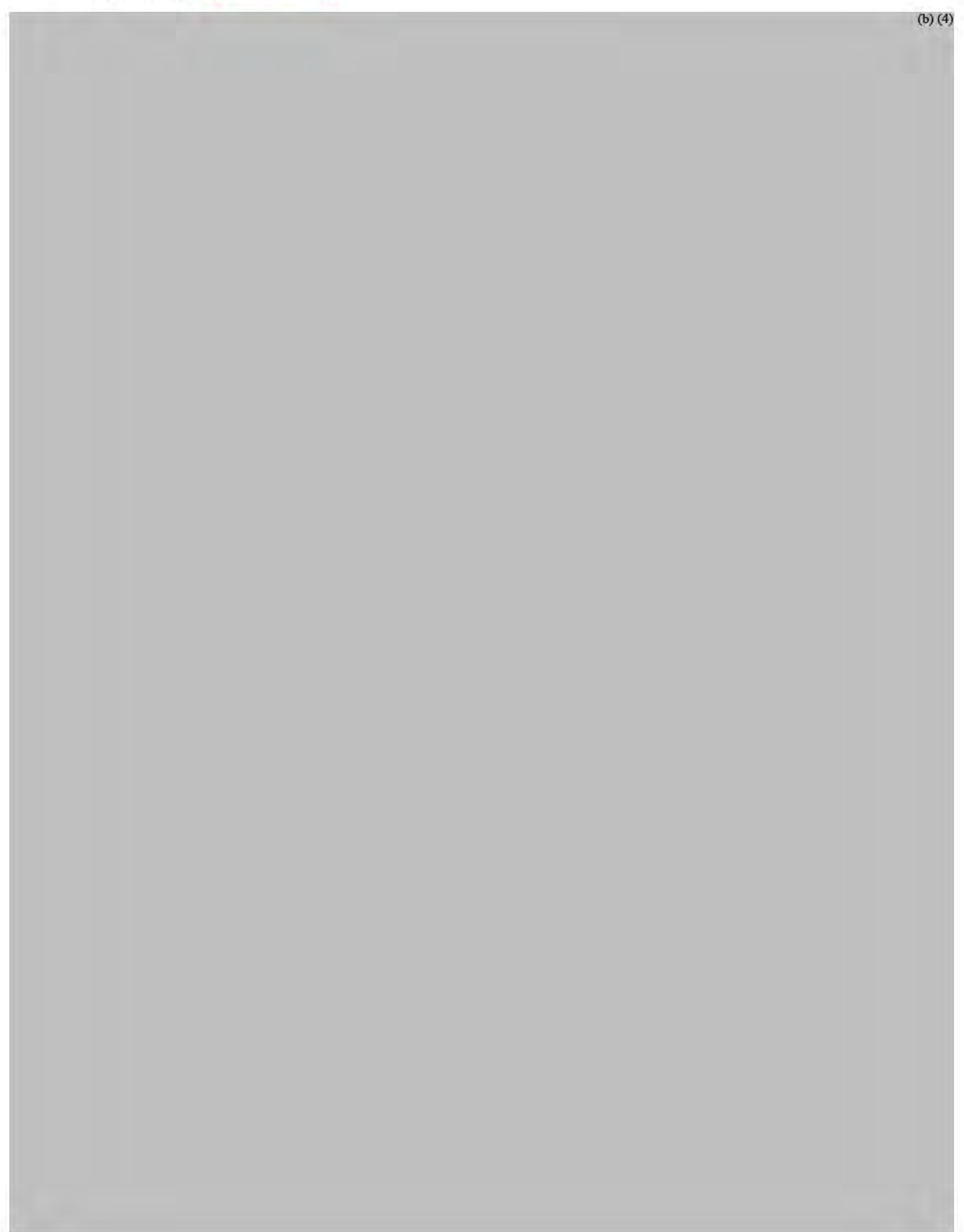
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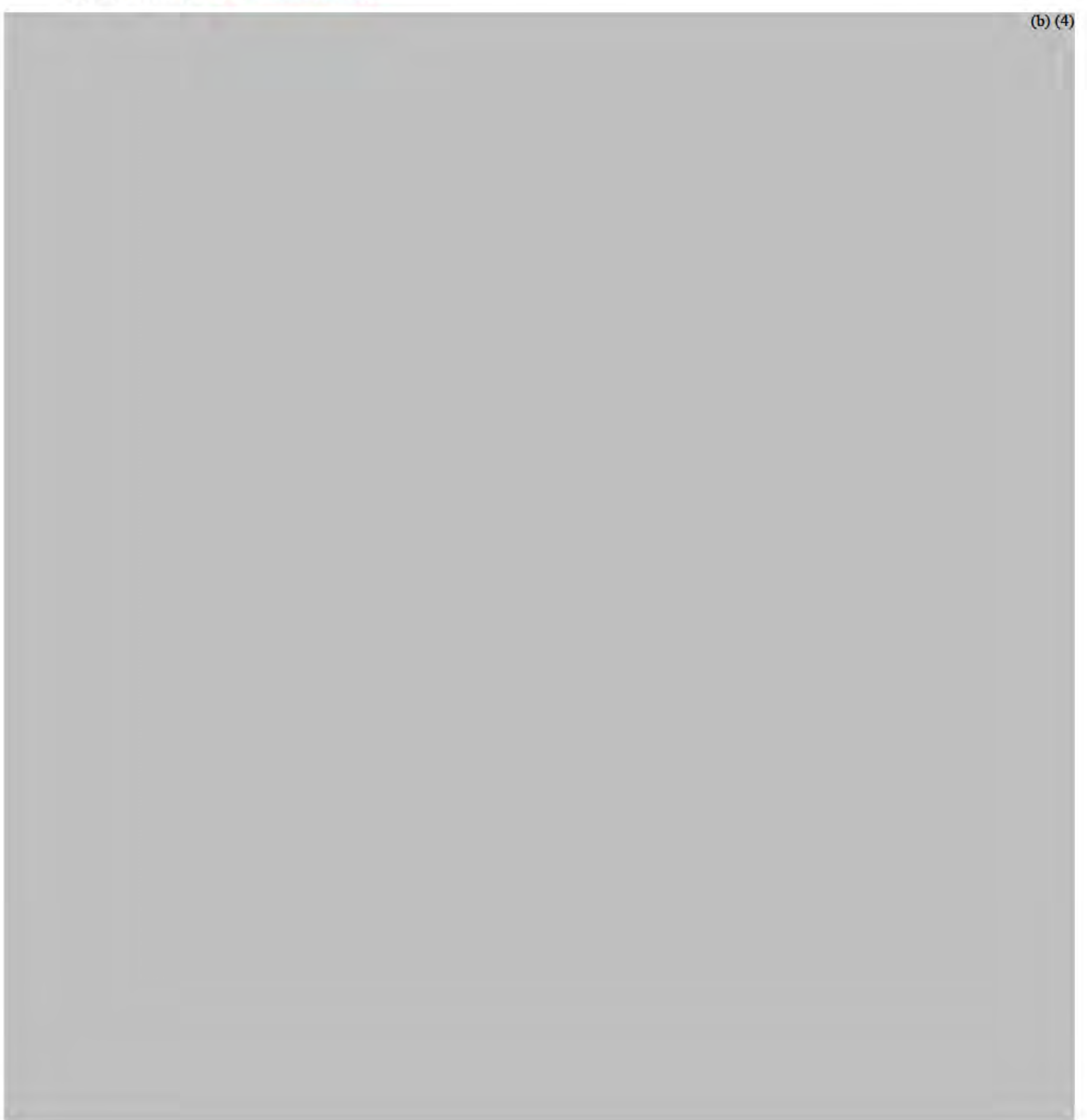
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Open Reading Frame Analysis of the Genetically Modified Site

The open reading frame analysis for the modified site on *Corynebacterium glutamicum* KCCM80058 (CONFIDENTIAL)

REPORT DATE: November 20, 2018

CJ BLOSSOM PARK

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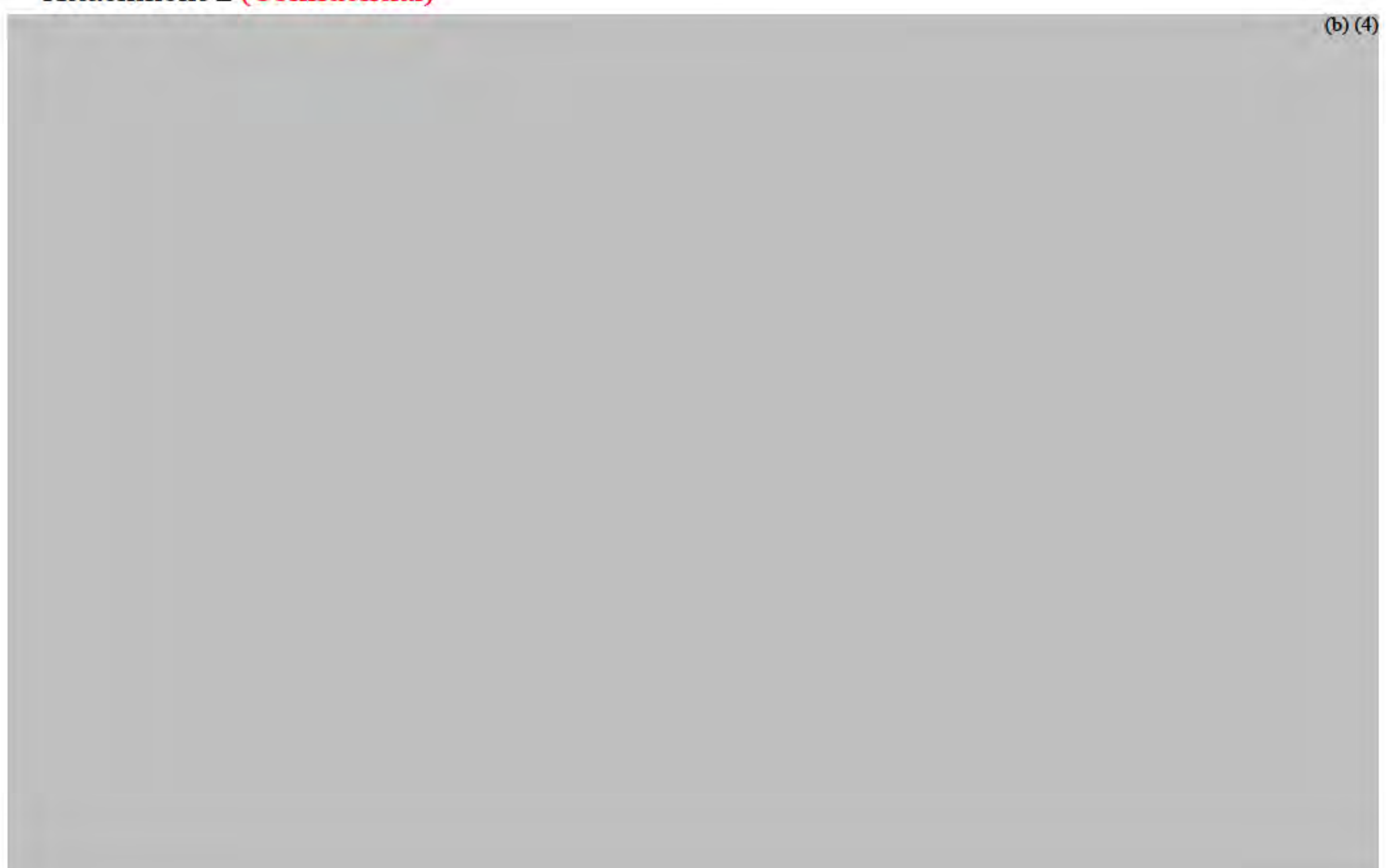
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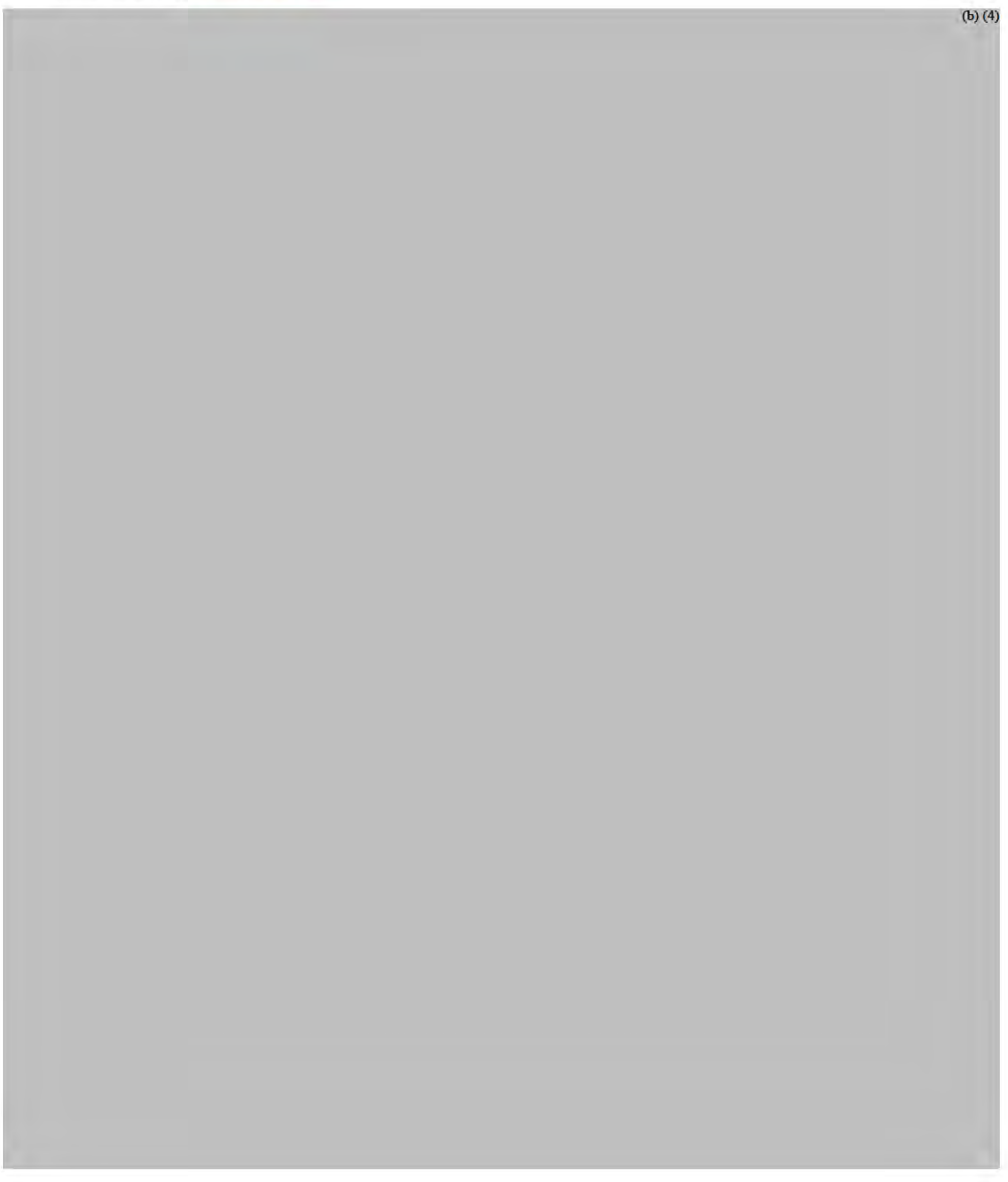
Open Reading Frame Analysis for the Full Genome Sequence of *Corynebacterium glutamicum* KCCM80058 (CONFIDENTIAL)

REPORT DATE: November 20, 2018

CJ BLOSSOM PARK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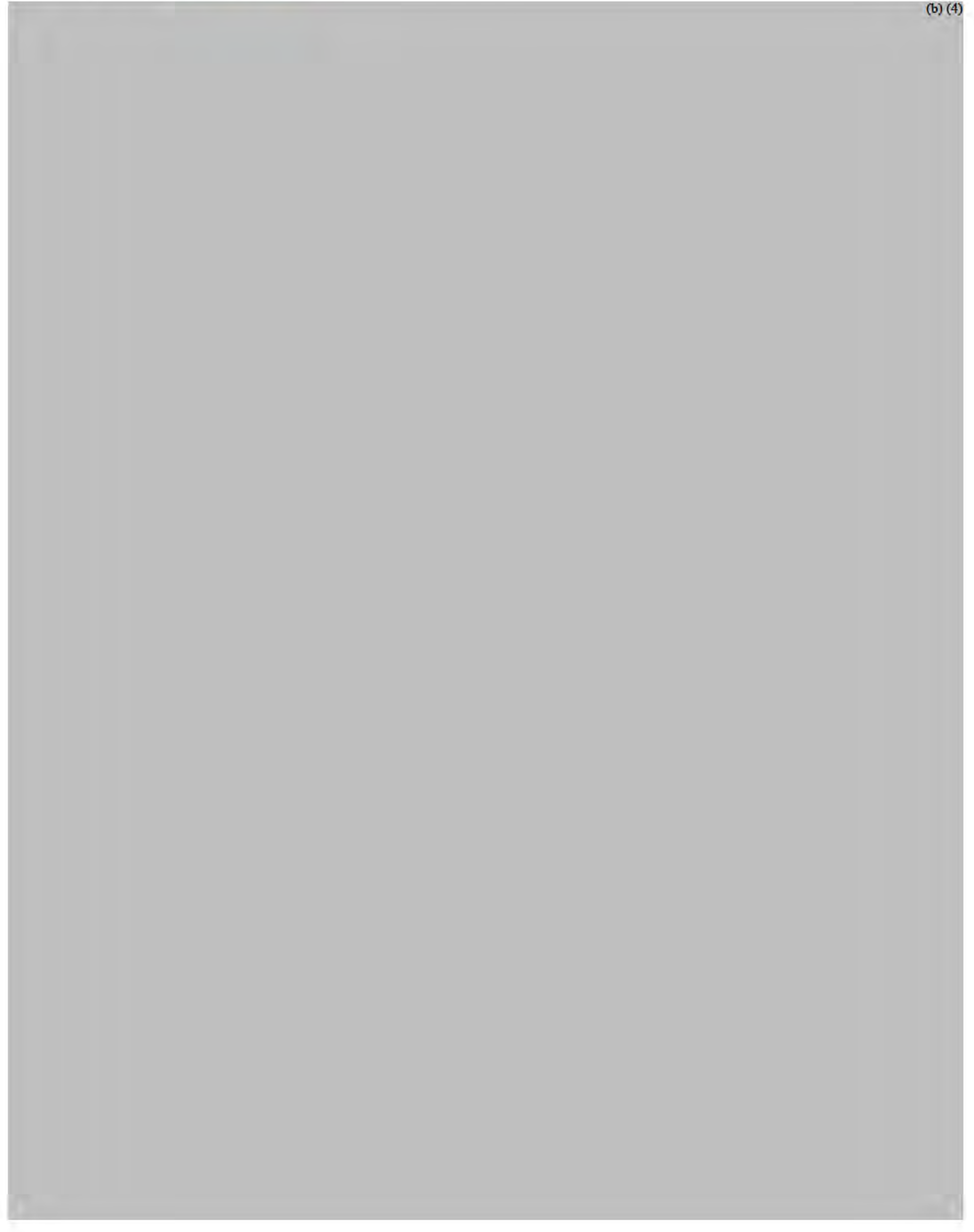

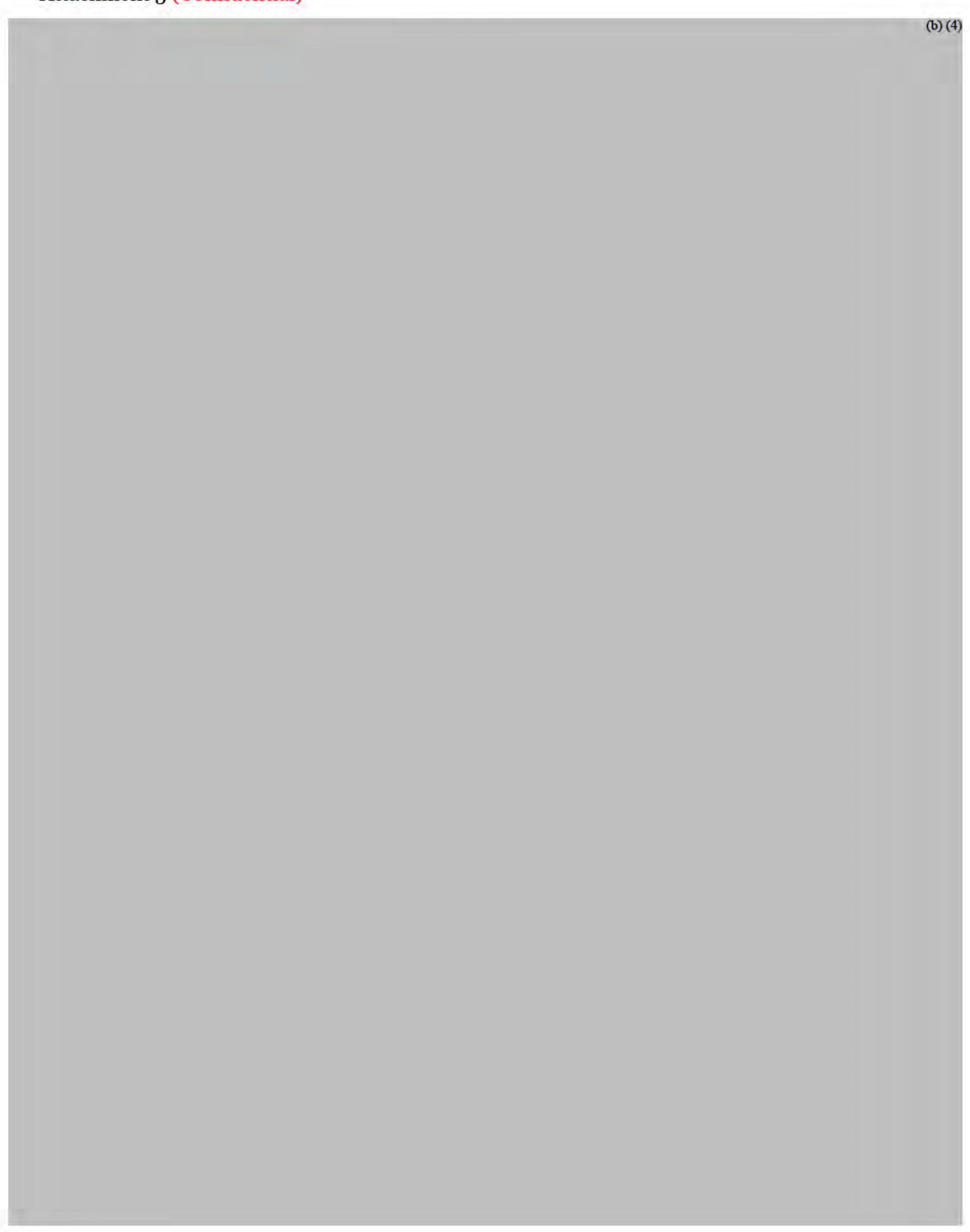
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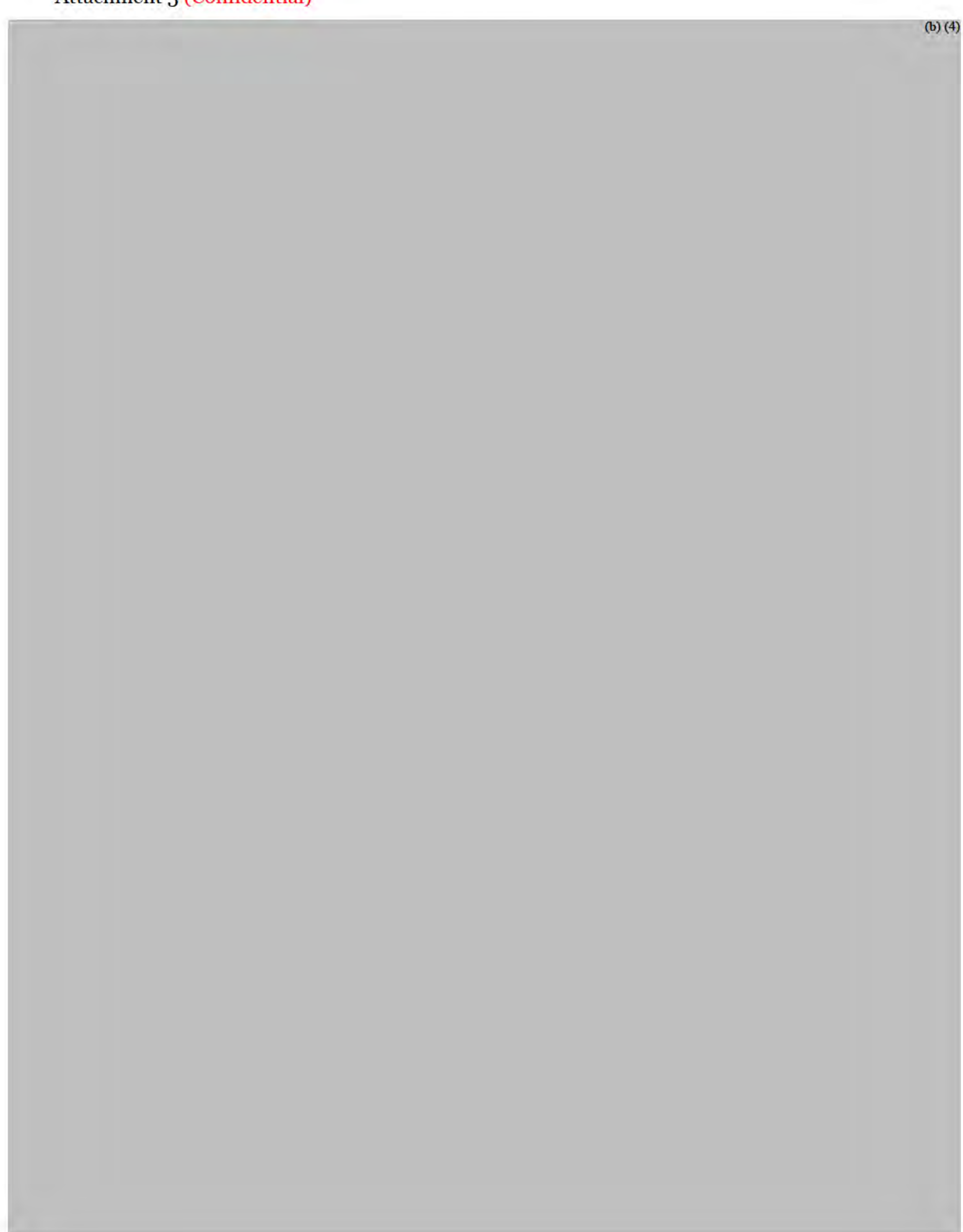
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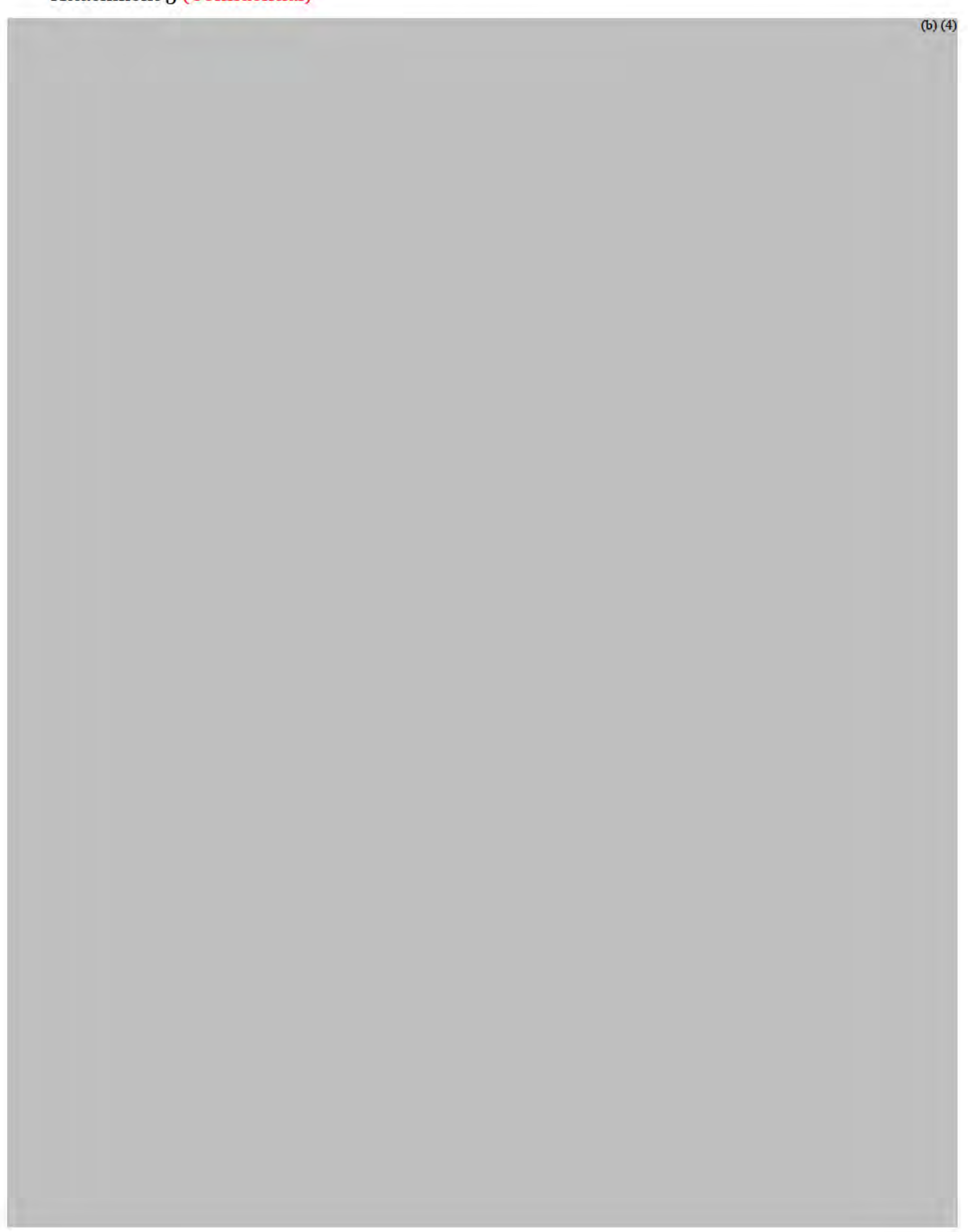
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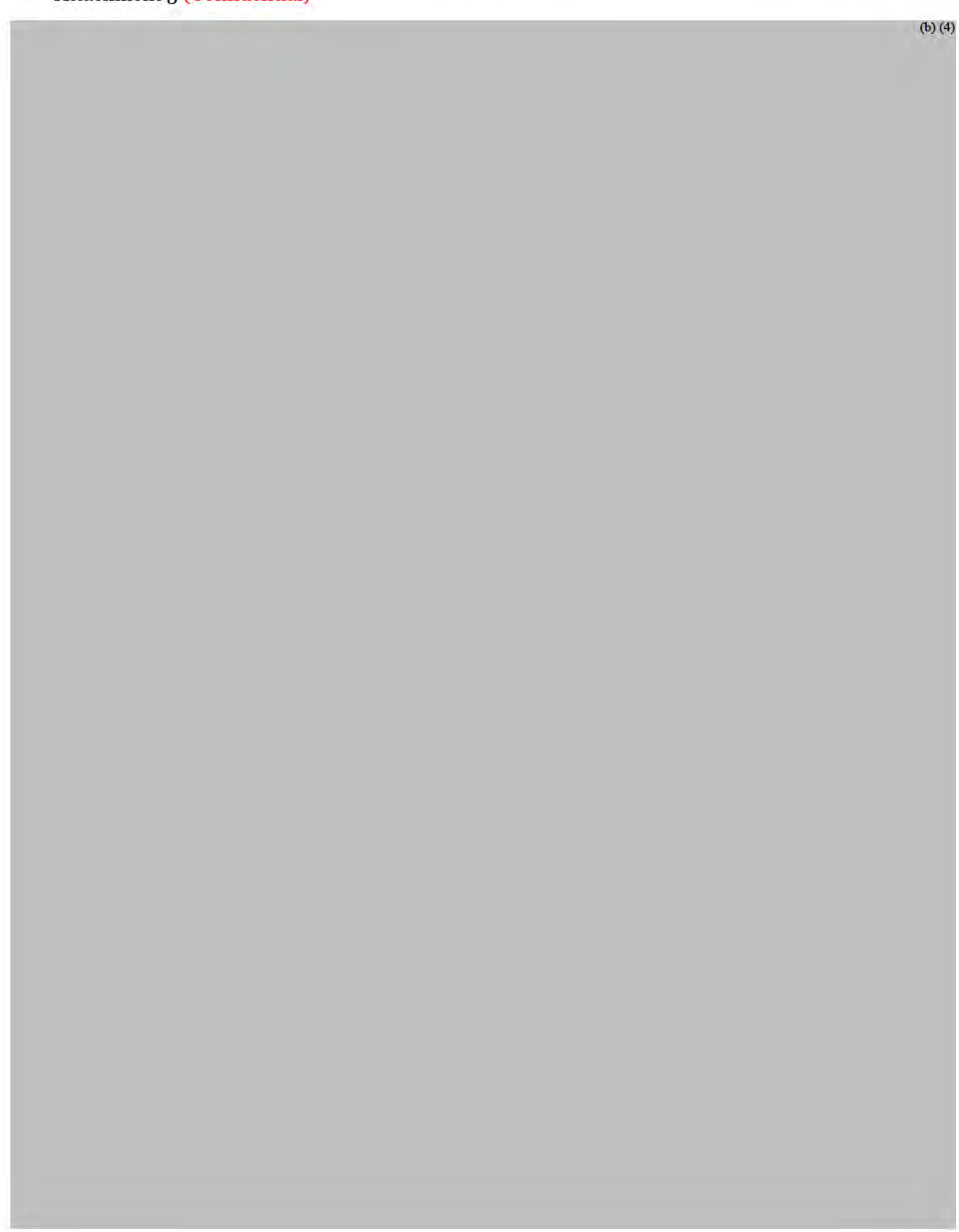
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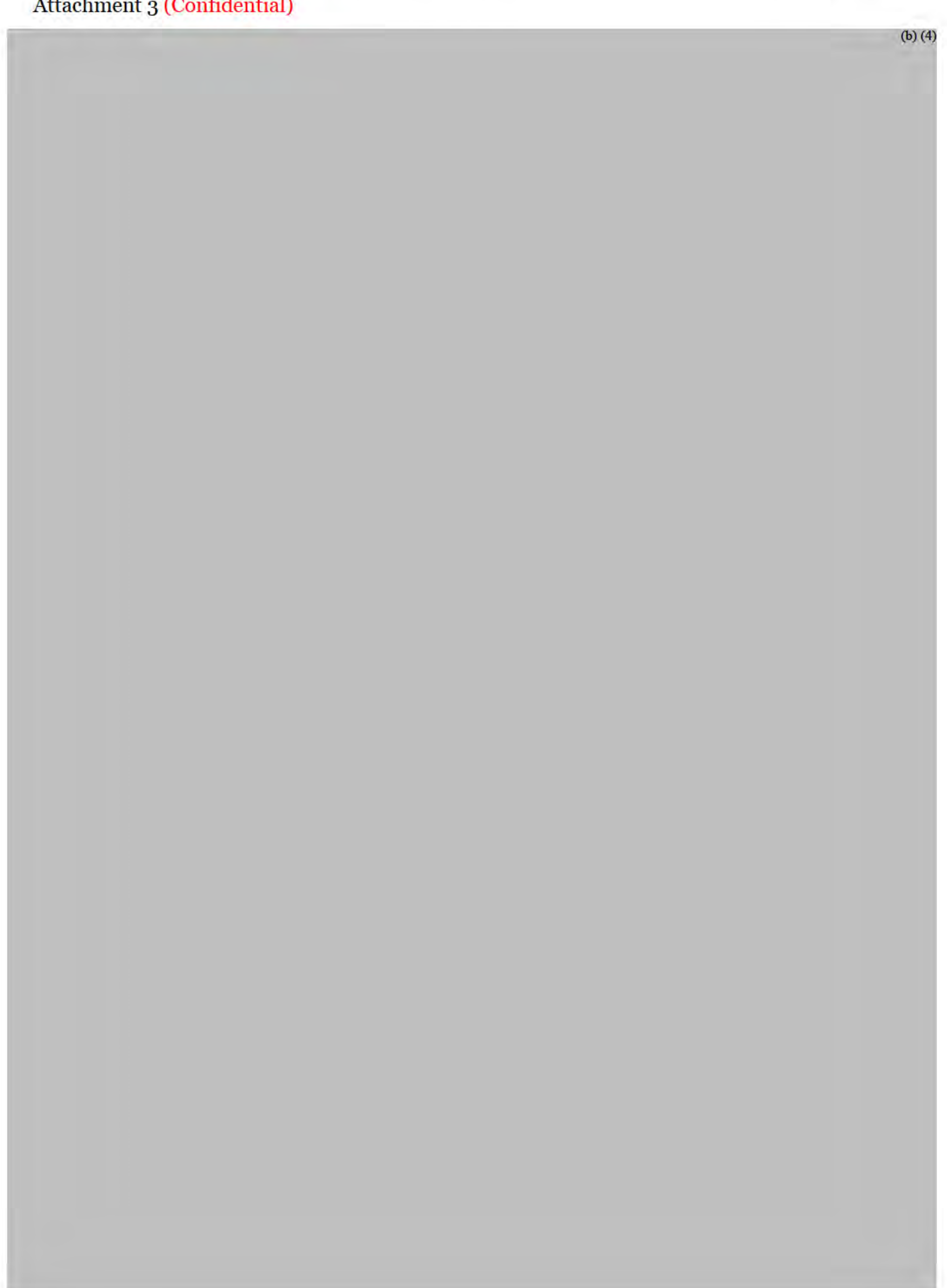

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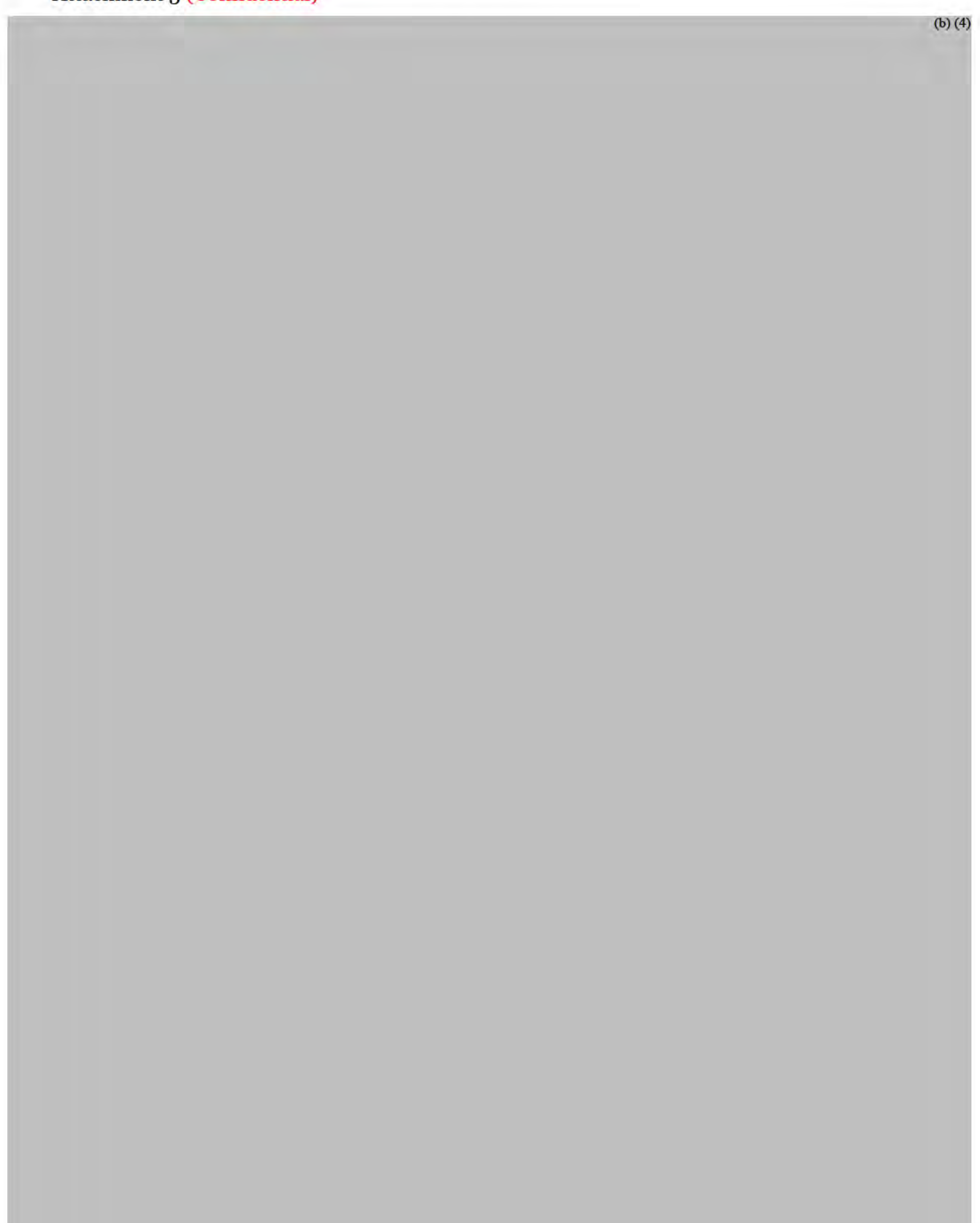
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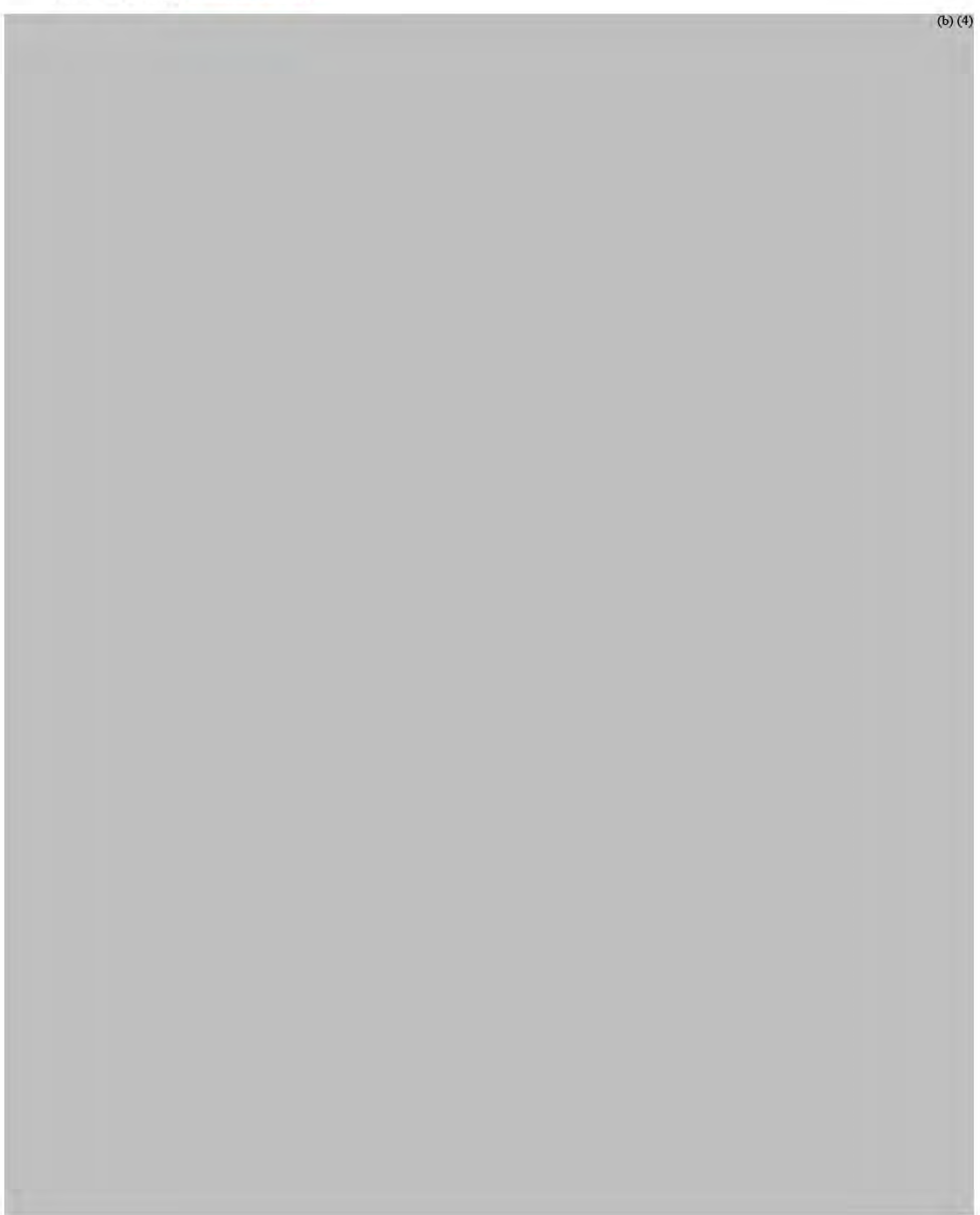

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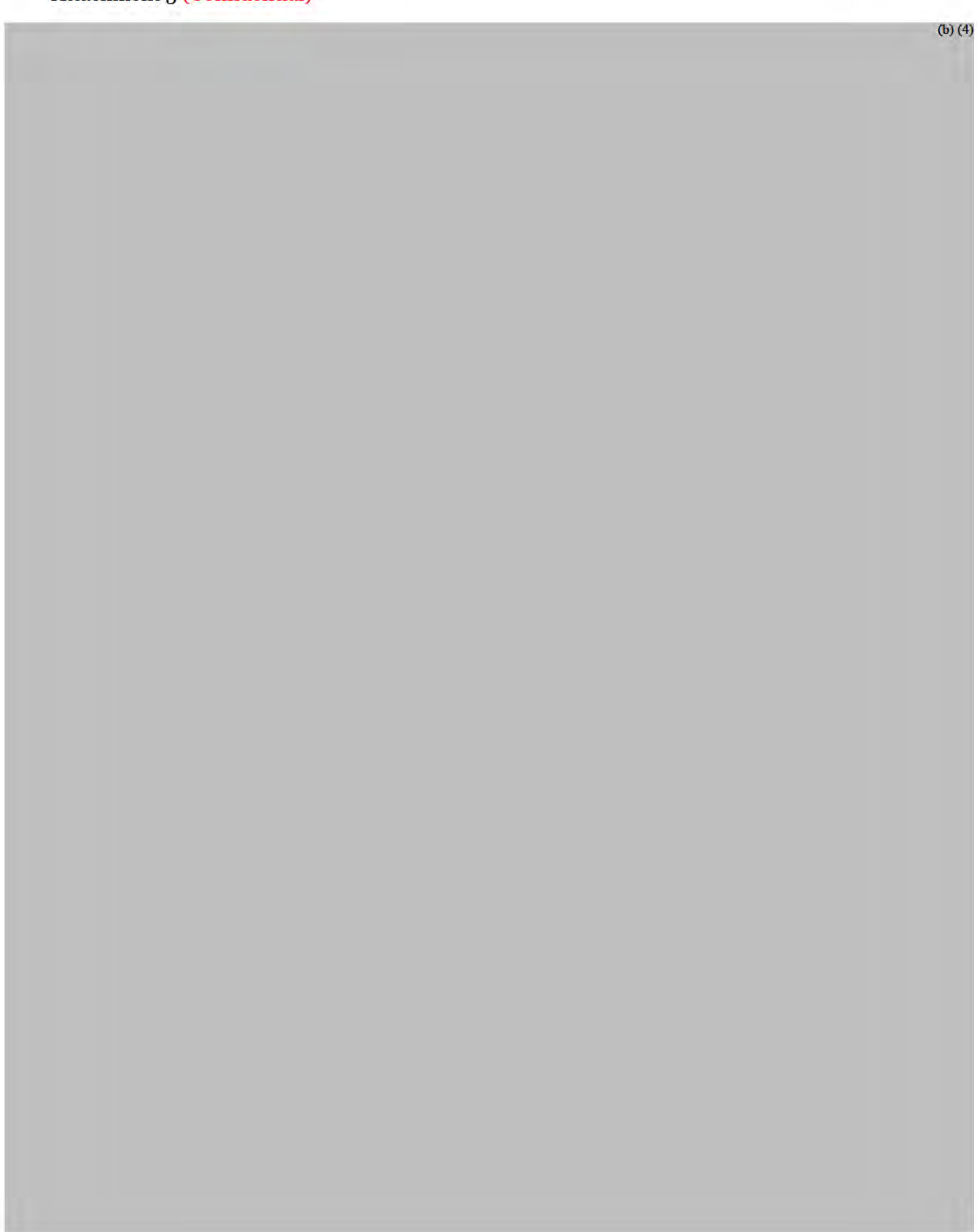
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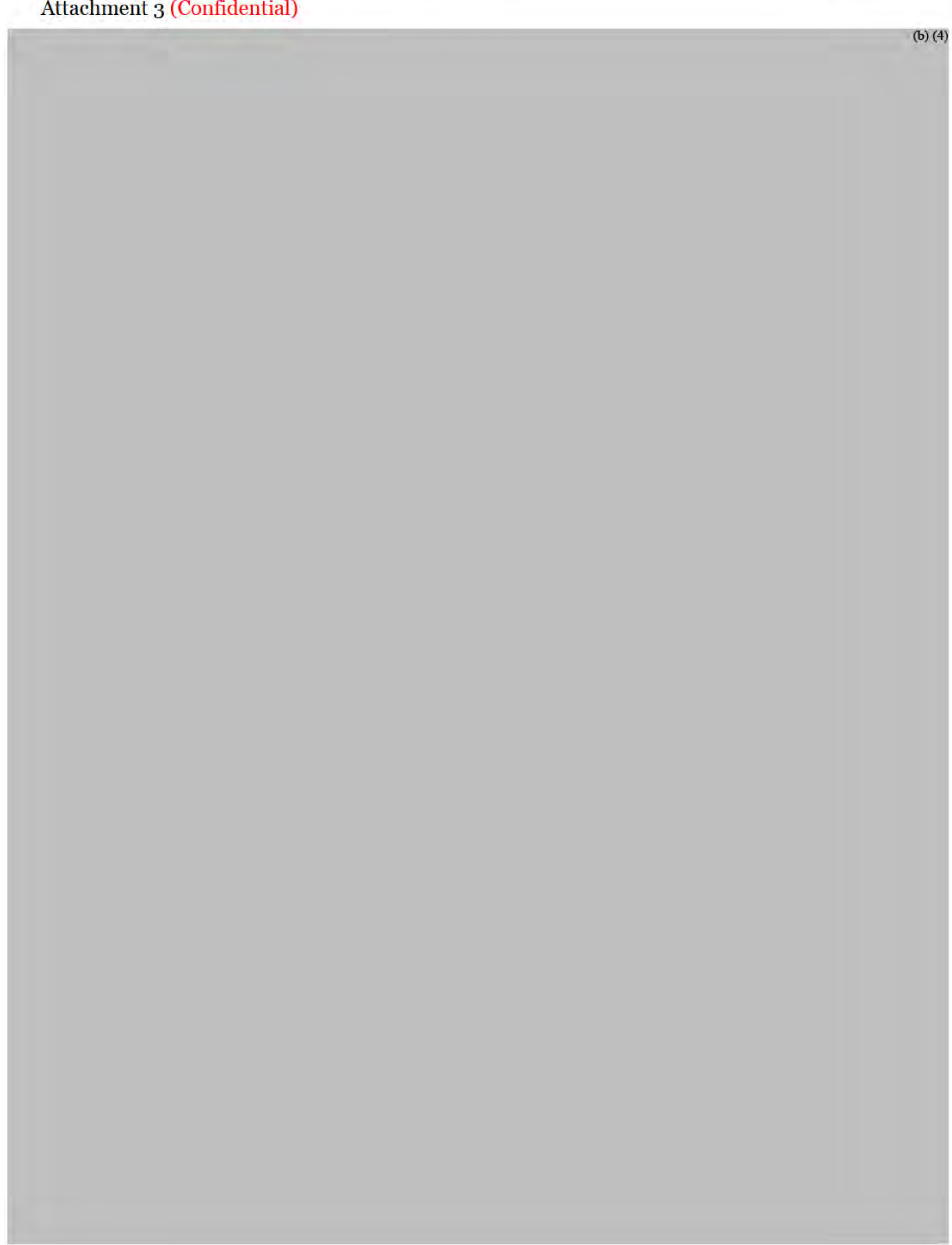
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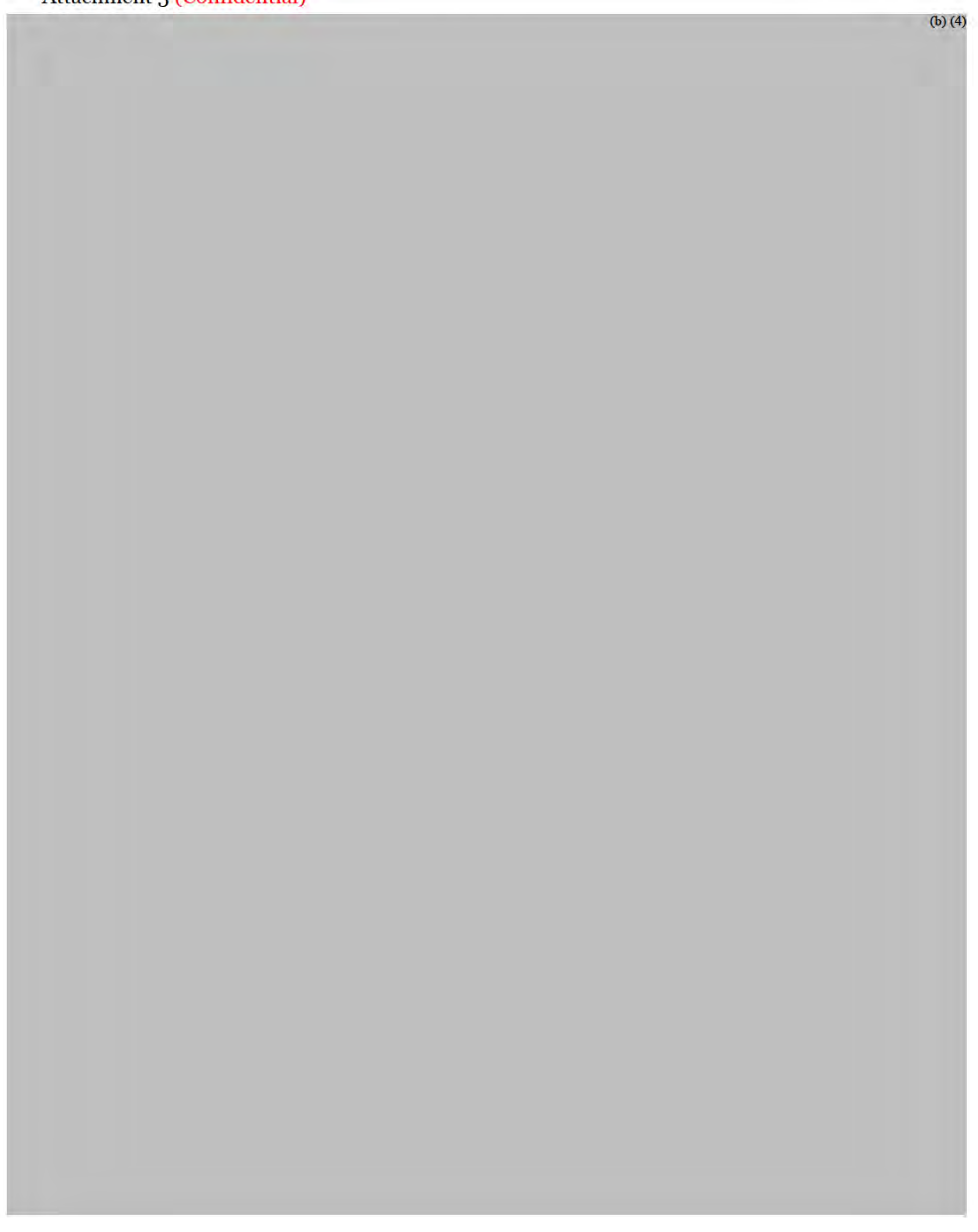
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Antibiotic resistance of the Production strain

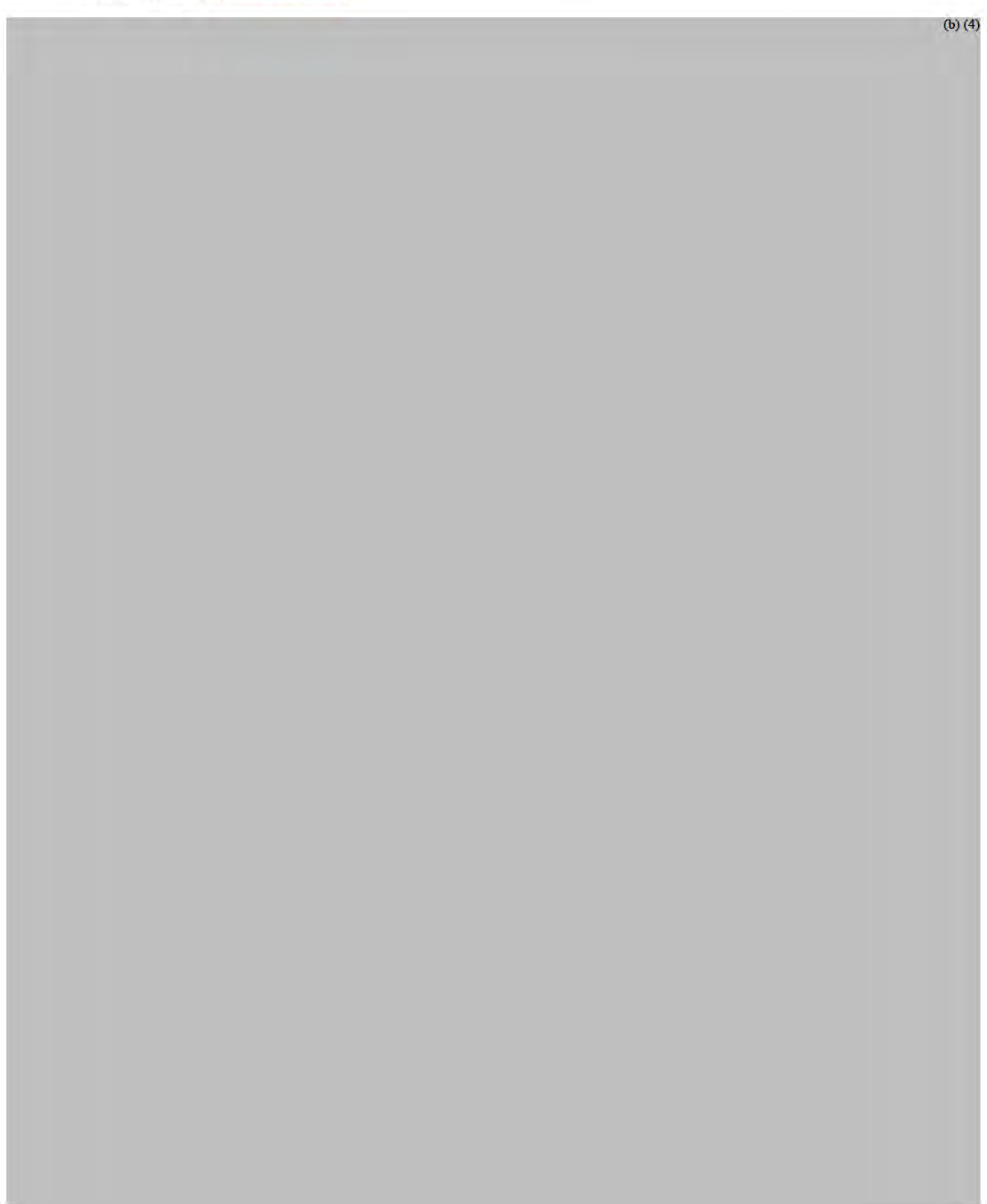
Determination of Antibiotic Minimal Inhibitory Concentration (MIC) of the Production Strain, *Corynebacterium glutamicum* KCCM80058

(Confidential)

ORIGINAL FINAL REPORT DATE: November 09, 2018

CJ Blossom Park

TITLE: Determination of Antibiotic Minimal Inhibitory Concentration (MIC) of the Production Strain, *Corynebacterium glutamicum* KCCM80058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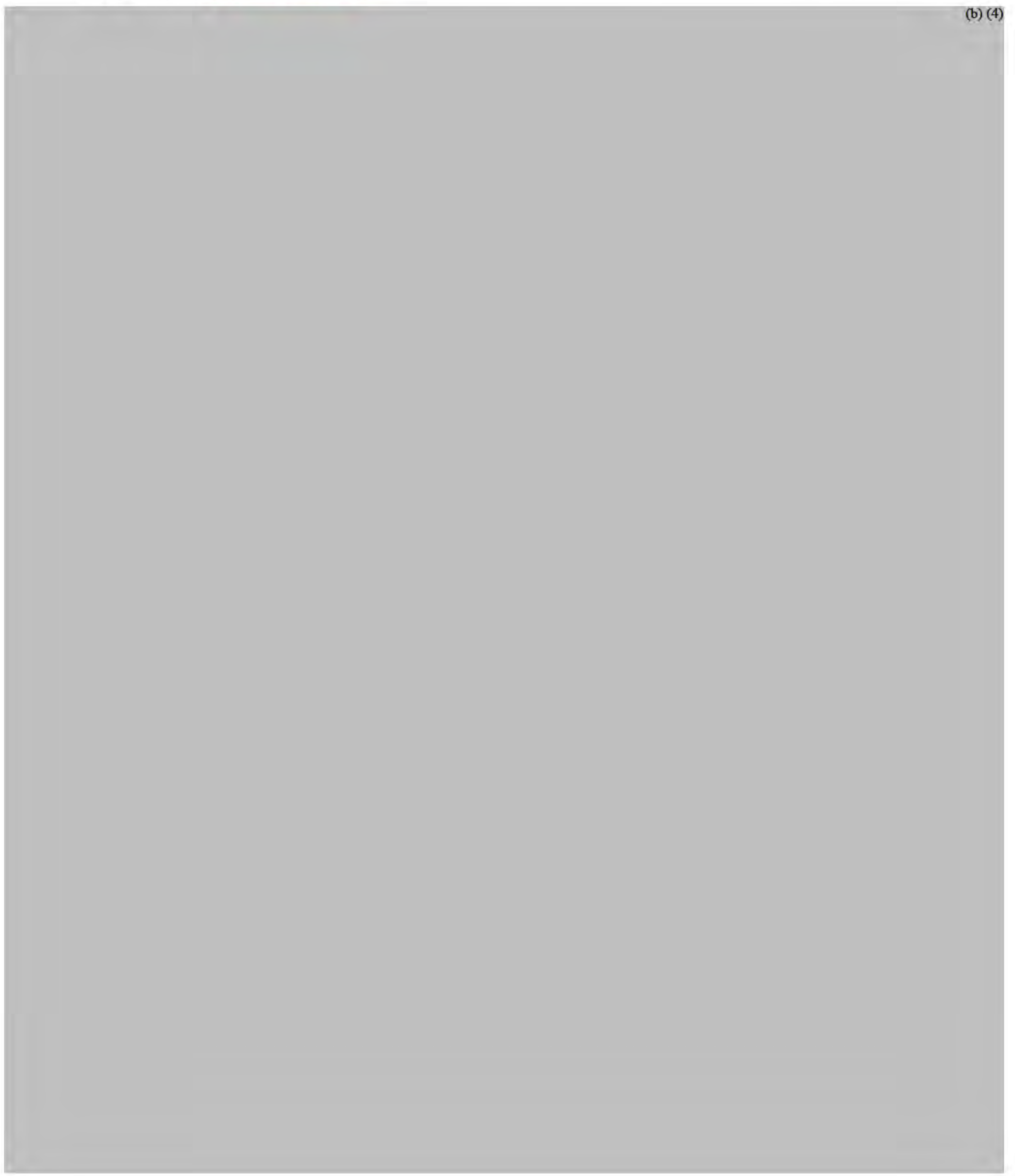
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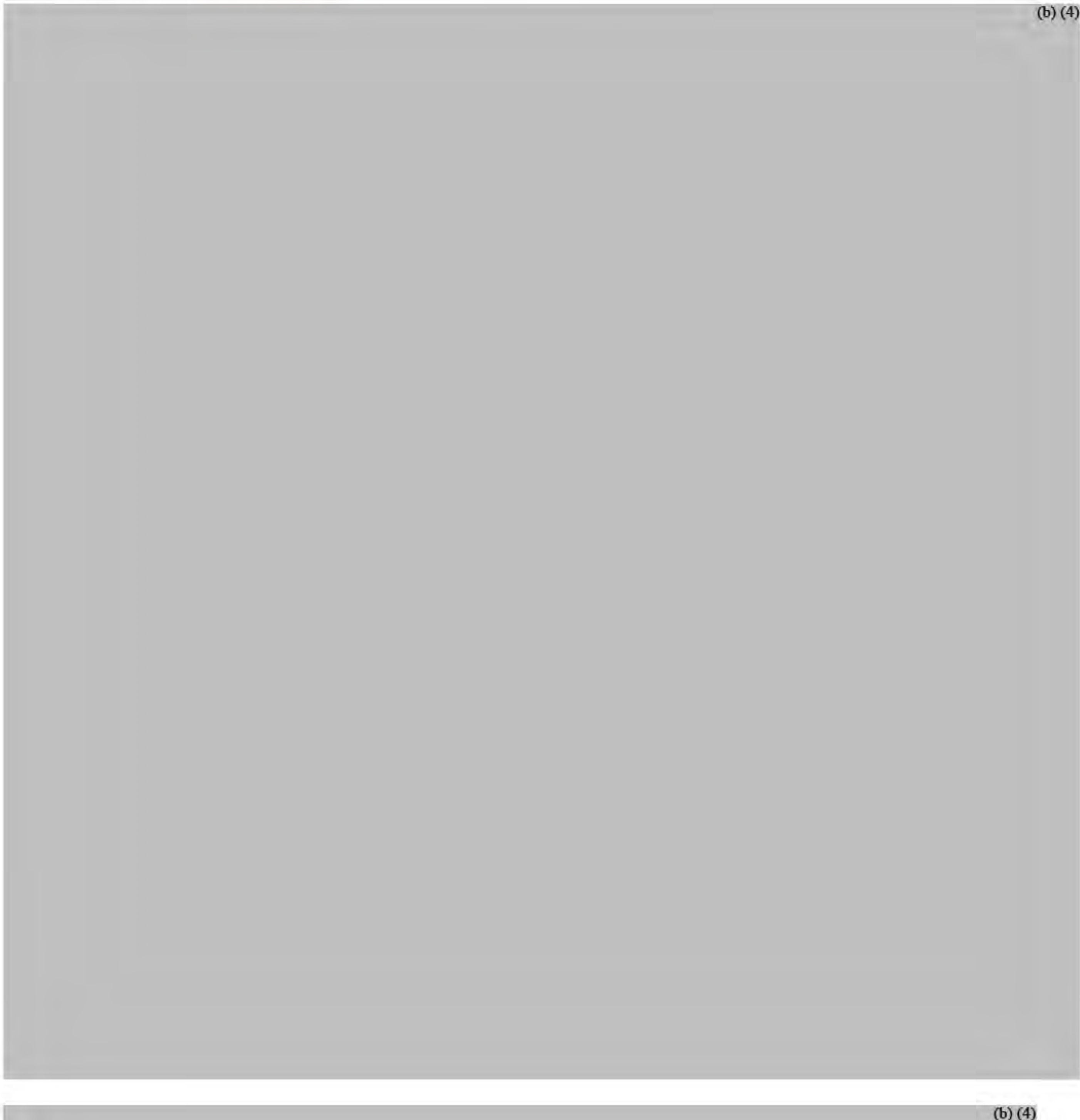
Detection of the Residual Production Strain in Dried L-Valine Fermentation Product

(Confidential)

ORIGINAL FINAL REPORT DATE: October 02, 2019

CJ Blossom Park



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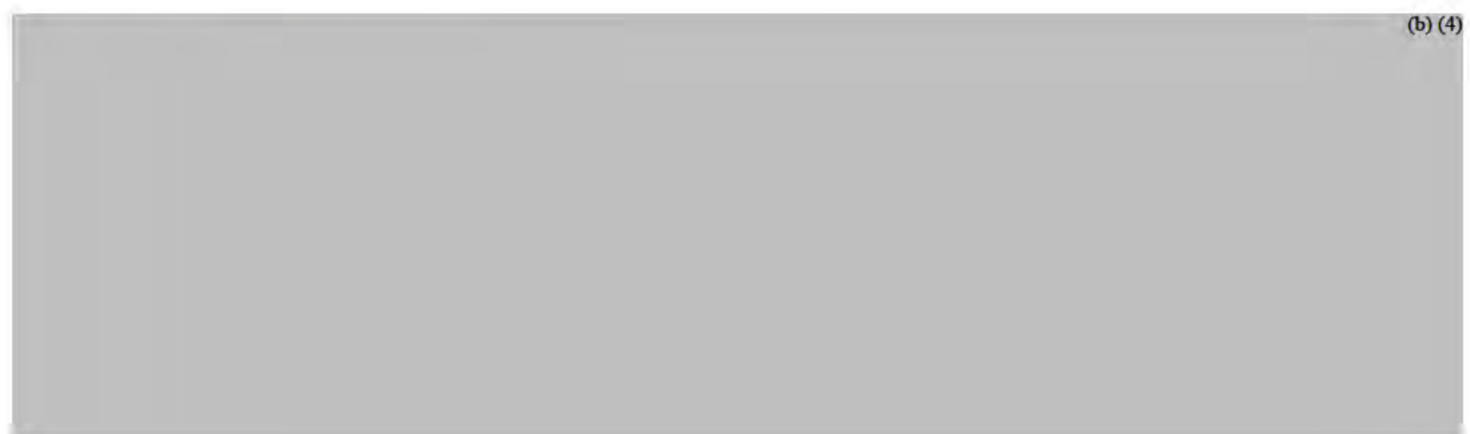
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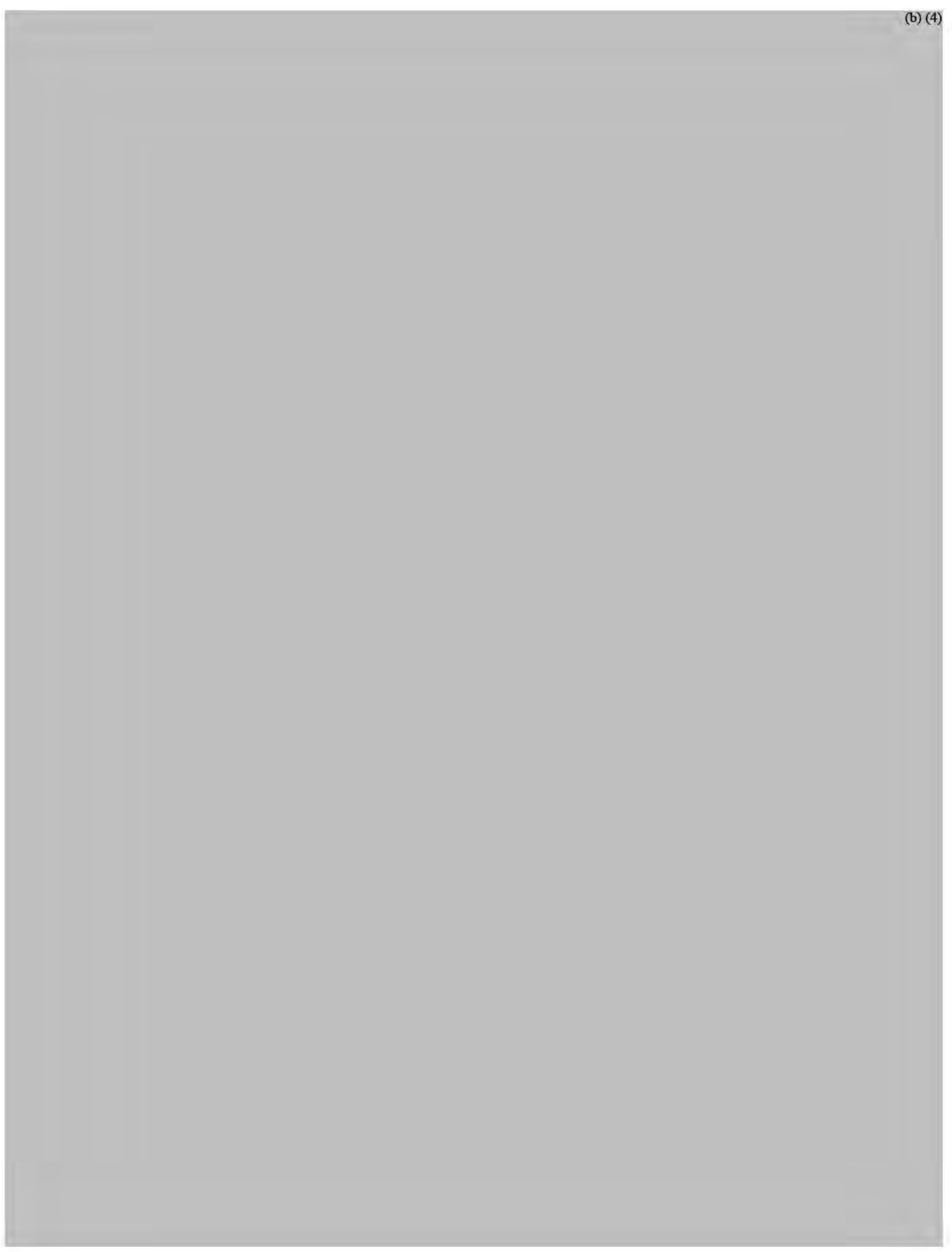
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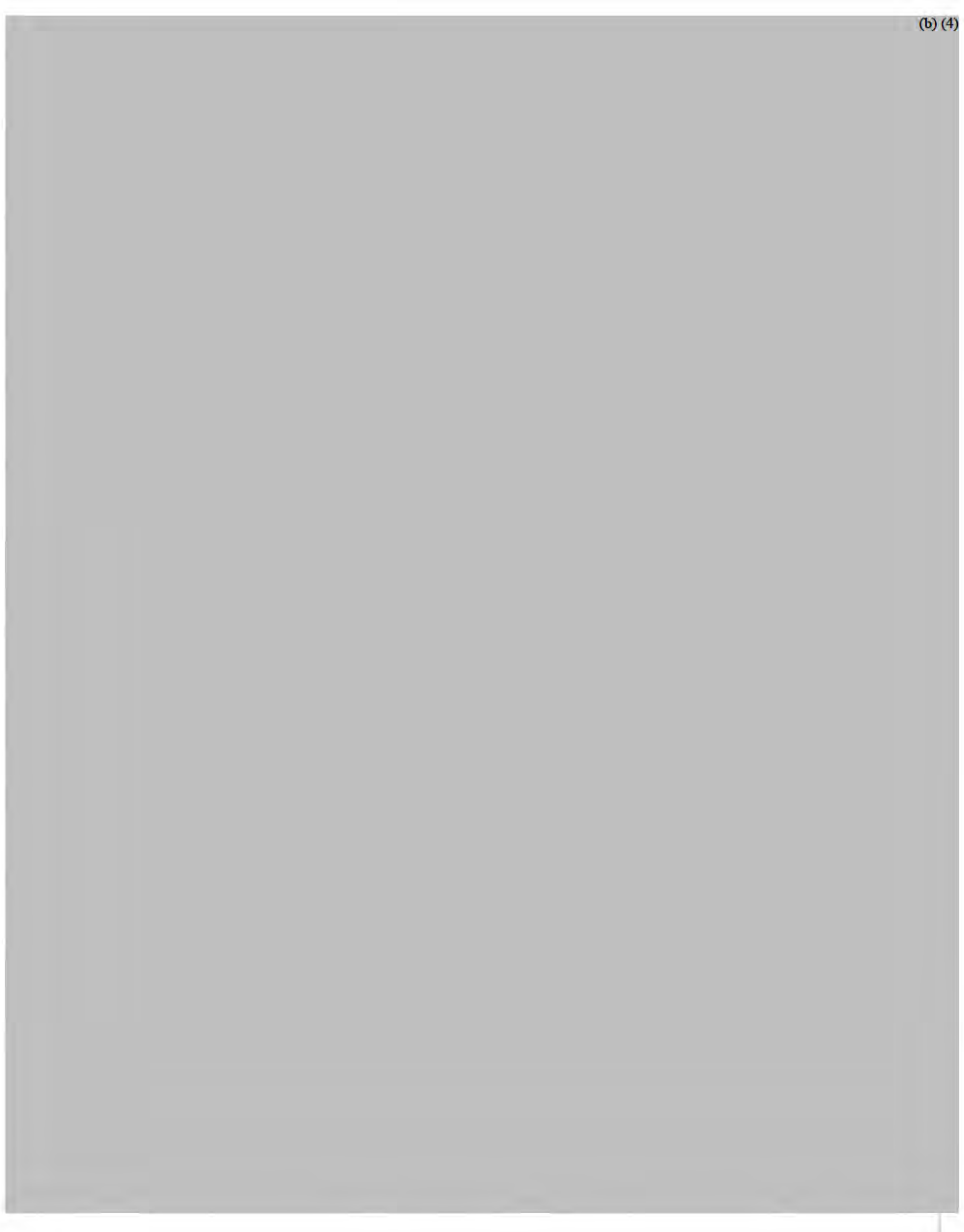
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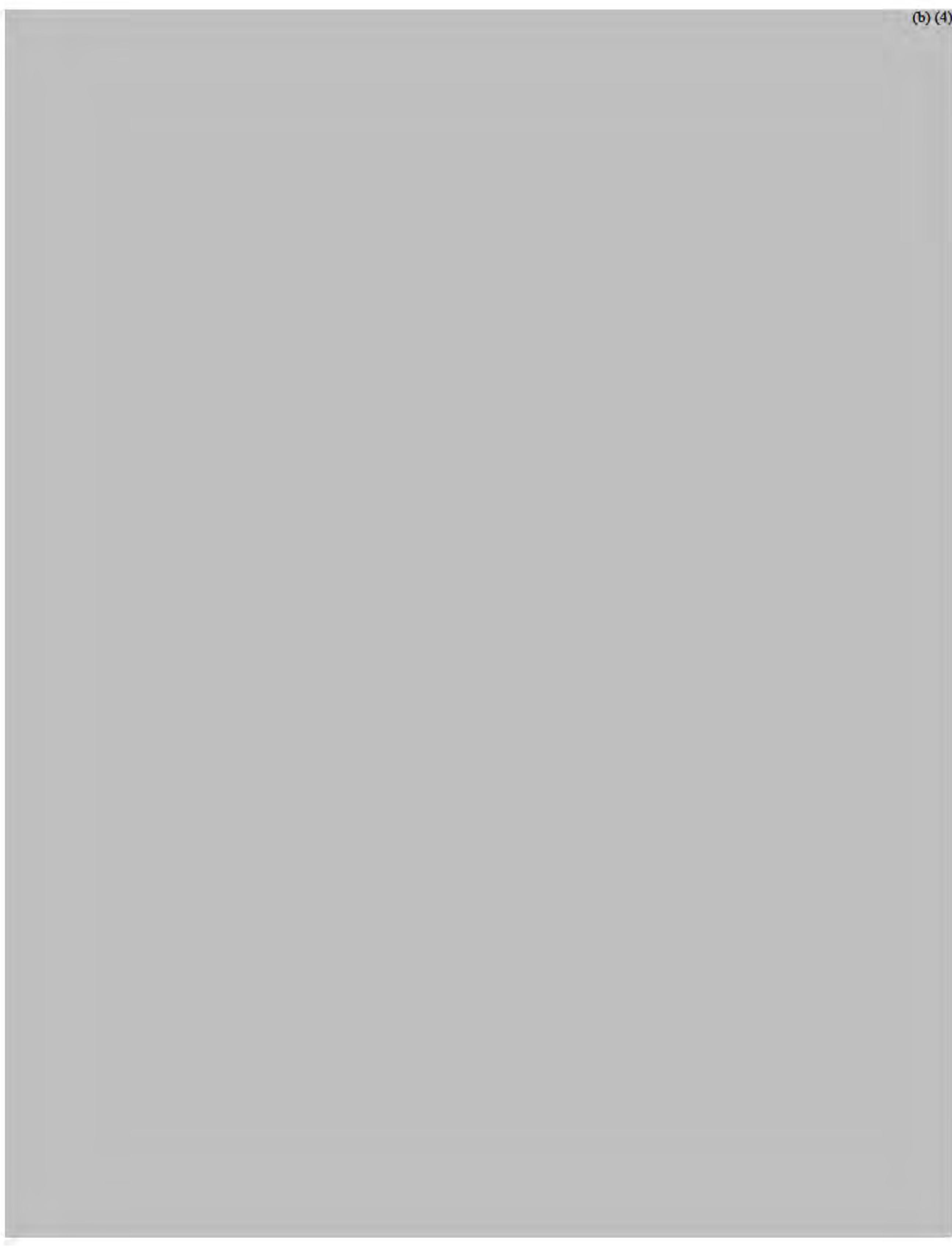
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APPENDIX 3. PRE-FERMENTATION INFORMATION (CONFIDENTIAL)

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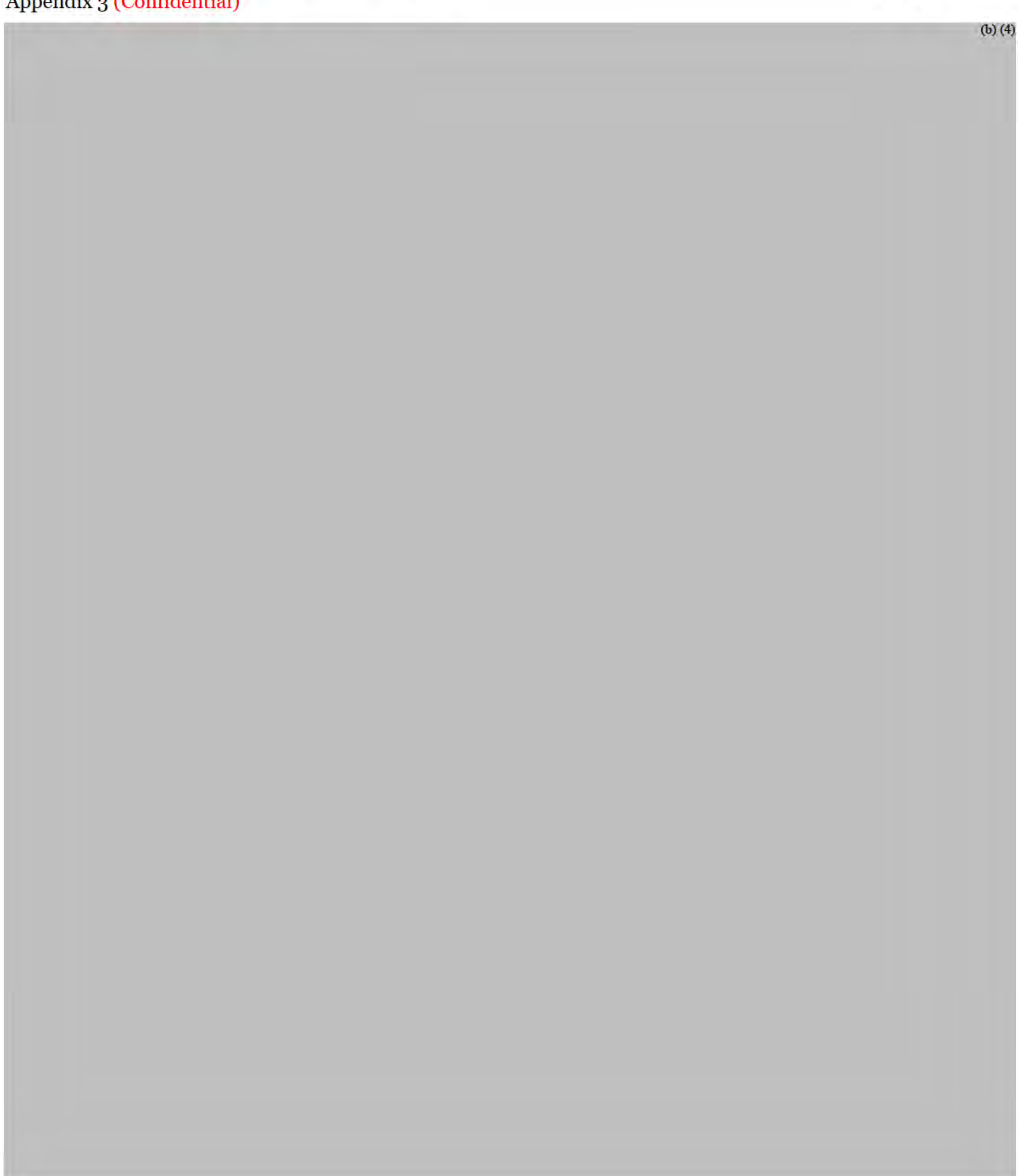
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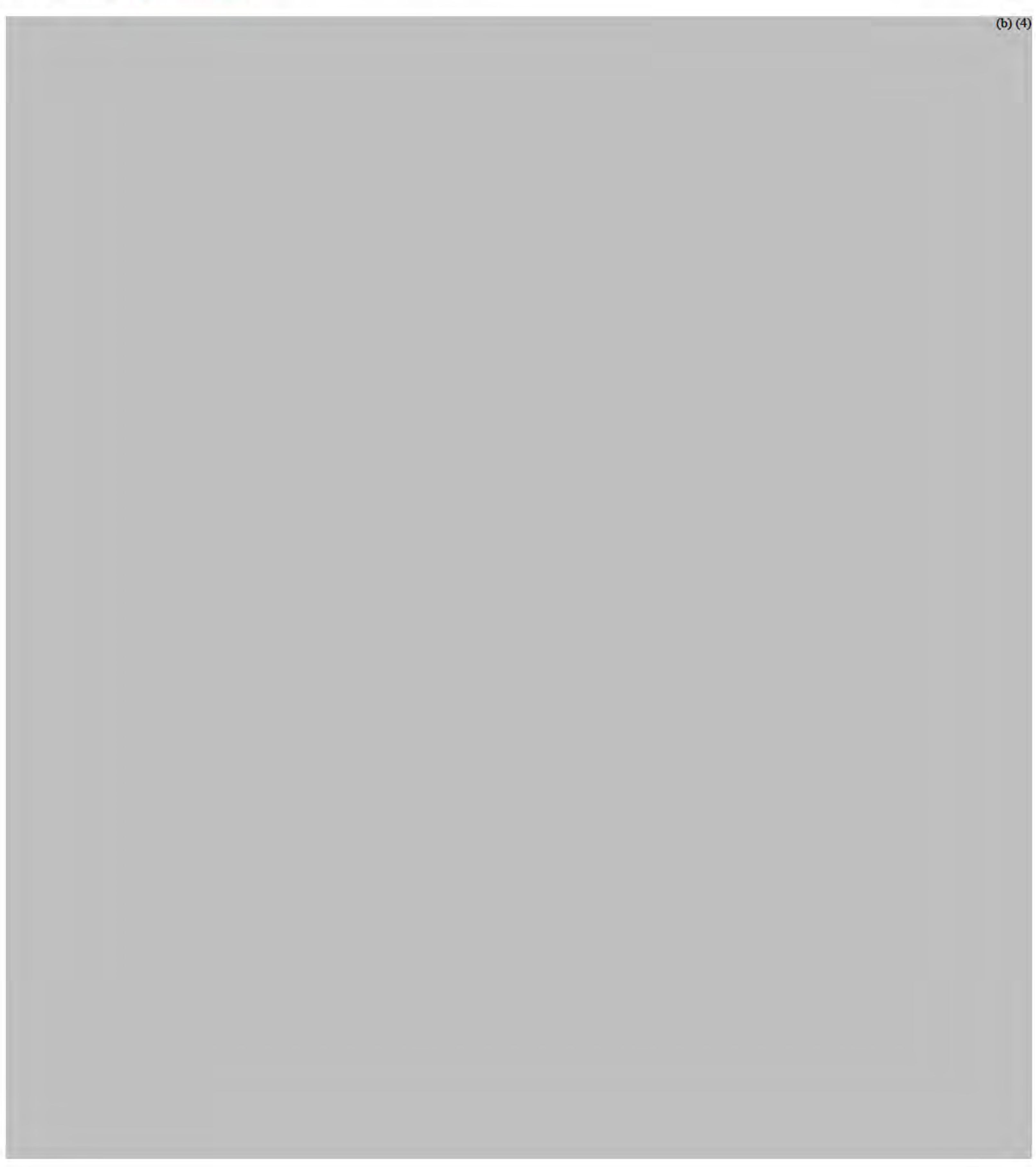
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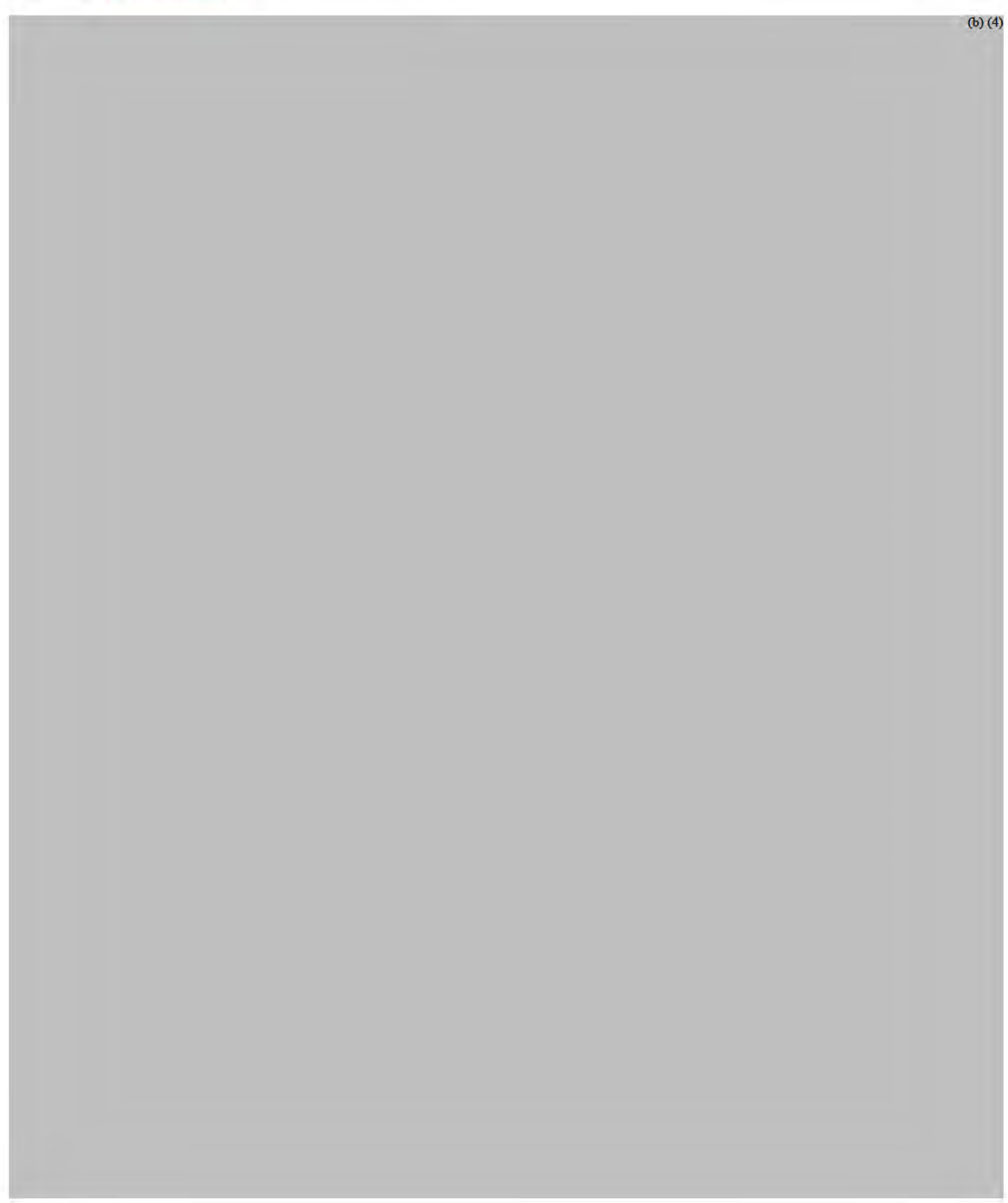
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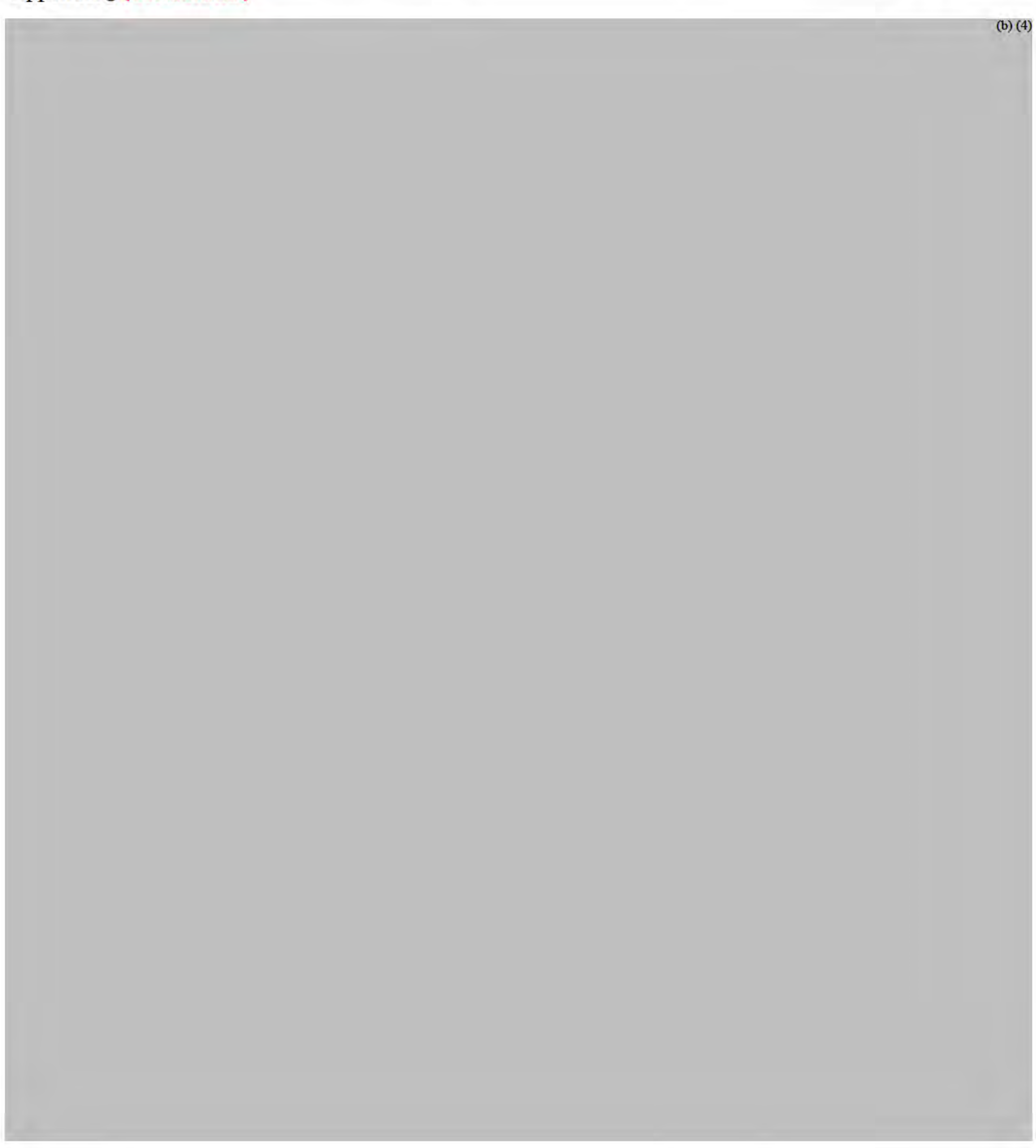
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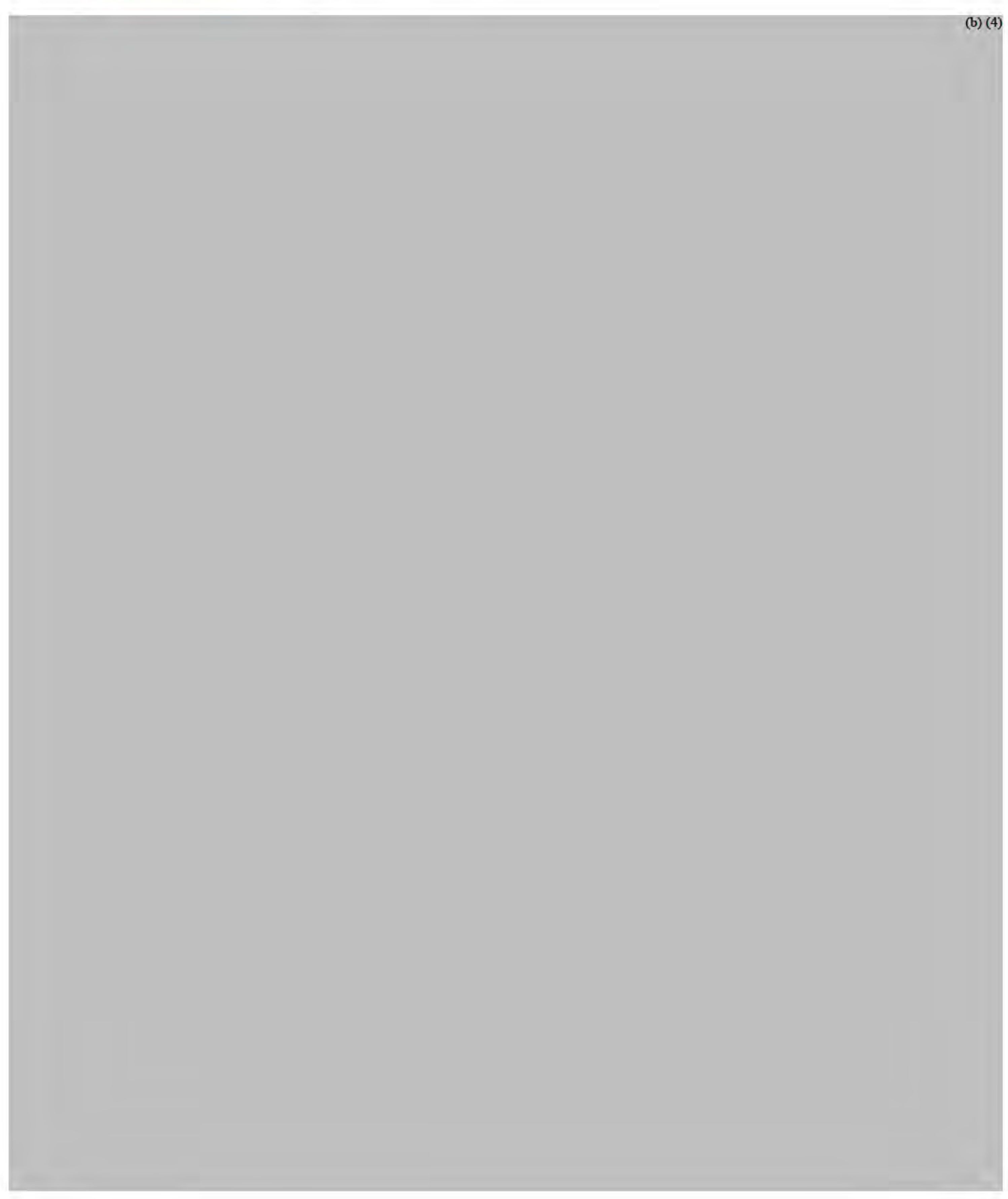
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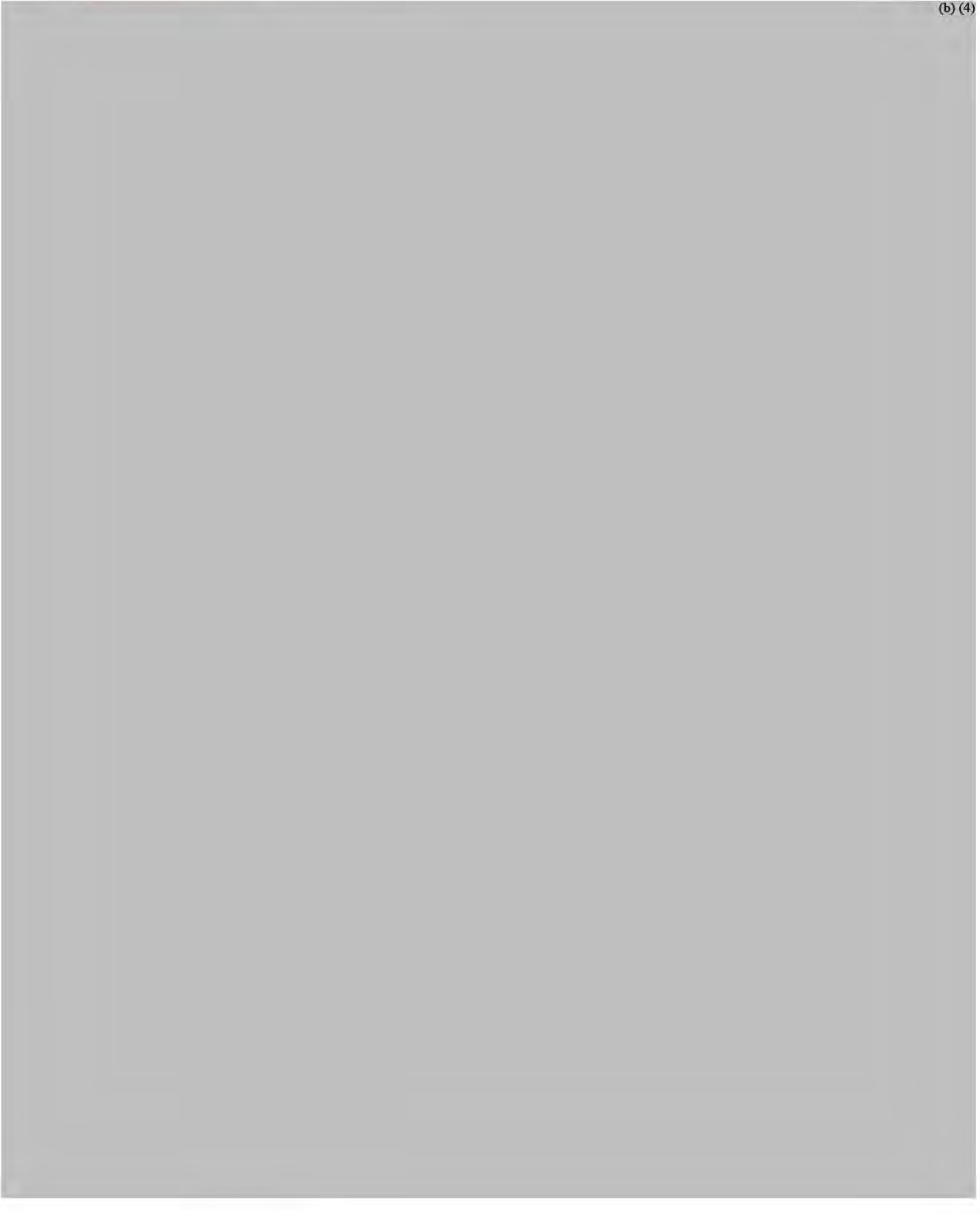
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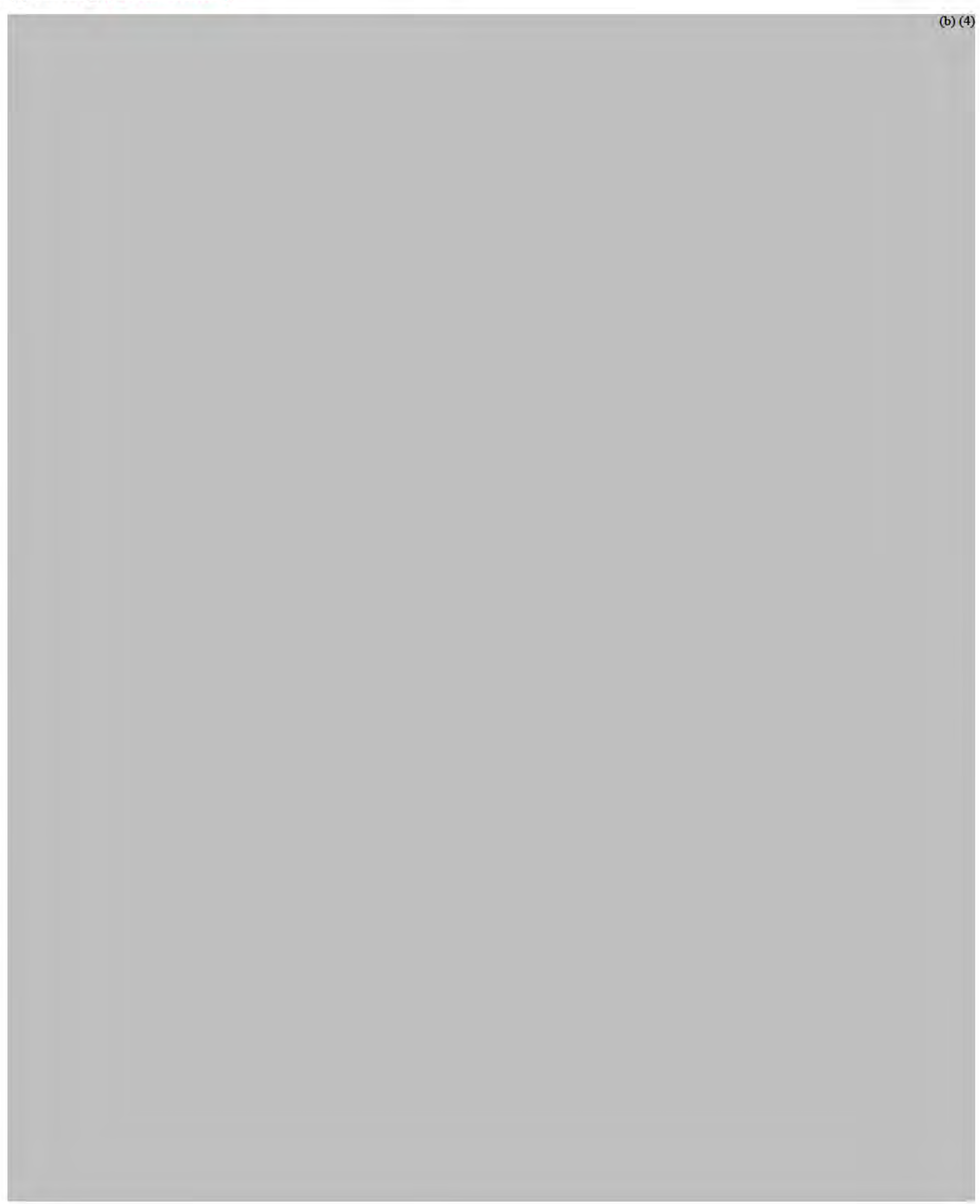
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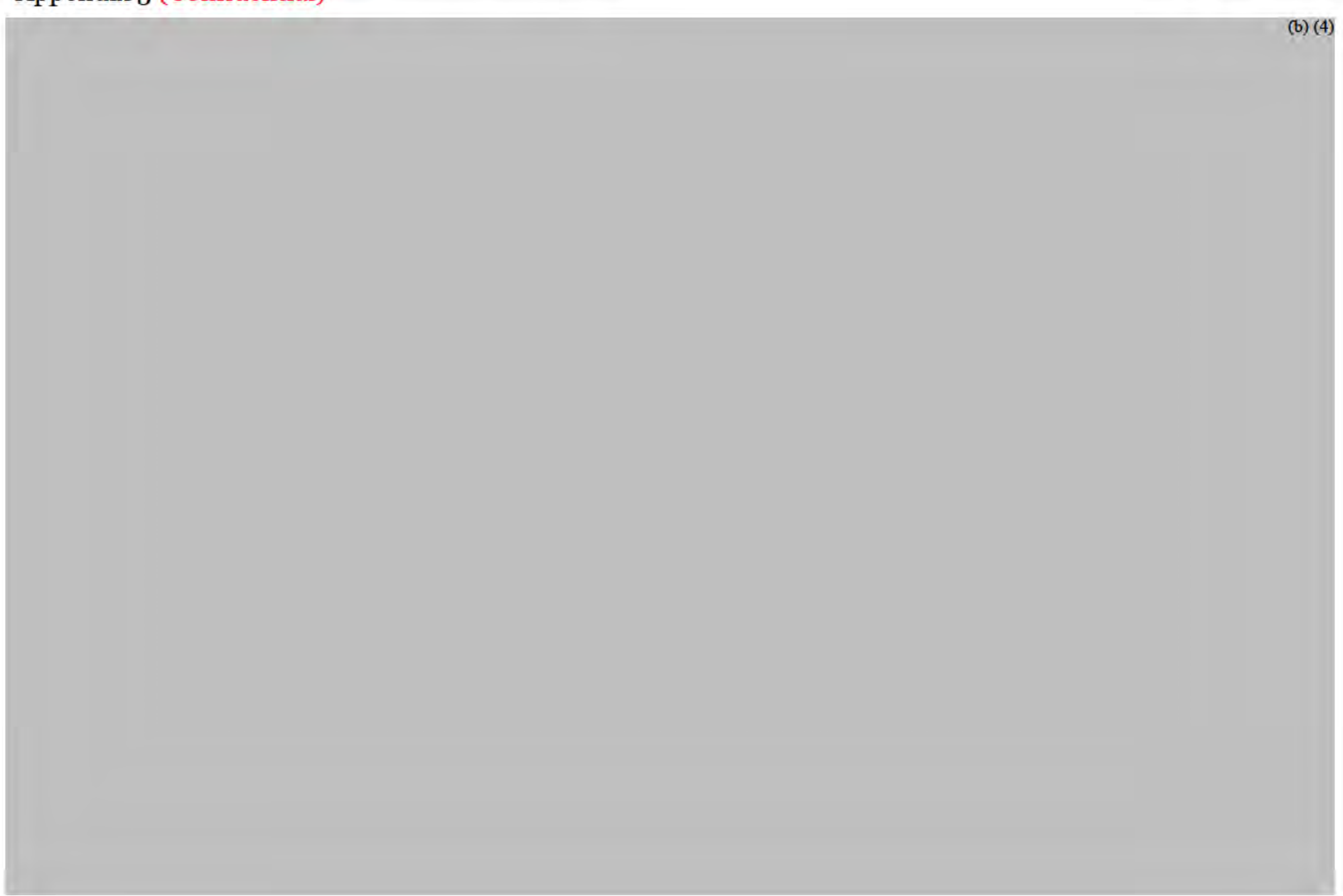
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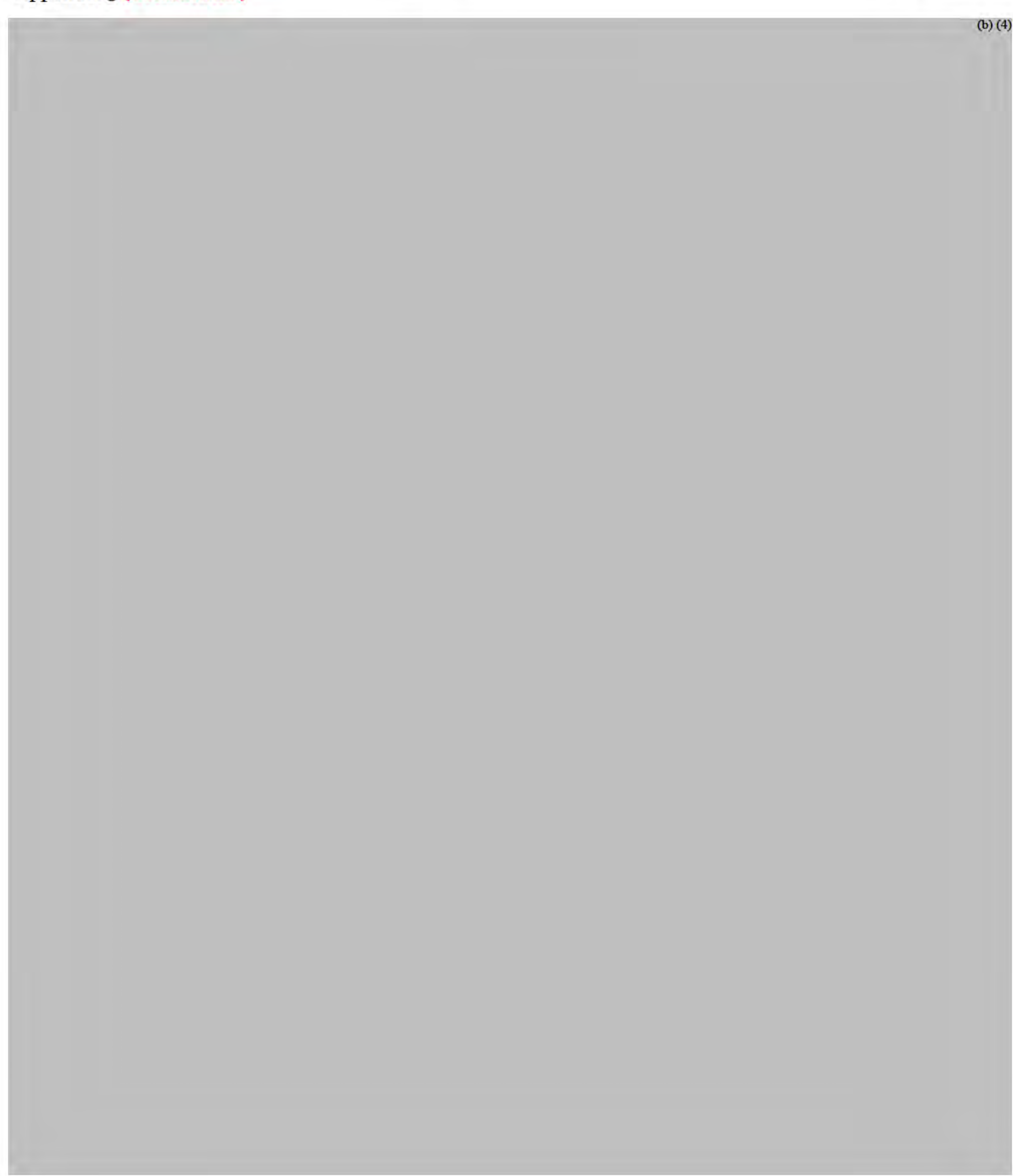

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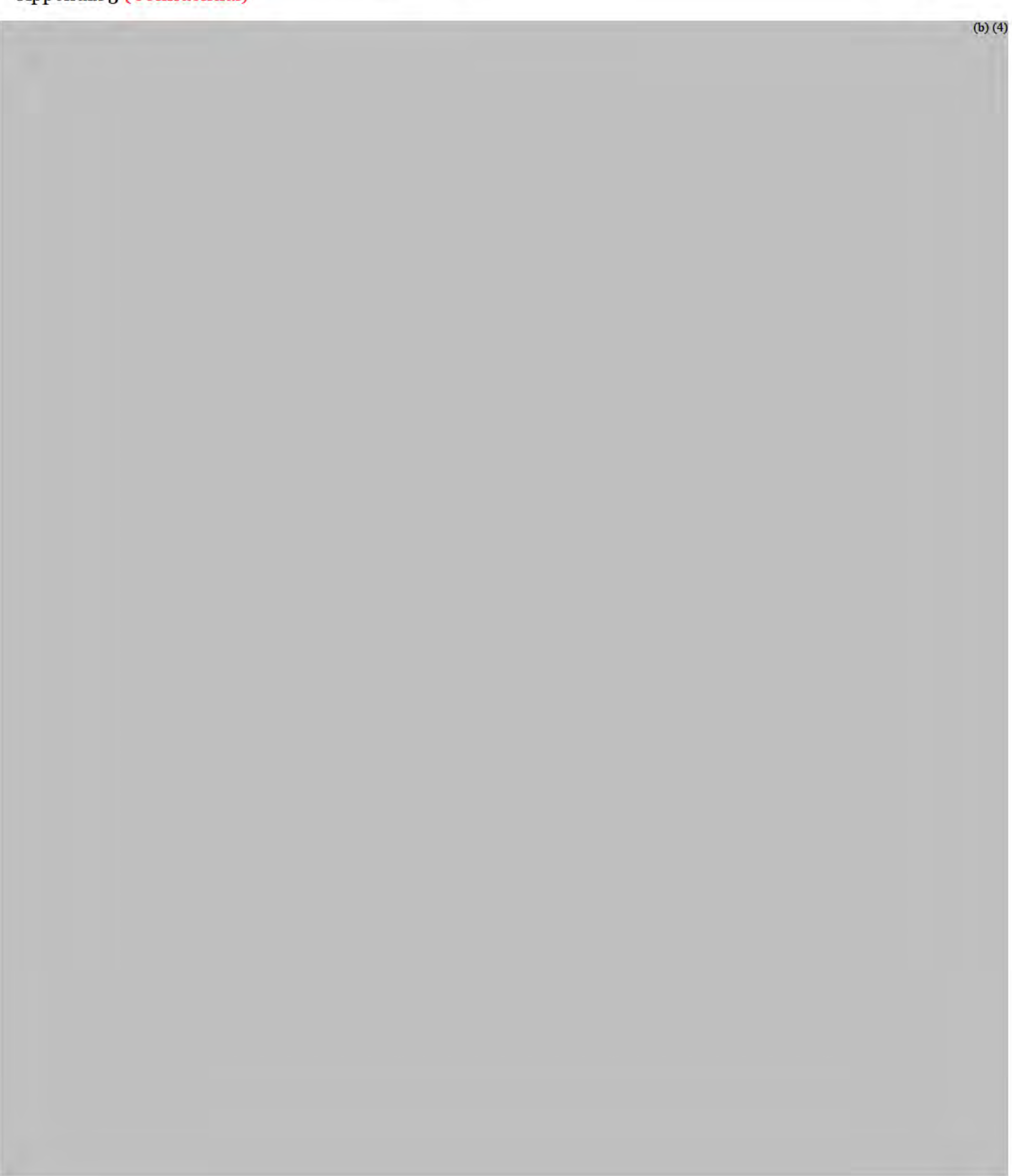

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Eggeling, Lothar, Michael Bott. 2005. Handbook of Corynebacterium glutamicum. Taylor and Francis group, LLC, p. 37-56

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Microbial Inactivation Study

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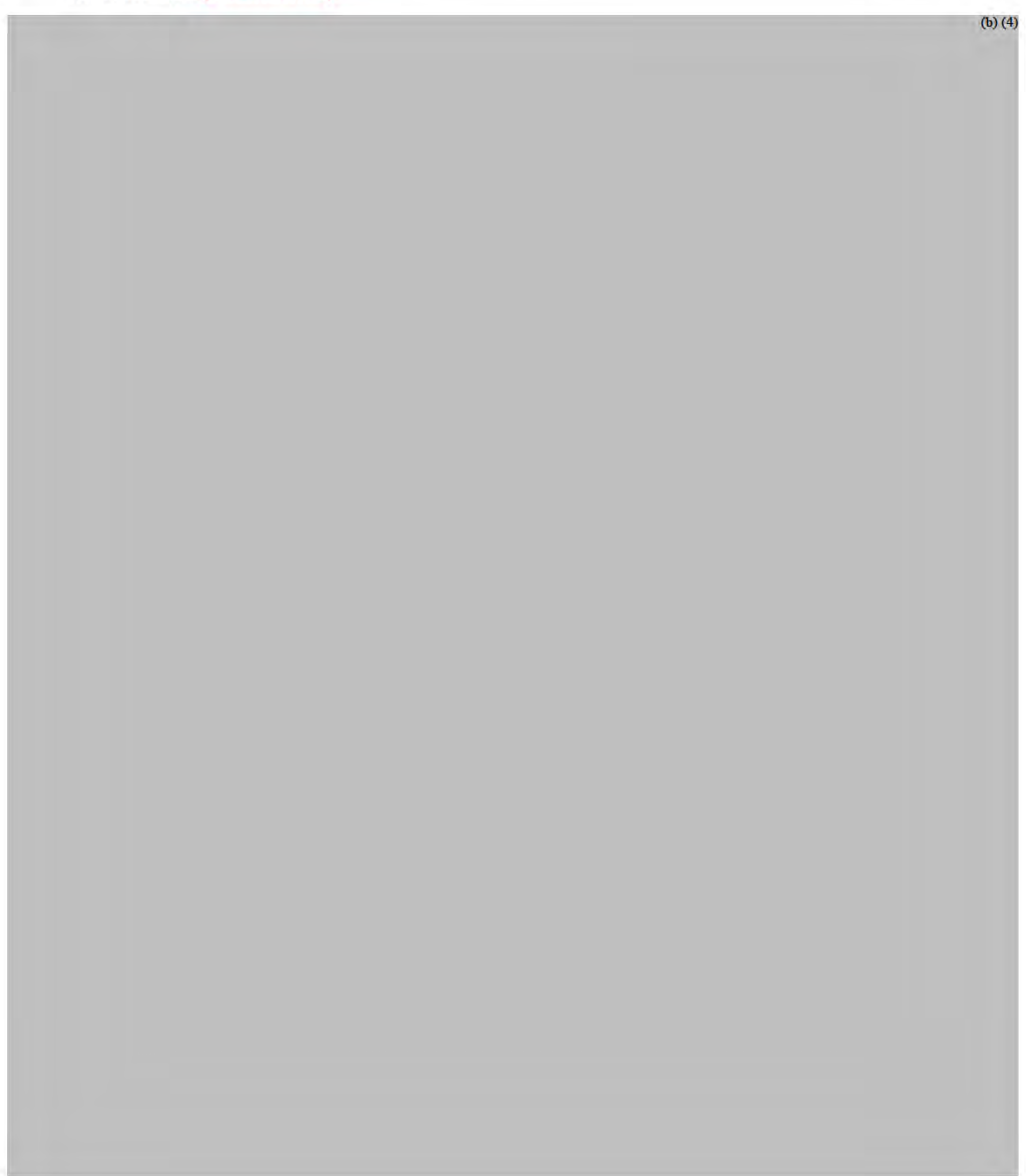
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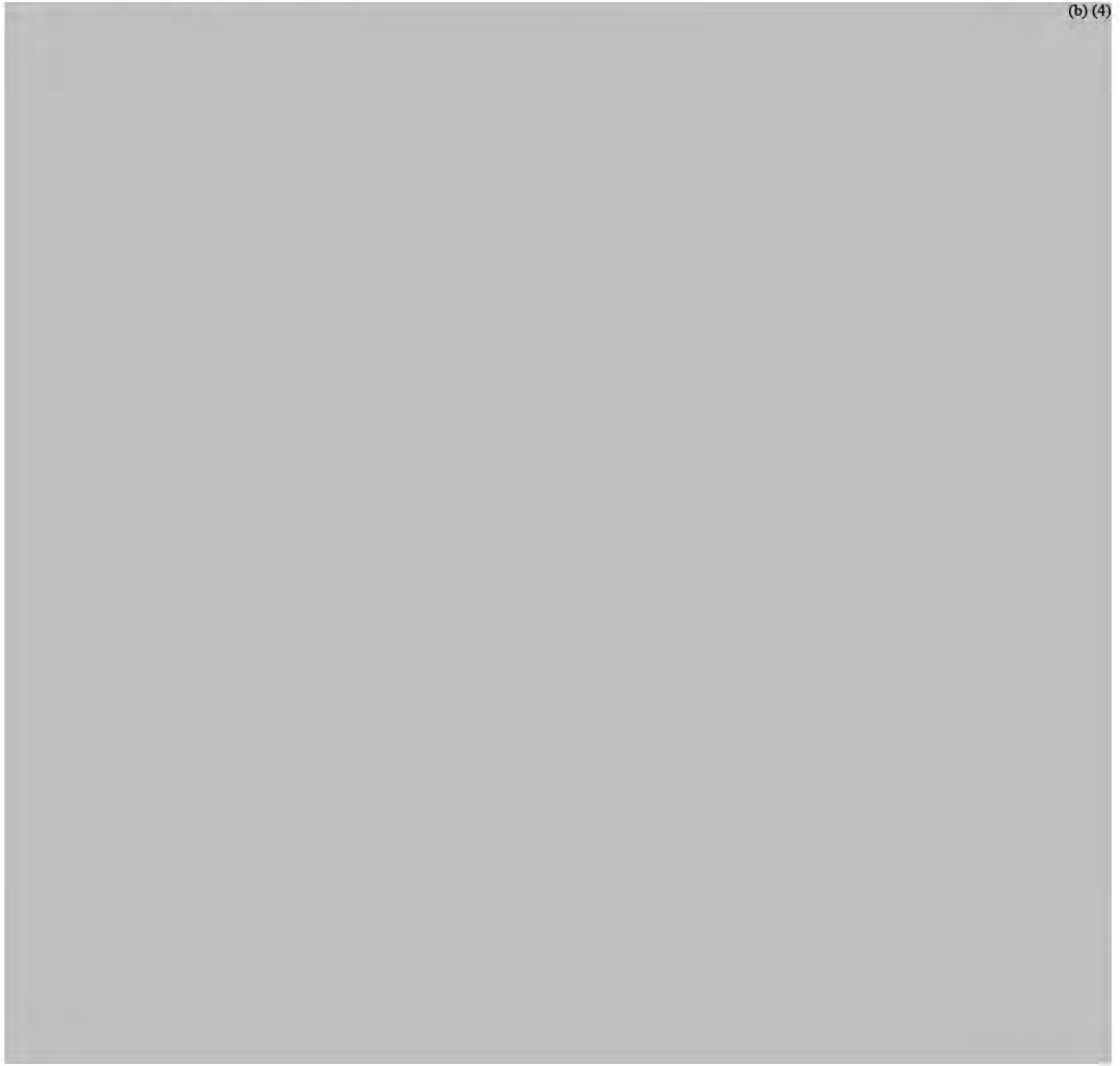

APPENDIX 4 - Manufacturing Process (CONFIDENTIAL)

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A. Manufacturing Process 125
B. Effect of Microbial Inactivation Procedures 128
C. List of Attachments 128

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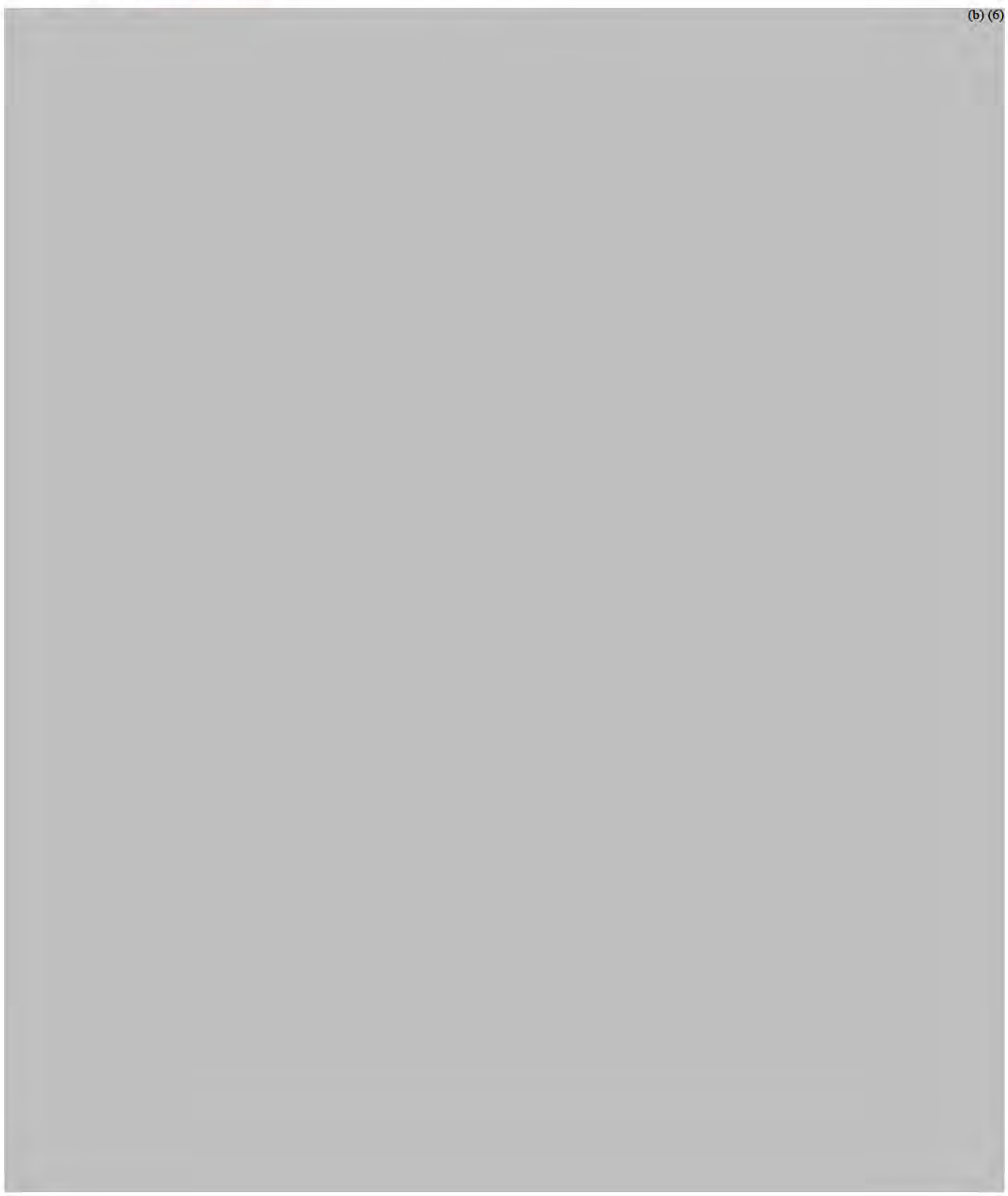
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APPENDIX 5 – Stability – 24months

STABILITY DATA

Stability Test Report

CJ CHEILJEDANG	Doc. No	Title	Page
	ST-2018-14	Stability test result record	130 / 3

Test Classification		Long Term Stability Test (24 month)		
Time Schedule		2016.04.14. ~ 2018.05.22		
SAMPLE	Sample name	Dried L-Valine Fermentation Product		
	Contents	Not less than 72% as L-valine		
	Batch(Lot)	GVAL160407	GVAL160408	GVAL160409
	Manufacturing date	2016.04.07	2016.04.08	2016.04.09
	Packaging	3 ply Kraft paper bag and 2 ply PE		
Test condition	Temp.	25±2℃		
	Humidity	60%RH±5%RH		
	Testing Frequency	3, 6, 9, 12, 18, 24 month		
Chamber Number		IB04		
Test items		Appearance, Assay, Loss on drying		
Test method		Appearance : Visual test		
		Loss on drying : AOAC 934.01		
		Valine content : HPLC (Refer to Validation report (Appendix 1- Attachment 2)		
Conclusion		No significant changes were observed during the twenty-four month test period. The study data supports product stability for twenty-four months.		

Written by	Reviewed by	Analytical Dep. Manager
정 다이	[signature]	[signature]
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Stability Test Report

CJ CHEILJEDANG	Doc. No	Title	Page
	ST-2018-14	Stability test result record	131 / 3

Batch No. GVAL160407

Test Items	Method	Specification	Initial	3 month	6 month	9 month	12 month	18 month	24 month
			2016.04.14	2016.07.17	2016.10.20	2017.01.25	2017.05.02	2017.11.15	2018.05.22
Appearance	Visual Test	Dark brown, free-flowing granules	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Loss on drying	AOAC 934.01	Not more than 5.0%	0.49%	(b) (4)					
L-valine	HPLC	Not less than 72.0%	72.12%						

Batch No. GVAL160408

Test Items	Method	Specification	Initial	3 month	6 month	9 month	12 month	18 month	24 month
			2016.04.14	2016.07.17	2016.10.20	2017.01.25	2017.05.02	2017.11.15	2018.05.22
Appearance	Visual Test	Dark brown, free-flowing granules	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Loss on drying	AOAC 934.01	Not more than 5.0%	0.67%	(b) (4)					
L-valine	HPLC	Not less than 72.0%	73.01%						

Batch No. GVAL160409

Test Items	Method	Specification	Initial	3 month	6 month	9 month	12 month	18 month	24 month
			2016.04.14	2016.07.17	2016.10.20	2017.01.25	2017.05.02	2017.11.15	2018.05.22
Appearance	Visual Test	Dark brown, free-flowing granules	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Loss on drying	AOAC 934.01	Not more than 5.0%	0.60%	(b) (4)					
L-valine	HPLC	Not less than 72.0%	72.75%						

APPENDIX 6 – Mash Feed Stability of Dried L-Valine Fermentation Product

Broiler Feed-Dried L-Valine Fermentation Product in Mash Feed Stability Report

Stability Test Report

	Doc. No.	Title	Page
	[illegible]	Stability Test Result Record	[illegible]

Dried L-Valine Fermentation Product

(b) (4), (b) (6)

1. Test Performance

1.1. Production of Broiler Feed-mixtures using three separate Dried L-Valine Fermentation Product Batches

The broiler feed mixtures containing Dried L-Valine Fermentation Product were prepared in a laboratory scale mixer with a three minute mixing time. The composition of the broiler feed mixture included 4970 g of broiler feed and 30 g of Dried L-Valine Fermentation Product. Each batch of Dried L-Valine Fermentation Product was mixed into the respective batch of broiler feed with an addition rate of 0.4 %. Four 100 gram samples were taken from each mixture. One of broiler feed test samples was sent directly to laboratory for analysis of L-valine content. The remaining test samples were stored in a climatic chamber at 25 °C and 60 % RH. Every four weeks, samples were taken out of the climatic chamber and sent to the laboratory for analysis. The broiler feed ingredient is listed in Table 1 according to supplier information. Table 2 shows the sample encoding of the stability samples. And the results of analysis are in Table 3.

Table l. Broiler Feed Ingredients (broiler grower: High tongtong 1[1])

Composition	
Maize	
Soybean meal	
Wheat	
Tallow	
Analytical components	**Percentage %**
Crude protein[2]	Not less than 20%
Crude fat[2]	Not less than 3.5%
Crude fiber[2]	Not more than 6.0%
Crude ash[2]	Not more than 9.0%
Calcium[2]	Not less than0.7%
Phophorous[2]	Not more than 1.2%
Metabolic Energy[2]	3.05 MKcal/kg
Methionine + Cystine+ Methionine hydroxy analogue (MHA) [2]	Not more than 0.84 %

[1] High tongtong 1: broiler grower, supplies from CJ Feed & Livestock

[2] The information of formulation and ingredients of the broiler feed is from supplier and other information was not identified.

Table 2. Broiler Feed-Amino Acid Mixture Stability Sample Identification

Batch No.	V-1	V-2	V-3
Stability samples	V-1-S-0	V-2-S-0	V-3-S-0
	V-1-S-1	V-2-S-1	V-3-S-1
	V-1-S-2	V-2-S-2	V-3-S-2
	V-1-S-3	V-2-S-3	V-3-S-3

Table 3. Stability Sample Valine Results

			Time in months			
Nominal value 0.40 %		Blank	Initial	1	2	3
Sample No.	Unit		S-0	S-1	S-2	S-3
Analysis method		EC 152/2009[1]				
V-1	%	N.D	0.53	(b) (4)		
V-2	%	N.D	0.46			
V-3	%	N.D	0.49			

[1] COMMISSION REGULATION (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Refer to Appendix 6, Attachment 1)

2. Conclusion

No significant changes were observed for the stability indicator test parameters. Therefore, the stability data supports a three month storage period for broiler feed-amino acid mash feed mixture.

FINAL REPORT

Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)

Study Number: 18-RA-0534

Sponsor: CJ BLOSSOM PAPK, BIO Research

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GLP Compliance Statement

Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)

This study was conducted in compliance with OECD Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17.

The study was performed following the approved protocol and SOPs in (b) (4) (b) (4), and the objective defined in the protocol was achieved. There were no events disturbing reliability of the study.

(b) (4) ____________ Sep 11, 2018

Date

Study director

Address: (b) (4)

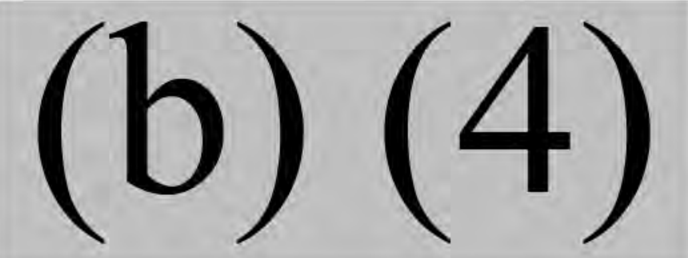
Contact:

E-mail:

(b) (4)

Signature

Sep 11, 2018
Date



Sep 11, 2018
Date

Hwasoo Kim, Sr.Manager
Sponsor's representative
CJ BLOSSOM PAPK, BIO Research

Sep. 10. 2018
Date

Quality Assurance Statement

Study number: 18-RA-0534

Title: Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)

Study period: Jul 05, 2018 – Sep 11, 2018

Sponsor: CJ BLOSSOM PARK, BIO Research

Items	Inspected on	Inspection results confirmed by Study Director on	Inspection results reported to Management on
Protocol	Jul 05, 2018	Jul 05, 2018	Jul 05, 2018
Animal receipt/Quarantine	Jul 11, 2018	Jul 11, 2018	Jul 12, 2018
Storage of test/reference article	Jul 17, 2018	Jul 17, 2018	Jul 18, 2018
	Jul 18, 2018	Jul 18, 2018	Jul 19, 2018
	Jul 19, 2018	Jul 19, 2018	Jul 20, 2018
Preparation of test/reference article	Jul 17, 2018	Jul 17, 2018	Jul 18, 2018
	Jul 18, 2018	Jul 18, 2018	Jul 19, 2018
	Jul 19, 2018	Jul 19, 2018	Jul 20, 2018
Administration and animal care	Jul 17, 2018	Jul 17, 2018	Jul 18, 2018
	Jul 18, 2018	Jul 18, 2018	Jul 19, 2018
	Jul 19, 2018	Jul 19, 2018	Jul 20, 2018
Observation and examination	Jul 17, 2018	Jul 17, 2018	Jul 18, 2018
	Jul 18, 2018	Jul 18, 2018	Jul 19, 2018
	Jul 19, 2018	Jul 19, 2018	Jul 20, 2018
	Jul 24, 2018	Jul 24, 2018	Jul 25, 2018
	Jul 25, 2018	Jul 25, 2018	Jul 26, 2018
	Jul 26, 2018	Jul 26, 2018	Jul 27, 2018
Necropsy	Jul 31, 2018	Jul 31, 2018	Jul 31, 2018
	Aug 01, 2018	Aug 01, 2018	Aug 01, 2018
	Aug 02, 2018	Aug 02, 2018	Aug 03, 2018
Raw data	Aug 14, 2018	Aug 21, 2018	Aug 22, 2018
Final report (draft)	Aug 14, 2018	Aug 21, 2018	Aug 22, 2018
Final report	Sep 11, 2018	Sep 11, 2018	Sep 11, 2018

Hereby, I do certify that the detailed methods in this final report was performed in accordance with the OECD Guideline for the Testing of Chemicals, TG 420 (17 Dec 2001) 'Acute Oral Toxicity – Fixed Dose Procedure' and the raw data obtained in this study were reflected accurately in this final report and this study was performed in accordance with the OECD Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17.

Date: Sep 11, 2018

(b) (4)

Quality Assurance Person (b) (4)

Quality Assurance Manager

Study Overview

Title	Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)
Objectives	The present study was performed to assess the toxicity of Granule valine in Sprague-Dawley rats following a single oral administration using an acute toxic method (Fixed Dose Procedure).
Regulatory guideline	OECD Guideline for Testing of Chemicals, TG 420 (17 Dec 2001) 'Acute Oral Toxicity – Fixed Dose Procedure'
Sponsor	CJ BLOSSOM PAPK, BIO Research 42nd street 55, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, Korea CJ Blossom Park, 16495 +82-31-8099-1902 (TEL), +82-31-8099-2914 (FAX) Sponsor's Representative: Hwasoo Kim
Test facility	(b) (4)

Study Schedule		
	Jul 05, 2018	Approval of protocol (study initiation)
	Jul 11, 2018	Animal acquisition (experimental initiation)
	Jul 17, 2018	Initiation of dosing
	Jul 19, 2018	Termination of dosing
	Jul 31, 2018	Initiation of necropsy
	Aug 02, 2018	Termination of necropsy (experimental completion)
	Aug 21, 2018	Submission of draft report
	Sep 11, 2018	Submission of final report (study completion)

(b) (4)

Responsible Staff Preparation/Storation of the test article: (b) (4)

Animal care:

Necropsy:

Archives:

Archives The protocol, protocol amendment, final report, raw data, sample of the test article and other relevant evidential documents will be retained as long as the quality of the preparation permits evaluation and stored in the Archives of (b) (4)

Further storage of above materials shall be consulted with the sponsor.

(b) (4)

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(b) (4)

Summary

This study was performed to assess the toxicity of Granule valine in Sprague-Dawley Rats following a single oral administration using the fixed dose procedure.

The starting dose was set at 300 mg/kg, and sighting study was conducted in which 1 female rat per group was sequentially administered at a dose level of 300 and 2000 mg/kg according to the fixed dose procedure. After a sighting study, a main study was conducted in which 4 female rats were administered at a dose level of 2000 mg/kg.

Mortalities, clinical signs, and body weight changes were monitored for 15 days, and then all animals were sacrificed and necropsy findings were observed. The summary of the results was as follows.

1. There were no mortalities.
2. There were no test article-related clinical signs.
3. There were no test article-related body weights changes.
4. No macroscopic abnormalities were observed at necropsy.

Based on the above results, when Granule valine was dosed to Sprague-Dawley rat by acute oral fixed dose procedure, GHS category of the test article was classification ‘5/unclassified’.

Materials and Methods

1. Test article and vehicle

1) Test article (Appendix 3)

Name:	Granule valine
Code No.:	C-2991
Lot No.:	GVAL180403
Date of receipt:	May 03, 2018
Amount:	13 g/pack × 1 pack
Appearance:	Brown Granule
Contents:	L-valine 71.87 %
Expiration date:	Apr 02, 2021
Storage conditions:	Room temperature, protect from light, protect from moisture
Supplier:	CJ BLOSSOM PAPK, BIO Research

2) Vehicle

Name:	Sterile distilled water for injection
Lot No.:	07S3B21
Storage condition:	Room temperature (refrigeration after preparation)
Supplier:	(b) (4)
Justification of selection:	The test article is suspended well in the vehicle and the vehicle is non-toxic to laboratory animals under the present experimental conditions.

2. Preparation and analysis of does formulation

1) Preparation of dose formulation

The test article was used without compensation for contents. The dose formulations for each dose were weighed after fracturing with a mortar, and suspension in the vehicle by vortex mixing.
The dose formulations were prepared on the day of administration.

2) Analysis of dose formulation

Analysis of dose formulation was not performed.

3. Test system and husbandry

1) Test system

(1) Animal information

Species and strain		Specific pathogen free (SPF) rats, Sprague-Dawley [Crl: CD(SD)]
Breeder / Supplier		(b) (4)
Justification of selection		The rats was used in this study are suitable for the toxicity test. The supplying system is well established, and the responses to drugs are stable. The basic data for toxicity test have been accumulated in abundance, therefore data can be used for the evaluation of the result. Only female rats were used according to the OECD guideline (TG 420) in this study.
Sex		Female
Number of animals	At receipt	13
	At dosing	6
Age of animals	At receipt	7 weeks
	At dosing	8 weeks
Body weight ranges	At receipt	160.87-172.77 g
	At dosing	176.02-187.24 g
Disposition of remnant animals		The remnant animals were euthanized.

(2) Quarantine and acclimation

The animals were examined for the external appearance and individually weighed at receipt, and acclimated under the laboratory conditions for 6-8 days. General clinical observations were made once a day and only healthy animals are used for study. According to the microbiological monitoring certificate provided by the supplier, there were no factors could affect the present study.

(3) Identification

Animals were identified by tail marking using a red oil-based marker during acclimation period and using a black oil-based marker during administration and observation periods.

Cages were identified by color-coded ID cards, and cage racks were given unique serial numbers. A log sheet was attached at the entrance of the animal room to identify the study.

(4) Animal experimentation ethics

(b) (4) received approval from AAALAC International for full accreditation in 2010. The present study was approved by Institutional Animal Care and Use Committee (IACUC) of (b) (4) (b) (4)

(b) (4)

2) Animal husbandry

(1) Environmental conditions and monitoring

This study was performed within Room No. 7 in the barriered animal facility area No. 2 of (b) (4). Environmental controls were set to maintain following conditions: temperature range of 23 ± 3 °C, relative humidity range of 55 ± 15 %, ventilation of 10–20 air changes/hr, 150-300 Lux of luminous intensity and a 12-hr light/12-hr dark cycle.

Throughout the study period, the temperature and humidity of animal room were measured every hour with a computer-based automatic sensor, and the environmental conditions such as ventilation frequency and luminous intensity were monitored on a regular basis. During the study period the room was maintained at a mean daily temperature of 22.7-23.4 °C, and a mean daily relative humidity of 52.3-61.2 %, and there were no deviations that could affect this study.

(2) Diet, water and contaminants

Animals were offered irradiation-sterilized pellet diet for lab animal (b) (4) (b) (4) purchased from (b) (4) (b) (4) *ad libitum*. According to the certificates on diet component and contaminant supplied by diet provider, there was no factor that could affect results of this study.

Tap water disinfected by ultraviolet sterilizer and ultrafiltration were given via polycarbonate water bottle, *ad libitum*. Examination of water was performed by an authorized (b) (4) (b) (4) (b) (4), and the quality satisfied the standards for the drinking water.

(3) Cages and housing density

No more than 3 animals were housed in a stainless steel cage (W 215 × L 355 × H 200 mm) during acclimation period and no more than 2 animals were housed during the dosing and observation period.

(4) Husbandry

Water was checked daily and water bottle was changed at least once a week. Other management was conducted corresponding to SOPs of Chemon Inc.

(b) (4)

4. Group identification, selection of dose, grouping and administration

1) Group identification

<Sighting study>

Group	Sex	No. of animal	Animal ID	Dose volume (mL/kg)	Dose (mg/kg)
G1	F	1	1	10	300
G2	F	1	2	10	2000

<Main study>

Group	Sex	No. of animal	Animal ID	Dose volume (mL/kg)	Dose (mg/kg)
G3	F	4	3-6	10	2000

2) Selection of dose

According to OECD Guideline for Testing of Chemicals, TG 420, the starting dose was set at 300 mg/kg since there was no available information on the toxicity of the test article.

3) Process of the study

The administrations were done as shown in the Test Procedure attached in this report (Annex 1-2).

4) Grouping

After the acclimation period healthy animals were weighed and their body weights were ranked. Then the animals which weigh close to the mean body weight were selected and distributed randomly as shown in the 'group identification' table.

5) Administration

Route and justification	Oral administration, the anticipated clinical route was selected.
Frequency and duration	Once a day, single dose. Dose was given no later than 11:54.
Dose volume	Dose volume was calculated based on the fasted body weight measured on the day of administration.
Method	After an overnight fasting (about 19-20 hours), the dorsal skin of animal was held firmly and prepared test article was directly administered into stomach using a syringe tube with a feeding needle. Food was resupplied about 3-4 hours after administration.

(b) (4)

5. Observations and examinations

1) Clinical signs

All animals were observed at least once a day and type of sign and severity with date, if any, were recorded individually. After dosing, animals were continuously observed for 30 minutes and then observed hourly (4 hours in total). The day of the administration was designated as Day 1, and animals were observed until Day 15.

2) Body weight

Animals were weighed on Day 1 (before administration), 2, 4, 8 and 15 after administration.

3) Necropsy

On Day 15, all survivors were anesthetized by inhalation of CO_2 gas and terminated by exsanguination from the posterior vena cava and abdominal aorta, and all vital organs were observed macroscopically. Histopathological examination was not performed because there were no organs with macroscopic abnormalities.

6. Statistical analysis

No statistical analyses were done.

(b) (4)

Results

Mortalities (Table 1; Appendix 1-1)

There were no mortalities.

Clinical sign (Table 2; Appendix 1-1)

There were no test article-related clinical signs.

Body weight (Table 3; Appendix 1-2)

There were no test article-related changes.

Necropsy findings (Table 4; Appendix 1-3)

There were no macroscopic abnormalities.

(b) (4)

Discussion and conclusion

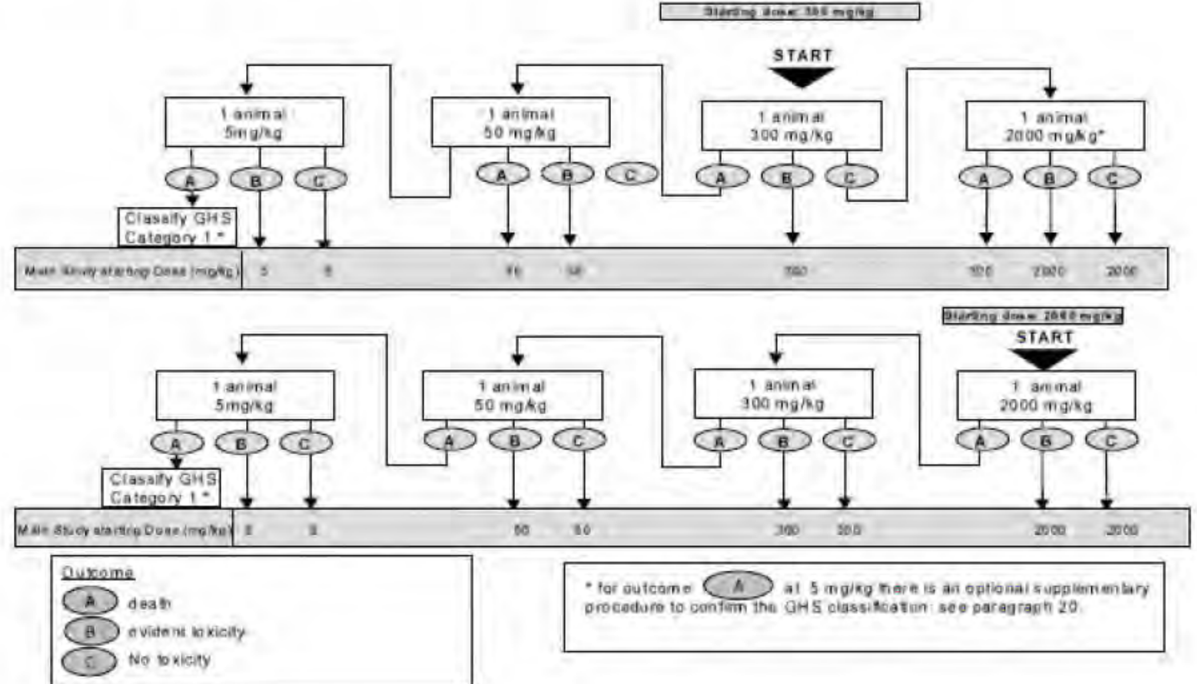
This study was performed to assess the toxicity of Granule valine Sprague-Dawley rats following a single oral administration using the fixed dose procedure.

There were no mortalities were observed, and no test article-related clinical signs, body weight changes and necropsy findings were observed.

Based on the above results, when Granule valine was dosed to Sprague-Dawley rat by acute oral fixed dose procedure, GHS category of the test article was classification '5/unclassified'.

(b) (4)

Annex 1. Flow chart for the sighting study



(b) (4)

Annex 2. Flow chart for the main study

Starting dose: 300 mg/kg

START

5 animals 5mg/kg — A B C

5 animals 50 mg/kg — A B C

5 animals 300 mg/kg — A B C

5 animals 2000 mg/kg* — A B C

Classify GHS Category 1 2 2 3 3 4 4 5 5/Unclassified

Starting dose: 2000 mg/kg

START

5 animals 5mg/kg — A B C

5 animals 50 mg/kg — A B C

5 animals 300 mg/kg — A B C

5 animals 2000 mg/kg — A B C

Classify GHS Category 1 2 2 3 3 4 4 5 5/Unclassified

Outcome
- A: ≥ 2 deaths
- B: ≥ 1 with evident toxicity and/or < 1 death
- C: No toxicity

Group size
The 5 animals in each main study group will include any animal tested at that dose level in the sighting study

Animal welfare override
If this dose level caused death in the sighting study, then no further animals will be tested. Go directly to outcome A

(b) (4)

Units and Abbreviation

Note: The following lists of codes, abbreviations and units are used by Chemon Inc. Some, but not necessarily all, of this information may be needed for this report.

%	Percent	**hr**	Hour
°	Degree	**min**	Minute
C	Celsius	**sec**	Second
L	Liter	**rpm**	Revolution per Minute
dL	Deciliter	**RCF**	Relative Centrifugal Force
mL	Milliliter	**SD**	Standard Deviation
μL	Microliter	**CV**	Coefficient of Variation
g	Gram	**RE**	Relative Error
kg	Kilogram	**RH**	Relative Humidity
mg	Milligram	**M**	Male
μg	Microgram	**F**	Female
ng	Nanogram	**NA**	Not Applicable
m	Meter	**N**	Number
cm	Centimeter	**SPF**	Specific Pathogen Free
mm	Millimeter	**TK**	Toxicokinetic
μm	Micrometer	**PK**	Pharmacokinetic
ppm	Parts per million	**AUC**	Area Under the Curve
ppb	Parts per billion	**C_{max}**	Maximum Concentration
wk	Week	**T_{max}**	Time at Maximum Concentration
d	Day	**$t_{1/2}$**	Half-life
GLP	Good Laboratory Practice Regulation	**SOP**	Standard Operating Procedures
QAU	Quality Assurance Unit	**ICH**	International Council on Harmonization
OECD	Organization for Economic Co-operation and Development	**SPSS**	Statistical Package for the Social Sciences
IACUC	Institutional Animal Care and Use Committee	**LC-MS/MS**	Liquid Chromatography-Tandem Mass Spectrometry
HPLC	High-Performance Liquid Chromatography	**DKBT**	Diplomated Korean Board of Toxicology

(b) (4)

TABLES

Table 1. Mortalities

MORTALITIES										FEMALE
GROUPS (mg/kg)	No. DEAD/ No. DOSED	DAYS AFTER DOSE								
		1	2	3	4	5	6	7	8	9-15
G1 (300)	0 / 1	0	0	0	0	0	0	0	0	0
G2 (2000)	0 / 1	0	0	0	0	0	0	0	0	0
G3 (2000)	0 / 4	0	0	0	0	0	0	0	0	0

(b) (4)

Table 2. Clinical signs

CLINICAL SIGNS				FEMALE
DAYS	SIGNS	GROUPS (mg/kg)		
		G1 (300)	G2 (2000)	G3 (2000)
1-14	Normal	1 / 1	1 / 1	4 / 4
15	Normal	1 / 1	1 / 1	4 / 4
	Terminal sacrifice	1 / 1	1 / 1	4 / 4

The day of administration was designated Day 1.
Number of animals with the sign / Number of animals examined.

Table 3. Body weights

DAYS	BODY WEIGHTS (g) GROUPS (mg/kg)		FEMALE
	G1 (300)	G2 (2000)	G3 (2000)
1	176.02	181.45	184.18±2.70
2	192.18	200.42	207.22±2.52
4	201.49	210.52	214.19±2.99
8	217.67	222.11	223.27±3.75
15	238.23	231.95	238.13±6.78
GAIN	62.21	50.50	53.95±7.93
N	1	1	4

The day of administration was designated Day 1.
Data are expressed as mean ± S.D.
Gain is body weight on Day 15 - body weight on Day 1.

(b) (4)

Table 4. Necropsy findings

NECROPSY FINDINGS				FEMALE
ORGANS	FINDINGS	GROUPS (mg/kg)		
		G1 (300)	G2 (2000)	G3 (2000)
No gross findings		1	1	4
N		1	1	4

(b) (4)

APPENDIX 1. INDIVIDUAL DATA

(b) (4)

Appendix 1-1. Clinical signs

		CLINICAL SIGNS	FEMALE
GROUPS (mg/kg)	ANIMAL ID	SIGNS	OBSERVED ON
G1 (300)	1	Normal Terminal sacrifice	DAY 1 (0.5-4 hrs), 2-15 DAY 15
G2 (2000)	2	Normal Terminal sacrifice	DAY 1 (0.5-4 hrs), 2-15 DAY 15
G3 (2000)	3	Normal Terminal sacrifice	DAY 1 (0.5-4 hrs), 2-15 DAY 15
	4	Normal Terminal sacrifice	DAY 1 (0.5-4 hrs), 2-15 DAY 15
	5	Normal Terminal sacrifice	DAY 1 (0.5-4 hrs), 2-15 DAY 15
	6	Normal Terminal sacrifice	DAY 1 (0.5-4 hrs), 2-15 DAY 15

The day of administration was designated Day 1.

(b) (4)

Appendix 1-2. Body weights

BODY WEIGHTS (g)							FEMALE
GROUPS (mg/kg)	ANIMAL ID	Day 1	Day 2	Day 4	Day 8	Day 15	GAIN
G1 (300)	1	176.02	192.18	201.49	217.67	238.23	62.21
G2 (2000)	2	181.45	200.42	210.52	222.11	231.95	50.50
G3 (2000)	3	187.24	205.07	211.87	225.72	242.51	55.27
	4	185.59	210.85	216.39	223.91	228.15	42.56
	5	182.39	206.19	217.13	217.79	239.58	57.19
	6	181.49	206.78	211.37	225.65	242.27	60.78

The day of administration was designated Day 1.
Gain is body weight on Day 15 - body weight on Day 1.

(b) (4)

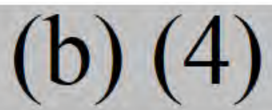
Appendix 1-3. Necropsy findings

NECROPSY FINDINGS			FEMALE
ANIMAL ID	FATE	LOCATION	FINDINGS
G1 (300 mg/kg)			
1	Terminal sacrifice		No gross findings
G2 (2000 mg/kg)			
2	Terminal sacrifice		No gross findings
G3 (2000 mg/kg)			
3	Terminal sacrifice		No gross findings
4	Terminal sacrifice		No gross findings
5	Terminal sacrifice		No gross findings
6	Terminal sacrifice		No gross findings

(b) (4)

APPENDIX 2. PROTOCOL AND PROTOCOL AMENDMENT

(b) (4)



(b) (4)

PROTOCOL

Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)

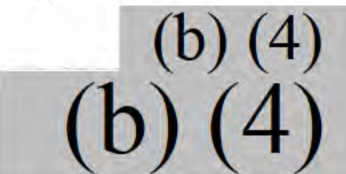
Study number: 18-RA-0534

Approval

(b) (4)

(b) (4)

Jul 05, 2018
Date



(b) (4)

Jul 05, 2018
Date

Hwasoo Kim, Sr.Manager
Sponsor's representative
CJ BLOSSOM PAPK, BIO Research

Jul 12 2018
Date

(b) (4)

(b) (4)

(b) (4)

Title Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)

Objectives The present study will be performed to assess the toxicity of Granule valine in Sprague-Dawley rats following a single oral administration using an acute toxic method (Fixed Dose Procedure).

Regulatory guideline OECD Guideline for Testing of Chemicals, TG 420 (17-Dec 2001) 'Acute Oral Toxicity – Fixed Dose Procedure'

Sponsor CJ BLOSSOM PARK, BIO Research

55, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, Republic of Korea CJ Blossom Park, 16495

+82-31-8099-1902 (TEL), +82-31-8099-2914 (FAX)

Test facility (b) (4)

Schedules

Jul 11, 2018	Animal acquisition (experimental initiation)
Jul 17, 2018	Initiation of administration
Jul 31, 2018	Initiation of necropsy
Aug 06, 2018	Completion of necropsy due date (experimental completion)
Aug 24, 2018	Submission of draft report (due date)

Any changes in the proposed schedule will be described in the final report without amendments.

2

(b) (4)

(b) (4)

Responsible staff Preparation /Storage of the test article: (b) (4)

Animal care:

Necropsy:

Archives:

Archives [SOP-AC-001~007]

The protocol (amendment and deviation, if any), final report, raw data, sample of the test article, specimens and other relevant evidential documents will be retained as long as the quality of the preparation permits evaluation and stored in the Archives of (b) (4)

Further storage of above materials shall be consulted with the sponsor.

GLP compliance OECD Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17

The amendment and deviation of the protocol are documented and are approved by the study director, management and sponsor after the review of Quality Assurance Unit (QAU).

The QAU of (b) (4) inspects solely throughout the progression of study.

Final report [SOP-TO-007]

The final report will be consisted of the cover page suggesting the content of the protocol, statement of study director, quality assurance statement, contents, summary, material and method, results of the observation and measurement, discussion and conclusion and reference, and pictures, tables, appendices, annexes etc. will be included if necessary.

(b) (4)

(b) (4)

1. Test article and vehicle

1) Test article [SOP-TA-001]

Name:	Granule valine
Code No.:	C-2991
Lot No.:	(b) (4)
Date of receipt:	May 03, 2018
Amount:	13 g/pack × 1 pack
Appearance:	Brown Granule
Contents:	L-valine 71.87 %
Expiration date:	Apr 02, 2021
Storage conditions:	Room temperature, protect from light, protect from moisture
Supplier:	CJ BLOSSOM PAPK, BIO Research

2) Vehicle

Name:	Sterile distilled water for injection
Lot No.:	(b) (4)
Storage condition:	Room temperature (refrigeration after preparation)
Supplier:	(b) (4)
Justification of selection:	The test article is suspended well in the vehicle and the vehicle is non-toxic to laboratory animals under the present experimental conditions.

2. Preparation and analysis of dose formulation

1) Preparation of dose formulation [SOP-TA-002]

The test article will be used without the compensation for contents. The dose formulations for each dose will be weighed after fracturing with a mortar, and suspension in the vehicle by vortex mixing.

The dose formulations will be prepared on the day of administration.

2) Analysis of dose formulation [SOP-AS-011]

Analysis of dose formulation will be not performed.

(b) (4)

(b) (4)

3. Test system and housing environment

1) Test system

(1) Animal information (b) (4)

Species and strain		Specific pathogen free (SPF) rats, Sprague-Dawley [Crl: CD(SD)]
Breeder / Supplier		(b) (4)
Justification of selection		The rats will be used in this study are suitable for the toxicity test. The supplying system is well established, and the responses to drugs are stable. The basic data for toxicity test have been accumulated in abundance, therefore data can be used for the evaluation of the result. Only female rats will be used according to the OECD guideline (TG 420) in this study.
Sex		Female
Number of animals	At receipt	13
	At dose	11*
Age of animals	At receipt	7 weeks
	At dose	Over 8-9* weeks
Body weight range at dose		Within mean body weight(g) ± 20 %
Disposition of remnant animals		The remnant animals will be treated according on SOPs of (b) (4)

*The actual number and age of animals at dose will be presented in the final report.

(2) Quarantine and acclimation (b) (4)

The animals will be examined for the external appearance and individually weighed at receipt, and acclimated under the laboratory conditions at least over 6 days. General clinical observations will be made once a day and only healthy animals are used for study.

(3) Identification (b) (4)

Animals will be individually by tail marking using a red oil-based marker during acclimation period and using a black oil-based marker during treatment and observation periods.

Cages will be identified by color-coded ID cards, and cage racks will be given unique serial numbers. A log sheet will be attached on the entrance of the animal room to identify the study.

(4) Animal experimentation ethics [SOP-VC-001]

(b) (4) received approval from AAALAC International for full accreditation in 2010. The present study was approved by Institutional Animal Care and Use Committee (IACUC) of (b) (4) (b) (4)

(b) (4)

(b) (4)

2) Animal husbandry

(1) Environmental conditions and monitoring (b) (4)

This study will be performed within Room No. 7 in the barrier animal facility area No. 2 of Research bldg. #3, (b) (4) Environmental controls will be set to maintain following conditions: temperature range of 23 ± 3 °C, relative humidity range of 55 ± 15 %, ventilation of 10–20 air changes/hr, 150-300 Lux of luminous intensity and a 12-hr light/12-hr dark cycle.

Throughout the study period, the temperature and humidity of animal room will be measured every hour with a computer-based automatic sensor, and the environmental conditions such as ventilation frequency and luminous intensity will be monitored on a regular basis.

(2) Diet, water and contaminants (b) (4)

Animals will be offered irradiation-sterilized pellet diet for lab animal [Teklad Certified Irradiated Global 18 % Protein Rodent Diet (b) (4) *ad libitum*.

Groundwater disinfected by ultraviolet sterilizer and ultrafiltration will be given via water bottle, *ad libitum*.

Examination about contaminant will be performed according to SOPs o (b) (4)

(3) Cages and housing [SOP-BE-008]

No more than 3 animals will be housed in a stainless steel cage (W 215 × L 355 × H 200 mm) during acclimation period and no more than 2 animals will be housed during the administration and observation period.

(4) Husbandry (b) (4)

Water will be checked daily and water bottles will be changed at least once a week. Other management will be conducted corresponding to SOPs of (b) (4).

(5) Grouping (b) (4)

In the acclimation period, healthy animals will be weighed and their body weights will be ranked. Then the animals which weigh close to the mean body weight will be selected and distributed randomly as shown in the 'group identification' table.

4. Group identification, selection of dose and administration

1) Group identification

<Sighting study>

Group	Sex	No. of animal	Animal ID	Dose volume (mL/kg)	Dose (mg/kg)
G1	F	1	1	10	300
#	F	#	#	10	#
#	F	#	#	10	#

6

(b) (4)

(b) (4)

<Main study>

Group	Sex	No. of animal	Animal ID	Dose volume (mL/kg)	Dose (mg/kg)
#	F	4	#	TBD	#
#	F	4	#	TBD	#

#The actual, group, No. of animal, animal ID, dose will be written in the final report.

2) Selection of dose [SOP-GT-006]

According to OECD Guideline for Testing of Chemicals, TG 420, the starting dose was set at 300 mg/kg since there was no available information on the toxicity of the test article.

3) Procedures of the study [SOP-GT-008]

The administrations will be as shown in the Test Procedure attached in this protocol (Annex1-2).

4) Administration [SOP-AT-001]

Route and justification	Oral administration, the anticipated clinical route is selected.
Frequency and duration	Once a day, single dose. Dose will be given no later than 14:00
Dose volume	Dose volume will be calculated based on the fasting body weight measured on the day of administration.
Method	After fasting overnight (About 16-20 hours), the dorsal skin of animals will be fixed and test article will be directly administered into stomach using a syringe tube with a feeding needle. Food will be offered about 3-4 hours after administration.

5. Observations and examinations

1) Clinical signs and mortality (b) (4)

All animals will be observed for clinical signs at least once a day. On the day of administration, the animals will be observed continuously during the first 30 minutes and then hourly until 4 hours after the administration. If adverse symptoms worsen, the animal will be isolated for preventing cannibalism. Moribund animals, or animals obviously in pain or showing of severe and enduring distress will be weighed, humanely killed and necropsied, and will be considered in the interpretation of the test results in the same way as animals that died on test. The day of administration will be designated as Day 1, and clinical signs will be observed until Day 15.

2) Body weight (b) (4)

Animals are weighed on Day 1(Before administration), 2, 4, 8 and 15 after administration.

(b) (4)

(b) (4)

3) **Necropsy** (b) (4)

On Day 15, all animals are euthanized by CO_2 gas, and then terminated by exsanguinations from the posterior vena cava and abdominal aorta. All organs of the rats found dead during the experimental period are also macroscopically observed. Organs showing evidence of gross pathology (The death animals on administration day will not be preserved) are preserved in adequate solution according to the SOPs of (b) (4) for the microscopic examination. If necessary, a histopathological examination will be conducted in consultation with the sponsor.

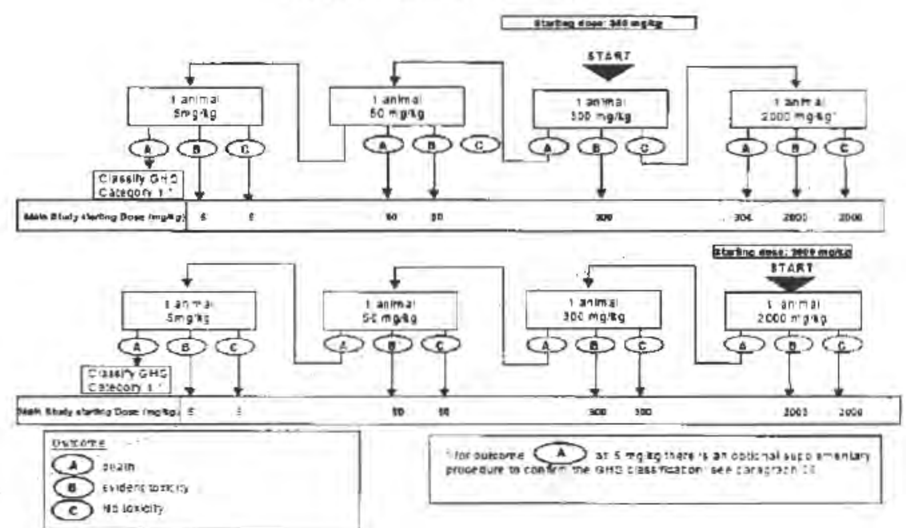
6. Statistical analyses (b) (4)

No statistical analyses will be done.

(b) (4)

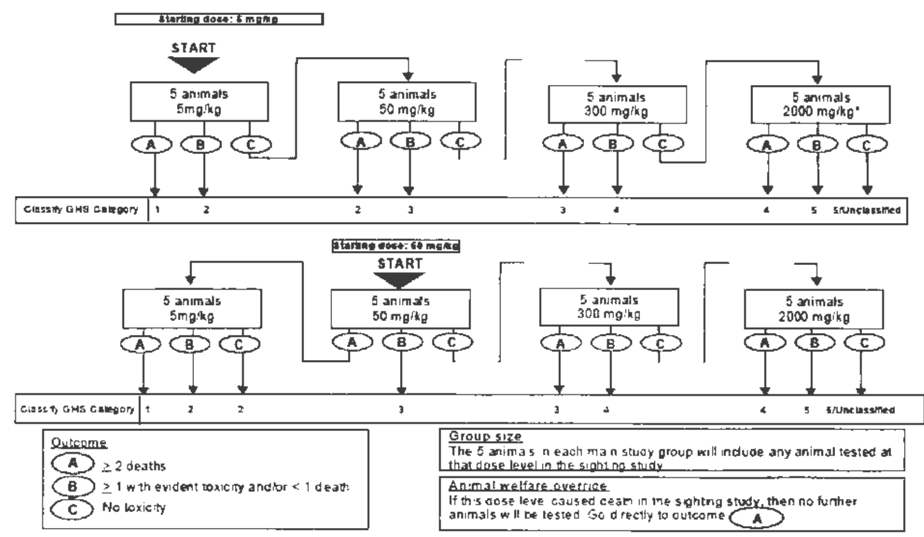
(b) (4)

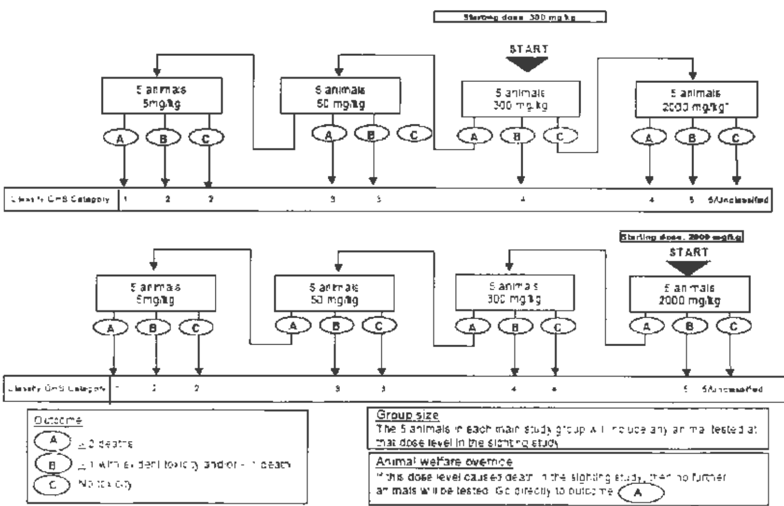
Annex 1. Flow chart for the sighting study



(b) (4)

Annex 2. Flow chart for the main study





(b) (4)

(b) (4)

Units and Abbreviation

Note: The following lists of codes, abbreviations and units are used by (b) (4)
Some, but not necessarily all, of this information may be needed for this protocol.

%	Percent	**hr**	Hour
°	Degree	**min**	Minute
C	Celsius	**sec**	Second
L	Liter	**rpm**	Revolution per Minute
dL	Deciliter	**RCF**	Relative Centrifugal Force
mL	Milliliter	**SD**	Standard Deviation
μL	Microliter	**CV**	Coefficient of Variation
g	Gram	**RE**	Relative Error
kg	Kilogram	**RH**	Relative Humidity
mg	Milligram	**M**	Male
μg	Microgram	**F**	Female
ng	Nanogram	**NA**	Not Applicable
m	Meter	**N**	Number
cm	Centimeter	**SPF**	Specific Pathogen Free
mm	Millimeter	**TK**	Toxicokinetic
μm	Micrometer	**PK**	Pharmacokinetic
ppm	Parts per million	**AUC**	Area Under the Curve
ppb	Parts per billion	**C_{max}**	Maximum Concentration
wk	Week	**T_{max}**	Time at Maximum Concentration
d	Day	**$t_{1/2}$**	Half-life
GLP	Good Laboratory Practice Regulation	**SOP**	Standard Operating Procedures
QAU	Quality Assurance Unit	**ICH**	International Council on Harmonization
OECD	Organization for Economic Co-operation and Development	**SPSS**	Statistical Package for the Social Sciences
IACUC	Institutional Animal Care and Use Committee	**LC-MS/MS**	Liquid Chromatography-Tandem Mass Spectrometry
HPLC	High-Performance Liquid Chromatography	**DKBT**	Diplomated Korean Board of Toxicology

ii

(b) (4)

Protocol Amendment Form
(시험계획서 변경/개정 기록지)

Study Title: Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)

Study No.: 18-RA-0534 Amendment No.: 1

Amendment to the Protocol:
(변경/개정 내용)

1. Page 6, 3, 2), (2) Diet, water and contaminants

Before amendment	Groundwater disinfected by ultraviolet sterilizer and ultrafiltration will be given via water bottle, *ad libitum*.
After amendment	Tap water disinfected by ultraviolet sterilizer and ultrafiltration will be given via water bottle, *ad libitum*.

내용점검필 2018. 08. 0 9 (주)켐온QAU

Reason for the Amendment:
(변경/개정 사유)

1. Changes due to typos.

Impact on Study:
(시험에 미치는 영향)

1. None

Approved by:

Study Direct(b) (4) — Date: Aug 09, 2018

Management (b) (4) — Date: Aug 09, 2018

Hwanoo Cun — Sponsor's representative — Date: Aug 20. 2018

(b) (4) (b) (4) TO-006-S03

(b) (4)

Protocol Amendment Form
(시험계획서 변경/개정 기록지)

Study Title: Acute Oral Toxicity Study of Granule valine in Sprague-Dawley Rats (Fixed Dose Procedure)

Study No.: 18-RA-0534   Amendment No.: 2

Amendment to the Protocol:
(변경/개정 내용)

1. Page 2, Sponsor

Before	55, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, Republic of Korea CJ Blossom Park, 16495
After	42th street 55, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, Korea CJ Blossom Park, 16495

Reason for the Amendment:
(변경/개정 사유)

1. Change due to request from sponsor.

Impact on Study:
(시험에 미치는 영향)

1. None

내용점검필 2018. 09. 06 (주)켐온QAU

Approved by:

(b) (4) — Date: Sep 06. 2018

(b) (4) — Date: Sep 06, 2018

Hwasoo Kim — Sponsor's representative — Date: Sep 10. 2018

(b) (4)

Chemon Inc.   TO-006-S03

(b) (4)

APPENDIX 3. CERTIFICATE OF ANALYSIS

(b) (4)

CJ Research Institute of Biotechnology

55 Gwanggyo-ro 42beon-gil, Yeongtong-gu, Suwon-si, Gyeonggi-do, Korea CJ Blossom Park
TEL : 031) 8099-2450 FAX : 031) 8099-2918

CJ CHEILJEDANG

Certificate of analysis

Certificate No	2018-PR-093	Receipt No.	2018-AN-067
Client		Date of Receipt	2018-04-09
Client Name		Date of Test	2018-04-12
Client Tel		Use of Report	Reference test
Client Address			

Test Sample	Granule valine
Manuf. Date	2018.04.03
Expiry Date	2021 04.02
Lot. No	GVAL180403
Quantity (kg)	

Test Item(s)	Test Result	Test method
L-valine	71.87%	HPLC
Loss on drying	0.53%	AOAC 934.01
Residue on ignition	2.74%	AOAC 942.05

- Information
- Temperature : (22-28) ℃, Relative Humidity : (30-60) %
- N.D : not detected (not quantifiable)
- The results shown in this test report refer only to the sample tested unless otherwise stated
  The Test Report cannot be reproduced, except in full

Tested by (b) (4)
Approved by Technical Manager (b) (4)

April. 25, 2018

CJ Research Institute of Biotechnology, BIO) Analysis Team

CJ BIO AD form 100-01 REV.01

FINAL REPORT

Bacterial Reverse Mutation Assay with Granule Valine

Study Number: 18-VG-0736

Sponsor: CJ BLOSSOM PARK, BIO Research

Nonclinical Research Institute, Chemon Inc.

(b) (4)

(b) (4)

GLP Compliance Statement

Bacterial Reverse Mutation Assay with Granule Valine

This study was conducted in accordance with OECD principles of Good Laboratory Practice (b) (4)

The study was performed following schedules of the approved protocol and SOPs in (b) (4) (b) (4) and the study objectives defined in the protocol were achieved. There were no circumstances that may have affected the reliability of the data.

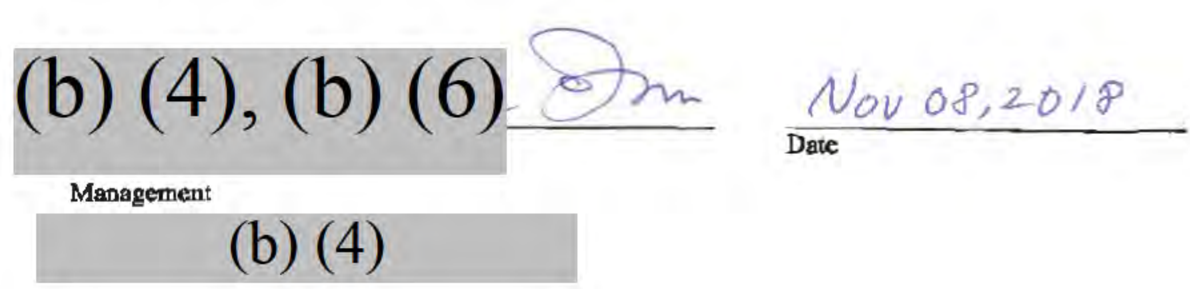
(b) (4), (b) (6) Nov 08, 2018
(b) (4), (b) (6) Date
Study director

Address: (b) (4)

Contact: (b) (4)
E-mail:

(b) (4)

Signature Page



CJ BLOSSOM PARK, BIO Research

Quality Assurance Statement

Study Number: 18-VG-0736

Title: Bacterial Reverse Mutation Assay with Granule Valine

Study period: Sep 19, 2018- Nov 08, 2018

Sponsor: CJ BLOSSOM PARK, BIO Research

Items	Inspected on	Inspection results confirmed by Study Director on	Inspection results reported to Management on
Protocol	Sep 17, 2018	Sep 19, 2018	Sep 20, 2018
Preparation of media and Inoculation of strains	Oct 01, 2018	Oct 01, 2018	Oct 02, 2018
Storage of test /reference article	Oct 02, 2018	Oct 02, 2018	Oct 04, 2018
Preparation of test /reference article	Oct 02, 2018	Oct 02, 2018	Oct 04, 2018
Status of bacterial strains	Oct 02, 2018	Oct 02, 2018	Oct 04, 2018
Identification	Oct 02, 2018	Oct 02, 2018	Oct 04, 2018
Chemical treatment	Oct 02, 2018	Oct 02, 2018	Oct 04, 2018
Scoring plates	Oct 04, 2018	Oct 04, 2018	Oct 05, 2018
Raw data	Oct 26, 2018	Oct 29, 2018	Oct 30, 2018
Final report (draft)	Oct 26, 2018	Oct 29, 2018	Oct 30, 2018
Final report	Nov 08, 2018	Nov 08, 2018	Nov 08, 2018

Hereby, I do certify that the detailed method in this final report was performed in accurately with OECD Guideline for Testing of Chemicals TG 471 (1997) 'Bacterial Reverse Mutation Test' and the raw data obtained in this study were reflected accurately in the final report and this study was performed in accordance with OECD Principles of Good Laboratory Practice (1997) (b) (4)

Date: Nov 08, 2018

(b) (4)

Quality Assurance Person (b) (4), (b) (6)

Quality Assurance Manager

(b) (4)

Study overview

Title

Bacterial Reverse Mutation Assay with Granule Valine

Objective

The objective of this study was to evaluate the test article, Granule Valine, for its ability to induce reverse mutation in the four histidine-requiring TA strains of *Salmonella typhimurium* and a tryptophan-requiring strain *Escherichia coli* WP2 *uvr*A.

Regulatory guideline

OECD Guideline for Testing of Chemicals TG 471 (1997) 'Bacterial Reverse Mutation Test'

Sponsor

CJ BLOSSOM PARK, BIO Research
42nd street 55, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, Korea,
CJ Blossom Park, 16495
+82-31-8099-1902 (TEL), +82-31-8099-2914 (FAX)
Sponsor's representative: (b) (4), (b) (6)

Test Facility

(b) (4)

Schedule

Sep 19, 2018: Approval of protocol (study initiation)
Oct 01, 2018: Inoculation of test strains (experiment initiation)
Oct 02, 2018: Chemical treatment
Oct 04, 2018: Scoring plates (experiment completion)
Oct 29, 2018: Submission of draft report
Nov 08, 2018: Submission of final report (study completion)

Contributing Scientists

Preparation/Storage of the Test article: (b) (4), (b) (6)
Archives:
Cell lines management:

(b) (4)

Archives The protocol, protocol amendment, final report, raw data, sample of test article and other relevant evidential documents will be stored in the Archives of (b) (4) until the reliability of the study results can be evaluated by the relevant regulatory authority (at least 5 years). Further storage of above materials shall be consulted with the sponsor.

(b) (4)

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(b) (4)

Summary

The test article, Granule Valine, was evaluated for its potential to induce reverse mutation in the four histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and a tryptophan auxotroph strain of *Escherichia coli* WP2 *uvr*A in the presence and absence of exogeneous metabolic activation system.

The metabolic activation system consisting of the cofactor-supplemented post-mitochondrial fraction (S9) of liver homogenate from rats pretreated with Aroclor 1254 was used. The test strains were exposed to the test article using the direct plate incorporation method.

Test article for treatment was suspended in sterile distilled water for injection and serial dilutions were made. The dose ranges are presented in the table below. Concurrent negative and positive controls were also included, and triplicate plates were used for each dose.

Test strains	S9 mix	Dose (μg/plate)				
TA strains	+/-	50	150	500	1500	5000
WP2 *uvr*A	+/-	50	150	500	1500	5000

No substantial increases in numbers of revertants per plate were observed in any of the test strains following treatment with the test article at any dose level. There was no indication of cytotoxicity over the range of doses tested.

The mean revertant of the positive control for each test strain exhibited a clear increase over the mean revertant of the negative control for that strain.

The results indicate that the test article, Granule Valine, was not mutagenic in this bacterial assay system.

(b) (4)

Materials and Methods

1. Test and reference articles

1) Test article (Appendix 5)

Name:	Granule Valine
Code No.:	C-2991
Lot No.:	(b) (4)
Date of receipt:	Jun 21, 2018
Amount:	11 g / tube × 1 tube
Appearance:	Brown Granule
Content:	L-valine 71.87%
Expiration date:	Apr 02, 2021
Storage conditions:	Room temperature, protect from moisture
Supplier:	CJ BLOSSOM PARK, BIO Research

2) Vehicle (negative control article)

Name:	Sterile distilled water for injection
Lot No.:	(b) (4)
Storage condition:	Room temperature (Refrigeration after opening)
Supplier:	(b) (4)
Justification of selection	The vehicle was selected according to the preliminary preparation.

3) Positive control articles

Positive control articles used in this study are listed in the following table. These positive control articles are among those recommended in the OECD guideline TG 471.

Metabolic activation	Positive controls (Abbr.)	CAS No.	Test Strains	Dose (μg/plate)
+	2-Aminoanthracene (2-AA)	(b) (4)	TA100	1
			TA1535	2
			TA1537	1
			WP2 *uvr*A	6
	Benzo[a]pyrene (B[a]P)		TA98	1
-	Sodium azide (SA)		TA100	0.5
			TA1535	0.5
	2-Nitrofluorene (2-NF)		TA98	2
	4-Nitroquinoline-1-oxide (4NQO)		WP2 *uvr*A	0.5
	Acridine Mutagen ICR 191(ICR-191)		TA1537	0.5

(b) (4)

Name	Supplier	Item No.	Lot No.	Date of Received	Storage Condition
2-AA	(b) (4)	A38800	(b) (4)	Aug 16, 2017	11 to 30 °C
B[a]P		48564		Mar 09, 2017	11 to 30 °C
SA		S8032		May 30, 2017	11 to 30 °C
2-NF		N16754		Aug 16, 2017	11 to 30 °C
4NQO		N8141		Aug 16, 2017	Below -15 °C
ICR-191		I3636		Aug 16, 2017	-1 to 10 °C

2. Preparation and analysis of dose formulation

1) Preparation of dose formulations

The test article was used without compensation for content. The test article was weighed and mixed with vehicle by using a vortex mixer to make the highest dose. The highest dose was diluted with the same vehicle to make lower doses. The preparation was done just before treatment.

2) Preparation of positive control articles

Frozen stock solutions of SA which has been prepared with sterile distilled water for injection (b) (4) was kept at below -15 °C. Stock solutions of 2-AA, B[a]P, 2-NF, 4NQO and ICR-191 prepared with DMSO (b) (4) (b) (4) ≥99.9 %) were kept frozen below -50 °C (B[a]P) and -15 °C (2-AA, 2-NF, 4NQO and ICR-191), respectively. The stock solutions were thawed just before the treatment.

3) Analysis of dose formulation

The dose formulation was not analyzed for concentration and stability.

3. Test system

1) Test system justification

The histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 (Maron and Ames, 1983) and a tryptophan auxotroph strain of *Escherichia coli* WP2 *uvr*A (Green and Muriel, 1976) were used. These test strains are among those recommended by the test guideline of OECD TG 471. These strains have been shown to be sensitive to the mutagenic activity of a wide range of chemical classes. The specific genotypes of the test strains and detectable mutations are listed below.

(b) (4)

Test strains	*his*/*trp* mutation	Additional mutation	Plasmid	Detection of mutation
TA100	*his*G46	*rfa* *uvr*B	pKM101	Base-pair substitution
TA1535	*his*G46	*rfa* *uvr*B	-	Base-pair substitution
TA98	*his*D3052	*rfa* *uvr*B	pKM101	Frame-shift
TA1537	*his*C3076	*rfa* *uvr*B	-	Frame-shift
WP2 *uvr*A	*trp*E	*uvr*A	-	Base-pair substitution

The *rfa* mutation in TA strains results in the partial loss of the lipopolysaccharide (LPS) barrier of cell wall and the mutation make the barrier more permeable to certain classes of large molecules. The *uvr*A or *uvr*B is essential for excision repair system of the test strain. Mutations of these genes result in a deficient DNA repair system and greatly enhance the sensitivity of these strains to some mutagens. The presence of plasmid pKM101 further increases the sensitivity of these strains to some mutagens.

2) Source of test strains and media

Source of test strains

Test strains were obtained from (b) (4) and subcultured in the (b) (4).

Culturing broth

The broth used to grow the test strains for mutagenicity assay was 2.5 % Oxoid Nutrient Broth No. 2 prepared in distilled water.

Minimal glucose agar (bottom agar) plates

The minimal glucose agar (25 mL per 15 x 90 mm petri dish) was Vogel-Bonner medium E supplemented with 1.5 % Bacto agar (Difco) and 2 % glucose. The minimal glucose agar for the WP2 *uvr*A strain was supplemented with additional 0.25 ml/L of 0.1 % L-tryptophan. Gamma ray-sterilized petri dishes were used.

Top agar

Top agar for selection of revertants was prepared with 0.6 % Bacto agar (Difco) and 0.5 % NaCl. The top agar for *Salmonella* strains was supplemented with 10 mL of 0.5 mM histidine/biotin solution per 100 mL.

3) Storage of test strains and phenotypic characterization

Frozen stocks of test strains

Frozen stock cultures for long-term storage were prepared from fresh overnight cultures. DMSO was added to the cultures (90 μL/mL) as a cryopreservative, and aliquots of cultures were stored at below -70 °C.

(b) (4)

Master plates

The frozen stocks were thawed and cultured for 10 hours to prepare master plates of test strains. A part of each bacterial culture was used for the confirmation of genotypes. After confirming the genetic characteristics of the strains, then the master plates were used as the source of bacteria for mutagenicity assays.

Verification of genetic characteristics

The following genetic characteristics of the strains were verified according to the methods of Maron and Ames (1983).

Phenotypes	Test strains
histidine requirement	*Salmonella typhimurium* TA strains
presence of *uvr*B mutation	*Salmonella typhimurium* TA strains
presence of R-factor	*Salmonella typhimurium* TA strains
presence of *rfa* mutation	*Salmonella typhimurium* TA strains
number of spontaneous revertant	*Salmonella typhimurium* TA strains and *E. coli* WP2 *uvr*A
tryptophan requirement	*E. coli* WP2 *uvr*A
presence of *uvr*A mutation	*E. coli* WP2 *uvr*A

4. Metabolic activation system (S9 mix)

1) S9 and cofactor

S9

Origin of S9: Aroclor 1254- induced male Sprague-Dawley rat liver

Supplier: (b) (4)

Item No.: (b) (4)

Lot No.: (b) (4)

Protein content: 40.5 mg/mL

Storage condition: In a freezer (below -15 °C)

Cofactor

Name: Cofactor-I

Supplier: (b) (4)

Item No.: (b) (4)

Lot No.: (b) (4)

Storage condition: Refrigeration (-1 to 10 °C)

(b) (4)

2) Preparation of S9 mix (per 1 mL, 5 % S9 v/v)

The S9 mix was prepared with S9 and cofactor solution just before use. The S9 mix contained 8 μmol $MgCl_2 \cdot 6H_2O$, 33 μmol KCl, 5 μmol G-6-P, 4 μmol NADPH, 4 μmol NADH, 100 μmol sodium phosphate buffer (pH 7.4) and 50 μL S9. Prepared S9 mix was placed in crushed ice.

5. Experimental procedures

1) Selection of dose range

Dose ranges of this study were selected based on the results of a range-finding test conducted on the test article using the five test strains in both the presence and absence of metabolic activation system with two plates per dose [(b) (4) a non-GLP study]. Six doses of test article ranging 8 to 5000 μg/plate were tested using the same methods of this study. The condition of the treatment mixtures and plates was checked for the formation of precipitation and cytotoxicity, if any. In the range-finding test, turbidity was observed in the treatment mixtures above 1000 μg/plate. Precipitation was observed in the plates above 1000 μg/plate at the time of colony counting. There were no significant increase or decrease in numbers of colony in all test strains at all doses

Therefore, the dose ranges for the present study were set as shown in the table below. Concurrent negative and positive controls will be also included, and triplicate plates were used for each dose.

Test strains	S9 mix	Dose (μg/plate)				
TA strains	+/-	50	150	500	1500	5000
WP2 *uvr*A	+/-	50	150	500	1500	5000

2) Plating procedures and scoring of plates

The test strains were exposed to the test article using the direct plate incorporation method.

A small amount of bacterial growth in each master plate was taken and transferred to a flask containing 20 mL of liquid medium (2.5 % Oxoid Nutrient Broth No. 2). Inoculated flasks were incubated for 10 hours in a shaker/incubator (37 ± 2 °C, 120 rpm). Overnight cultures were removed from incubation and the viable cell counts were determined by optical density (OD) at 600 nm, and the cultures were stored in a refrigerator until use.

For the plating assay, the followings were added to each sterile culture tube containing 2 mL of top agar held at 45 ± 2 °C in a dry bath: 0.5 mL of S9 mix (or sodium-phosphate buffer, pH 7.4 for the non-activating plates), 0.1 mL of bacterial culture and 0.1 mL of test article. The contents were vortexed for 2 - 3 second and overlaid onto the surface of the bottom agar.

Negative control plates were treated with 0.1 mL of vehicle instead of test article. The positive control plates were treated with positive control articles with the same method.

The sterility of the highest dose test article solution was checked by plating a 0.1 mL aliquot (mixed with 2 mL of top agar) on the minimal glucose agar. S9 mix was also checked for sterility by plating 0.5 mL with the same method.

After the top agar solidified, plates were inverted and incubated at 37 ± 2 °C for 50 ± 2 hours and then revertant colonies were counted with unaided eyes.

3) Identification of plates

Each plate was labeled with an oil-based pen to identify the study number, test strain, dose level and activation condition.

4) Observations

The turbidity and/or precipitation in the treatment mixture were checked with unaided eyes, and if settlement of fine particle was observed, it was considered as precipitation.

Revertant colonies were counted with unaided eyes. The condition of background lawn was scored relative to the negative control, and contamination and other abnormality of each plate were checked.

A dose level was considered to be cytotoxic if at least one of the following criteria was met:

(1) A clearing or diminution (reduction) of the background lawn that was accompanied by a substantial reduction in the number of revertants per plate.

(2) The presence of microcolonies (pinpoint colonies).

There is no common standard of 'decrease' for the number of revertants, so it was determined if the number of revertants per plate was less than 50 % of that of solvent control or when there is a reversal of an increasing trend of the number of colonies.

5) Presentation of the results

Mean revertant per plate and standard deviation were calculated from the triplicate plates per dose. The actual numbers of revertant were also presented. The 'increase factor' was calculated by dividing the value of treated plate by the value of negative control plate. The increase factors were rounded off to one decimal place.

6) Assay acceptance criteria

The assay was considered valid only if all of the following criteria were met.

(1) At least 0.5×10^8 CFU of bacteria/plate were plated.

(2) A minimum of three non-toxic dose levels were required to evaluate assay data.

(3) The mean number of spontaneous revertants per plate should be within the range presented in the following table.

Test strains	No. Revertant
TA100	75-200
TA1535	3-37
TA98	15-60
TA1537	4-31
WP2 *uvr*A	5-40

(4) The mean revertants per plate of a positive control for a respective test strain should be at least a 2-fold increase over the mean revertants per plate of the solvent control for that test strain. The integrity of the S9 mix should be demonstrated by increases of revertants for the positive control plates treated with B[a]P and with 2-AA.

(5) There should be no microbial colonies due to the contamination in the plates for sterility check of test article and S9 mix.

6. Statistics and evaluation of the results

1) Statistical analysis

No statistical analysis was done.

2) Evaluation of results

The result was regarded as positive if there was a dose-related increase over the range tested and/or a reproducible increase at one or more doses in the number of revertant per plate in at least one strain with or without metabolic activation system. A positive result indicates that the test substance induces point mutation in the test strain.

The result was regarded as negative if the result did not meet the positivity criteria. The negative result indicates that the test article is not mutagenic in the test strains. Biological relevance of the results was also considered for the evaluation of the results.

(b) (4)

Results

Dose formulations

The suspension or precipitation of test article were observed above 1.5 mg/mL in the vehicle.

Bacterial reverse mutation test (Table 1, Appendix 1 and Appendix 2)

Turbidity was observed above 1500 μg/plate when the prepared test article was mixed with the top agar. Precipitation was observed above 1500 μg/plate on the bottom agar at the time of plate scoring. There was no microbial colony due to contamination in any of the plates for sterility check of test article and S9 mix.

In TA100, TA1535, TA98 and TA1537, there were no substantial increases in numbers of colony at any dose level of test article both in the presence and absence of metabolic activation system. There were no signs of cytotoxicity at any dose level in any test strain.

In WP2 *uvr*A, there were no substantial increases in numbers of colony at any dose level of test article both in the presence and absence of metabolic activation system. There were no signs of cytotoxicity at any dose level.

The mean revertant of the positive control for each test strain exhibited a clear increase over the mean revertant of the negative control for that strain.

The viable cell counts of test strains were $0.68 - 1.45 \times 10^9$ (TA strains) and 1.49×10^9 (*E. coli*) CFU/mL, and more than 0.5×10^8 CFU of bacteria/plate were plated.

(b) (4)

Discussion and Conclusion

All criteria for a valid assay were met. For all of the test strains, in the presence and absence of S9 mix, there were no significant increases of the revertants per plate in all test strains, and the experimental results failed to meet the criteria for positivity.

Therefore, it was concluded that the test article, Granule Valine, did not induce reverse mutation in the test strains used in this study.

References

1) Basic Mutagenicity Tests: UKEMS Recommended Procedures, Edited by David J. Kirkland, Cambridge University Press, 1990. ISBN 0-521-39347-7.
2) Green, MHL and Muriel, WJ (1976): Mutagen testing using *trp*+ reversion in *Escherichia coli*., Mutat. Res., 38:3-32.
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4) Maron, DM and Ames, BN (1983): Revised methods for the *Salmonella* mutagenicity test, Mutat. Res., 113:173-215.
5) Vogel, HJ and Bonner, DM (1956): Acetylornithinase of *E. coli*: Partial purification and some properties, J. Biol. Chem., 218:97-106 (1956).
6) (b) (4).

Units and Abbreviations

Note: The following lists of codes, abbreviations and units are used by (b) (4)
Some, but not necessarily all, of this information may be needed for this report.

%	Percent
°	Degree
C	Celsius
L	Liter
mL	Milliliter
μL	Microliter
g	Gram
kg	Kilogram
mg	Milligram
μg	Microgram
ng	Nanogram
m	Meter
cm	Centimeter
mm	Millimeter
μm	Micrometer
nm	Nanometer
hr	Hour
min	Minute
sec	Second
rpm	Revolution per Minute
G-6-P	Glucose-6-phosphate
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
GLP	Good Laboratory Practice Regulation
MFDS	Ministry of Food and Drug Safety
OECD	Organization for Economic Co-operation and Development
QAU	Quality Assurance Unit
SD	Standard Deviation
SOP	Standard Operating Procedures
SPSS	Statistical Package for the Social Sciences
DKBT	Diplomated Korean Board of Toxicology

(b) (4)

TABLE

(b) (4)

Table 1. Reverse mutagenicity assay results –summary

Test Strain	Chemical Treated	Dose (μg/plate)	Colonies/plate [factor][a) With S9 mix		Without S9 mix	
TA100	Test article	0	166 ± 3		170 ± 2	
		50	177 ± 3	[1.1]	164 ± 3	[1.0]
		150	174 ± 2	[1.0]	184 ± 4	[1.1]
		500	182 ± 2	[1.1]	181 ± 2	[1.1]
		1500 T	173 ± 2	[1.0]	172 ± 4	[1.0]
		5000 T	179 ± 4	[1.1]	168 ± 3	[1.0]
TA1535	Test article	0	23 ± 2		21 ± 1	
		50	22 ± 2	[1.0]	21 ± 0	[1.0]
		150	26 ± 1	[1.2]	23 ± 2	[1.1]
		500	17 ± 1	[0.8]	22 ± 1	[1.1]
		1500 T	19 ± 1	[0.9]	23 ± 2	[1.1]
		5000 T	22 ± 1	[1.0]	25 ± 1	[1.2]
TA98	Test article	0	22 ± 1		21 ± 1	
		50	24 ± 1	[1.1]	21 ± 1	[1.0]
		150	24 ± 2	[1.1]	16 ± 1	[0.8]
		500	21 ± 1	[1.0]	18 ± 2	[0.9]
		1500 T	21 ± 2	[1.0]	17 ± 1	[0.8]
		5000 T	25 ± 2	[1.1]	22 ± 1	[1.0]
TA1537	Test article	0	14 ± 1		12 ± 1	
		50	11 ± 1	[0.8]	11 ± 2	[0.9]
		150	15 ± 1	[1.1]	9 ± 2	[0.8]
		500	11 ± 2	[0.8]	13 ± 2	[1.1]
		1500 T	12 ± 2	[0.9]	10 ± 1	[0.8]
		5000 T	15 ± 1	[1.1]	13 ± 1	[1.1]
E. coli WP2 *uvr*A	Test article	0	37 ± 2		39 ± 3	
		50	37 ± 2	[1.0]	38 ± 1	[1.0]
		150	38 ± 1	[1.0]	37 ± 1	[0.9]
		500	37 ± 2	[1.0]	37 ± 1	[0.9]
		1500 T	39 ± 1	[1.0]	32 ± 2	[0.8]
		5000 T	37 ± 2	[1.0]	42 ± 1	[1.1]
Positive controls						
TA100	2-AA	1.0	1720 ± 133	[10.4]		
TA1535	2-AA	2.0	155 ± 16	[6.9]		
TA98	B[a]P	1.0	176 ± 8	[7.9]		
TA1537	2-AA	1.0	213 ± 16	[15.6]		
WP2 *uvr*A	2-AA	6.0	122 ± 7	[3.3]		
TA100	SA	0.5			304 ± 39	[1.8]
TA1535	SA	0.5			265 ± 21	[12.8]
TA98	2-NF	2.0			194 ± 3	[9.2]
TA1537	ICR-191	0.5			188 ± 6	[15.3]
WP2 *uvr*A	4NQO	0.5			142 ± 17	[3.6]

Test article: Granule Valine

T: Turbidity in the treatment mixture

a) Three plates/dose were used. No. of colonies of treated plate/No. of colonies of negative control plate

Abbreviations

2-AA, 2-aminoanthracene; SA, sodium azide; B[a]P, benzo[a]pyrene; ICR-191, acridine mutagen ICR 191; 4NQO, 4-nitroquinoline N-oxide; 2-NF, 2-Nitrofluorene.

(b) (4)

APPENDICES

(b) (4)

Appendix 1. Reverse mutagenicity assay results – individual plate counts

Test Strain	Chemical Treated	Dose (μg/plate)	Colonies/plate (Status of background lawn[a])					
			With S9 mix			Without S9 mix		
TA100	Test article	0	168 (N)	163 (N)	166 (N)	169 (N)	172 (N)	170 (N)
		50	180 (N)	175 (N)	177 (N)	162 (N)	167 (N)	162 (N)
		150	173 (N)	176 (N)	172 (N)	182 (N)	189 (N)	181 (N)
		500	184 (N)	180 (N)	181 (N)	183 (N)	180 (N)	181 (N)
		1500 T	172 (P)	175 (P)	173 (P)	175 (P)	173 (P)	167 (P)
		5000 T	177 (P)	176 (P)	184 (P)	165 (P)	169 (P)	170 (P)
TA1535	Test article	0	21 (N)	23 (N)	24 (N)	21 (N)	20 (N)	21 (N)
		50	20 (N)	23 (N)	23 (N)	21 (N)	21 (N)	21 (N)
		150	26 (N)	26 (N)	27 (N)	24 (N)	24 (N)	21 (N)
		500	17 (N)	18 (N)	16 (N)	22 (N)	22 (N)	23 (N)
		1500 T	20 (P)	18 (P)	20 (P)	22 (P)	22 (P)	25 (P)
		5000 T	22 (P)	22 (P)	21 (P)	25 (P)	25 (P)	24 (P)
TA98	Test article	0	23 (N)	21 (N)	23 (N)	22 (N)	20 (N)	21 (N)
		50	25 (N)	23 (N)	23 (N)	21 (N)	22 (N)	21 (N)
		150	23 (N)	26 (N)	22 (N)	15 (N)	16 (N)	17 (N)
		500	22 (N)	21 (N)	21 (N)	17 (N)	17 (N)	20 (N)
		1500 T	20 (P)	23 (P)	21 (P)	18 (P)	16 (P)	17 (P)
		5000 T	26 (P)	25 (P)	23 (P)	22 (P)	23 (P)	21 (P)
TA1537	Test article	0	13 (N)	14 (N)	14 (N)	13 (N)	12 (N)	12 (N)
		50	11 (N)	10 (N)	12 (N)	12 (N)	11 (N)	9 (N)
		150	14 (N)	15 (N)	16 (N)	8 (N)	11 (N)	9 (N)
		500	11 (N)	13 (N)	9 (N)	11 (N)	15 (N)	13 (N)
		1500 T	10 (P)	11 (P)	14 (P)	10 (P)	10 (P)	9 (P)
		5000 T	15 (P)	15 (P)	14 (P)	12 (P)	13 (P)	14 (P)
E. coli WP2 *uvr*A	Test article	0	37 (N)	39 (N)	36 (N)	39 (N)	37 (N)	42 (N)
		50	35 (N)	38 (N)	37 (N)	38 (N)	39 (N)	37 (N)
		150	37 (N)	38 (N)	39 (N)	37 (N)	38 (N)	37 (N)
		500	35 (N)	37 (N)	39 (N)	36 (N)	38 (N)	37 (N)
		1500 T	39 (P)	40 (P)	38 (P)	32 (P)	31 (P)	34 (P)
		5000 T	39 (P)	37 (P)	35 (P)	41 (P)	42 (P)	42 (P)
Positive controls								
TA100	2-AA	1.0	1664 (N)	1624 (N)	1872 (N)			
TA1535	2-AA	2.0	149 (N)	144 (N)	173 (N)			
TA98	B[a]P	1.0	185 (N)	172 (N)	170 (N)			
TA1537	2-AA	1.0	217 (N)	196 (N)	227 (N)			
WP2 uvrA	2-AA	6.0	127 (N)	126 (N)	114 (N)			
TA100	SA	0.5				304 (N)	266 (N)	343 (N)
TA1535	SA	0.5				250 (N)	289 (N)	256 (N)
TA98	2-NF	2.0				195 (N)	196 (N)	190 (N)
TA1537	ICR-191	0.5				183 (N)	188 (N)	194 (N)
WP2 uvrA	4NQO	0.5				158 (N)	125 (N)	143 (N)

Test article: Granule Valine

T: Turbidity in the treatment mixture

a) Status of background lawn (BL) and plate

N, normal BL; R, reduced BL; A, absent or almost absent BL; E, enhanced BL; O, obscured BL by precipitation; P, precipitation of test article in plate; M, presence of microcolonies; C, contaminated plate.

Abbreviations

2-AA, 2-aminoanthracene; SA, sodium azide; B[a]P, benzo[a]pyrene; ICR-191, acridine mutagen ICR 191; 4NQO, 4-nitroquinoline N-oxide; 2-NF, 2-Nitrofluorene.

(b) (4)

Appendix 2. Viable cell counts of test strains and results of sterility tests

Test strain	Viable cell counts (10^9 CFU/mL)	Sterility of test article Solution (highest dose)	Sterility of S9 mix
TA100	1.36	No colony due to contamination	No colony due to contamination
TA1535	0.94		
TA98	1.45		
TA1537	0.68		
WP2 *uvr*A	1.49		

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Appendix 3. Historical control data

(Reverse mutation assays in the histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and a tryptophan auxotroph strain of *Escherichia coli* WP2 *uvr*A)

All negative (vehicle) controls [Jan 2006 − Dec 2017]

Strain	TA100		TA1535		TA98		TA1537		WP2 *uvr*A	
S9 mix	+	-	+	-	+	-	+	-	+	-
Min	95	86	5	5	15	11	3	4	13	10
Max	210	213	29	33	52	51	35	25	44	42
Mean	140	137	13	13	30	24	13	10	24	21
SD	25	24	4	4	7	6	4	3	5	5
Confidence	91	91	4.7	5.7	17	12	5.4	4.1	14	11
Intervals (95 %)	181	183	20	21	43	36	20	17	35	31
No. of plates	795	795	771	771	783	786	780	777	789	783

Sterile distilled water for Injection controls [Jan 2006 − Dec 2017]

Strain	TA100		TA1535		TA98		TA1537		WP2 *uvr*A	
S9 mix	+	-	+	-	+	-	+	-	+	-
Min	95	86	5	7	15	13	5	4	13	10
Max	210	213	27	27	52	51	35	24	44	42
Mean	139	137	12	13	30	24	13	10	25	21
SD	25	24	3	3	7	6	4	3	5	5
Confidence	90	90	5.3	6.2	17	12	5.5	4.4	14	11
Intervals (95 %)	187	184	18	19	44	37	20	16	35	31
No. of plates	396	396	381	381	387	390	384	384	393	390

Dimethyl sulfoxide controls [Jan 2006 − Dec 2017]

Strain	TA100		TA1535		TA98		TA1537		WP2 *uvr*A	
S9 mix	+	-	+	-	+	-	+	-	+	-
Min	95	88	6	5	15	11	3	4	13	10
Max	198	207	29	33	51	44	28	25	39	39
Mean	139	135	13	13	29	23	13	10	24	20
SD	26	24	4	4	6	6	4	3	5	5
Confidence	89	89	5.1	5.4	17	11	5.2	3.8	14	11
Intervals (95 %)	190	181	20	21	42	35	21	17	34	30
No. of plates	321	321	312	312	318	318	315	315	318	315

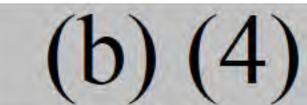
Positive controls [a] [Jan 2006 − Dec 2017]

Strain	TA100		TA1535		TA98		TA1537		WP2 *uvr*A	
S9 mix	+	-	+	-	+	-	+	-	+	-
Min	360	180	47	62	78	116	46	31	68	48
Max	2832	820	484	648	532	486	711	724	308	424
Mean	1106	465	160	296	212	290	158	175	142	164
SD	515	95	67	82	81	73	74	102	45	65
Confidence	95.5	278	28.22	134	53.4	146	12.3	-25	53.7	36.5
Intervals (95 %)	2116	651	93	457	371	435	304	374	229	291
No. of plates	567	768	744	744	606	498	753	651	558	756

a) See Table 1 for names of positive control articles and doses/plate

(b) (4)

Appendix 4. Protocol and protocol amendment



PROTOCOL

Bacterial Reverse Mutation Assay with Granule Valine

Study Number: 18-VG-0736 (b) (4)

Approval:

(b) (4), (b) (6) Sep 19 2018
Date

(b) (4), (b) (6) Sep 20, 2018
Date

Sep 28 2018
Date

Sponsor representative
CJ BLOSSOM PARK, BIO Research

ORIGINAL
2018 년 10 월 02 일
시험책임자 이 [illegible]

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(b) (4)

Title Bacterial Reverse Mutation Assay with Granule Valine

Objective The objective of this study is to evaluate the test article Granule Valine for its ability to induce reverse mutation in the four histidine-requiring TA strains of *Salmonella typhimurium* and a tryptophan-requiring strain *Escherichia coli* WP2 *uvr*A.

Regulatory guideline OECD Guideline for Testing of Chemicals TG 471 (1997) 'Bacterial Reverse Mutation Test'

Sponsor CJ BLOSSOM PARK, BIO Research
42nd street 55, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, Korea,
CJ Blossom Park, 16495
+82-10-8099-1902 (TEL), +82-31-8099-2914 (FAX)

Test Facility (b) (4)

Schedule

Oct 01, 2018	Inoculation of test strains (experimental initiation)
Oct 02, 2018	Chemical treatment
Oct 04, 2018	Scoring plates (experimental completion)
Oct 30, 2018	Draft report (expected data)

Contributing Scientists
Preparation/Storage of the Test article: (b) (4), (b) (6)
Archives:
Cell lines management:

(b) (4)

(b) (4)

Archives (b) (4)

The protocol (amendment and deviation, if any), raw data, sample of test article and other relevant evidential documents will be stored in the Archives (b) (4) (b) (4) until the reliability of the study results can be evaluated by the relevant regulatory authority (at least 5 tears).

Further storage of above materials shall be consulted with the sponsor.

GLP compliance OECD Principles of Good Laboratory Practice (19 (b) (4)

The amendments and deviation from the protocol (if any) will be documented, reviewed by Quality Assurance Unit (QAU), and approved by the study director, management and sponsor.

The QAU o (b) (4) inspects solely throughout the progression of study.

Final report (b) (4)

The final report will fully reflect the contents of the present protocol and consist of (but not limited to) cover page, statement of GLP compliance, quality assurance statement, synopsis, contents, summary, materials and methods, results, discussion and conclusion, references, tables and appendices.

(b) (4)

(b) (4)

1. Test and reference articles

1) Test article [SOP-TA-001]

Name: Granule Valine

Code No.: C-2991

Lot No.: (b) (4)

Date of receipt: Jun 21, 2018

Amount: 11 g / tube × 1 tube

Appearance: Brown Granule

Content: L-valine 71.87%

Expiration date: Apr 02, 2021

Storage conditions: Room temperature, protect from moisture

Supplier: CJ BLOSSOM PARK, BIO Research

2) Vehicle (Negative control)

Name: Sterile distilled water for injection

Lot No.: (b) (4)

Supplier: Room temperature (Refrigeration after opening)

Storage condition: (b) (4)

Justification of selection: The vehicle was selected according to the preliminary preparation.

3) Positive control article

Positive control articles for this study are listed in the following table. These positive control articles are among those recommended in the OECD guideline TG 471.

Metabolic activation	Positive controls (Abbr.)	CAS No.	Test Strains	Dose (μg/plate)
+	2-Aminoanthracene (2-AA)	613-13-8	TA100	1
			TA1535	2
			TA1537	1
			WP2 *uvrA*	6
	Benzo[a]pyrene (B[a]P)	50-32-8	TA98	1
-	Sodium azide (SA)	26628-22-8	TA100	0.5
			TA1535	0.5
	2-Nitrofluorene (2-NF)	607-57-8	TA98	2
	4-Nitroquinoline-1-oxide (4NQO)	56-57-5	WP2 *uvrA*	0.5
	Acridine Mutagen ICR 191 (ICR-191)	17070-45-0	TA1537	0.5

(b) (4)

Name	Supplier	Item No.	Lot No.	Date Received	Storage Condition
2-AA	(b) (4)	A38800	(b) (4)	Aug 16, 2017	11 - 30 °C
B[a]P		48564		Mar 09, 2017	11 - 30 °C
SA		S8032		May 30, 2017	11 - 30 °C
2-NF		N16754		Aug 16, 2017	11 - 30 °C
4NQO		N8141		Aug 16, 2017	Below -15 °C
ICR-191		I3636		Aug 16, 2017	-1 - 10 °C

2. Preparation and analysis of dose formulation

1) Preparation of dose formulations [SOP-TA-002]

The test article will be used without compensation for content. The test article will be weighed and mixed with vehicle by using a vortex mixer to make the highest dose. The highest dose will be diluted with the same vehicle to make lower doses. The preparation will be done just before treatment.

2) Preparation of positive control articles

Frozen stock solutions of SA which has been prepared with sterile distilled water for injection (b) (4) was kept at below -15 °C. Stock solutions of 2-AA, B[a]P, 2-NF, 4NQO and ICR-191 prepared with DMSO (b) (4) (b) (4) ≥99.9 %) were kept frozen below -50 °C (B[a]P) and -15 °C (2-AA, 2-NF, 4NQO and ICR-191), respectively. The stock solutions will be thawed just before the treatment.

3) Analysis of dose formulation

The dose formulation will not be analyzed for concentration and stability.

3. Test system

1) Test system justification

The histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 (Maron and Ames, 1983) and a tryptophan auxotroph strain of *Escherichia coli* WP2 *uvrA* (Green and Muriel, 1976) will be used. These test strains are among those recommended by the test guideline of the OECD TG 471. These strains have been shown to be sensitive to the mutagenic activity of a wide range of chemical classes. The specific genotypes of the test strains and detectable mutations are listed below.

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(b) (4)

Test strains	*his*/*trp* mutation	Additional mutation	Plasmid	Detection of mutation
TA100	*his*G46	*rfa* *uvr*B	pKM101	Base-pair substitution
TA1535	*his*G46	*rfa* *uvr*B	-	Base-pair substitution
TA98	*his*D3052	*rfa* *uvr*B	pKM101	Frame-shift
TA1537	*his*C3076	*rfa* *uvr*B	-	Frame-shift
WP2 *uvr*A	*trp*E	*uvr*A	-	Base-pair substitution

2) Source of test strains and media

Source of test strains

Test strains, obtained from (b) (4), (b) (4) and subcultured in the (b) (4) will be used.

Culturing broth (b) (4)

The test strains for mutagenicity assay will be grown in 2.5 % Oxoid Nutrient Broth No. 2 prepared in distilled water.

Minimal glucose agar (bottom agar) plates (b) (4)

The minimal glucose agar (25 mL per 15 x 90 mm petri dish) will be Vogel-Bonner medium E supplemented with 1.5 % Bacto agar (Difco) and 2 % glucose. The minimal glucose agar for the WP2 *uvr*A strain will be supplemented with additional 0.25 ml/L of 0.1 % L-tryptophan. Gamma ray-sterilized petri dishes will be used.

Top agar (b) (4)

Top agar for selection of revertants will be prepared with 0.6 % Bacto agar (Difco) and 0.5 % NaCl. The top agar for *Salmonella* strains will be supplemented with 10 mL of 0.5 mM histidine/biotin solution per 100 mL.

3) Storage of test strains and phenotypic characterization

Frozen stocks of test strains (b) (4)

Frozen stock cultures for long-term storage were prepared from fresh overnight cultures. DMSO was added to the cultures (90 μL/mL) as a cryopreservative, and aliquots of cultures were stored at below -70 °C.

Master plates (b) (4)

The frozen stocks were thawed and cultured for 10 hours to prepare master plates of test strains. A part of each bacterial culture was used for the confirmation of genotypes. After confirming the genetic characteristics of the strains, then the stored master plates are used as the source of bacteria for mutagenicity assays.

(b) (4)

(b) (4)

Verification of genetic characteristics (b) (4)

The following genetic characteristics of the strains were verified according to the methods of Maron and Ames (1983).

Phenotypes	Test strains
histidine requirement	*Salmonella typhimurium* TA strains
presence of *uvr*B mutation	*Salmonella typhimurium* TA strains
presence of R-factor	*Salmonella typhimurium* TA strains
presence of *rfa* mutation	*Salmonella typhimurium* TA strains
number of spontaneous revertant	*Salmonella typhimurium* TA strains and *E. coli* WP2 *uvr*A
tryptophan requirement	*E. coli* WP2 *uvr*A
presence of *uvr*A mutation	*E. coli* WP2 *uvr*A

4. Metabolic activation system (S9 mix)

1) S9 and cofactor

S9

Origin of S9: Aroclor 1254- induced male Sprague-Dawley rat liver

Supplier: (b) (4)

Item No.: (b) (4)

Lot No.: to be specified in the final report

Protein content: to be specified in the final report

Storage condition: In a freezer (below -15 °C)

Cofactor

Name: Cofactor-I

Supplier: (b) (4)

Item No.:

Lot No.: to be specified in the final report

Storage condition: Refrigeration (-1 to 10 °C)

2) Preparation of S9 mix (per 1 mL, 5 % S9 v/v) [SOP-MT-108]

The S9 mix will be prepared with S9 and cofactor solution just before use. The S9 mix will contain: 8 μmol $MgCl_2 \cdot 6H_2O$, 33 μmol KCl, 5 μmol G-6-P, 4 μmol NADPH, 4 μmol NADH, 100 μmol sodium phosphate buffer (pH 7.4) and 50 μL S9. Prepared S9 mix will be placed in crushed ice.

(b) (4)

(b) (4)

5. Experimental procedures

1) Selection of dose range (b) (4)

Dose ranges of this study were selected based on the results of a range-finding test conducted on the test article using the five test strains in both the presence and absence of metabolic activation system with two plates per dose [(b) (4), a non-GLP study]. Six doses of test article ranging 8 to 5000 μg/plate were tested using the same methods of this study. The condition of the treatment mixtures and plates was checked for the formation of precipitation and cytotoxicity, if any. In the range-finding test, turbidity was observed in the treatment mixtures above 1000 μg/plate. Precipitation was observed in the plates above 1000 μg/plate at the time of colony counting. There were no significant increase or decrease in numbers of colony in all test strains at all doses.

Therefore, the dose ranges for the present study were set as shown in the table below. Concurrent negative and positive controls will be also included, and triplicate plates will be used for each dose.

Test strains	S9 mix	Dose (μg/plate)				
TA strains	+/-	50	150	500	1500	5000
WP2 *uvrA*	+/-	50	150	500	1500	5000

2) Plating procedures and scoring of plates (b) (4)

The test strains will be exposed to the test article using the direct plate incorporation method.

A small amount of bacterial growth in each master plate will be taken and transferred to a flask containing 20 mL of liquid medium (2.5 % Oxoid Nutrient Broth No. 2). Inoculated flasks will be incubated for 10 hours in a shaker/incubator (37 ± 2 °C, 120 rpm). Overnight cultures will be removed from incubation and the viable cell counts will be determined by optical density (OD) at 600 nm, and the cultures will be stored in a refrigerator until use.

For the plating assay, the followings will be added to each sterile culture tube containing 2 mL of top agar held at 45 ± 2 °C in a dry bath: 0.5 mL of S9 mix (or sodium-phosphate buffer, pH 7.4 for the non-activating plates), 0.1 mL of bacterial culture and 0.1 mL of test article. The contents will be vortexed for 2 - 3 second and overlaid onto the surface of the bottom agar.

Negative control plates will be treated with 0.1 mL of solvent instead of test article. The positive control plates will be treated with positive control articles with the same method.

The sterility of the most concentrated test article dilution will be checked by plating a 0.1 mL aliquot (mixed with 2 mL of top agar) on the minimal glucose agar. S9 mix will be also checked for sterility by plating 0.5 mL with the same method.

After the top agar solidified, plates will be inverted and incubated at 37 ± 2 °C for 50 ± 2 hours and then revertant colonies will be counted with unaided eyes.

(b) (4)

3) Identification of plates

Each plate will be labeled with an oil-based pen to identify the study number, test strain, dose level and activation condition.

4) Observations

The turbidity and/or precipitation in the treatment mixture will be checked with unaided eyes, and if settlement of fine particle observed, it will be considered as precipitation.

Revertant colonies will be counted with unaided eyes. The condition of background lawn will be scored relative to the solvent control, and contamination and other abnormality of each plate will be checked.

A dose level will be considered to be cytotoxic if at least one of the following criteria is met:

(1) A clearing or diminution (reduction) of the background lawn that accompanied by a substantial reduction in the number of revertant per plate.

(2) The presence of microcolonies (pinpoint colonies).

There is no common standard of 'decrease', so it will be determined if the mean number of revertant per plate is less than 50 % of that of solvent control or when there is a reversal of an increasing trend of the number of colonies.

5) Presentation of the results

Mean revertant per plate and standard deviation will be calculated from the triplicate plates per dose. The actual numbers of revertant will be also presented. The 'increase factor' will be calculated by dividing the value of treated plate by the value of negative control plate. The increase factors will be rounded off to one decimal place.

6) Assay acceptance criteria

The assay will be considered valid only if all of the following criteria are met.

(1) At least 0.5×10^8 CFU of bacteria/plate were plated.

(2) A minimum of three non-toxic dose levels were required to evaluate assay data.

(3) The mean number of spontaneous revertants per plate should be within the range presented in the following table.

Test strains	No. Revertant/plate
TA100	75-200
TA1535	3-37
TA98	15-60
TA1537	4-31
WP2 *uvr*A	5-40

(b) (4)

(b) (4)

(4) The mean revertants per plate of a positive control for a respective test strain should be at least a 2-fold increase over the mean revertants per plate of the solvent control for that test strain. The integrity of the S9 mix should be demonstrated by increases of revertants for the positive control plates treated with B[a]P and with 2-AA.

(5) There should be no microbial colonies due to the contamination in the plates for sterility check of test artiele and S9 mix.

6. Statistics and evaluation of the results

1) Statistical analysis

No statistical analysis will be done.

2) Evaluation of results

The result will be regarded as positive if there was a dose-related increase over the range tested and/or a reproducible increase at one or more doses in the number of revertant per plate in at least one strain with or without metabolic activation system. A positive result indicates that the test substance induces point mutation in the test strain.

The result will be regarded as negative if the result does not meet the positivity criteria. The negative result indicates that the test substance is not mutagenic in the test strains. A confirmatory test may be performed if it is not possible to make a definite judgement.

Biologieal relevance of the results will be also considered for the evaluation of the results.

7. References

1) Basic Mutagenicity Tests: UKEMS Recommended Procedures, Edited by David J. Kirkland, Cambridge University Press, 1990. ISBN 0-521-39347-7.

2) Green, MHL and Muriel, WJ (1976): Mutagen testing using *trp*+ reversion in *Escherichia coli*., Mutat. Res., 38:3-32.

3) GREEN, MHL (1984) Mutagen testing using *trp*+ reversion in *Escherichia coli* in KILBEY, BJ, LEGATOR, M, NICHOLS, W and RAMEL, C (Eds.). *Handbook of Mutagenicity Test Procedures. Second edition*, p.161-187. Elsevier Science Publishers BV, Amsterdam.

4) Maron, DM and Ames, BN (1983): Revised methods for the Salmonella mutagenicity test, Mutat. Res., 113:173-215.

5) Vogel, HJ and Bonner, DM (1956): Acetylornithinase of E. coli: Partial purification and some properties, J. Biol. Chem., 218:97-106 (1956).

(b) (4).

(b) (4)

Units and Abbreviations

Note: The following lists of codes, abbreviations and units are used by (b) (4)
Some, but not necessarily all, of this information may be needed for this protocol.

%	Percent
°	Degree
C	Celsius
L	Liter
mL	Milliliter
µL	Microliter
g	Gram
kg	Kilogram
mg	Milligram
µg	Microgram
ng	Nanogram
m	Meter
cm	Centimeter
mm	Millimeter
µm	Micrometer
nm	Nanometer
hr	Hour
min	Minute
sec	Second
rpm	Revolution per Minute
G-6-P	Glucose-6-phosphate
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
GLP	Good Laboratory Practice Regulation
MFDS	Ministry of Food and Drug Safety
OECD	Organization for Economic Co-operation and Development
QAU	Quality Assurance Unit
SD	Standard Deviation
SOP	Standard Operating Procedures
SPSS	Statistical Package for the Social Sciences
DKBT	Diplomated Korean Board of Toxicology

(b) (4)

Protocol Amendment Form
(시험계획서 변경/개정 기록지)

Study Title: Bacterial Reverse Mutation Assay with Granule Valine

Study No.: 18-VG-0736 Amendment No.: 1

Amendment to the Protocol:
(변경/개정 내용)

1. Page 2, Sponsor

Before amendment	After amendment
+82-10-8099-1902 (TEL)	+82-31-8099-1902 (TEL)

Reason for the Amendment:
(변경/개정 사유)

1. Correction of typo.

Impact on Study:
(시험에 미치는 영향)

1. None.

Approved by:

St (b) (4) Date

M Date

Sp Date Nov. 01. 2018

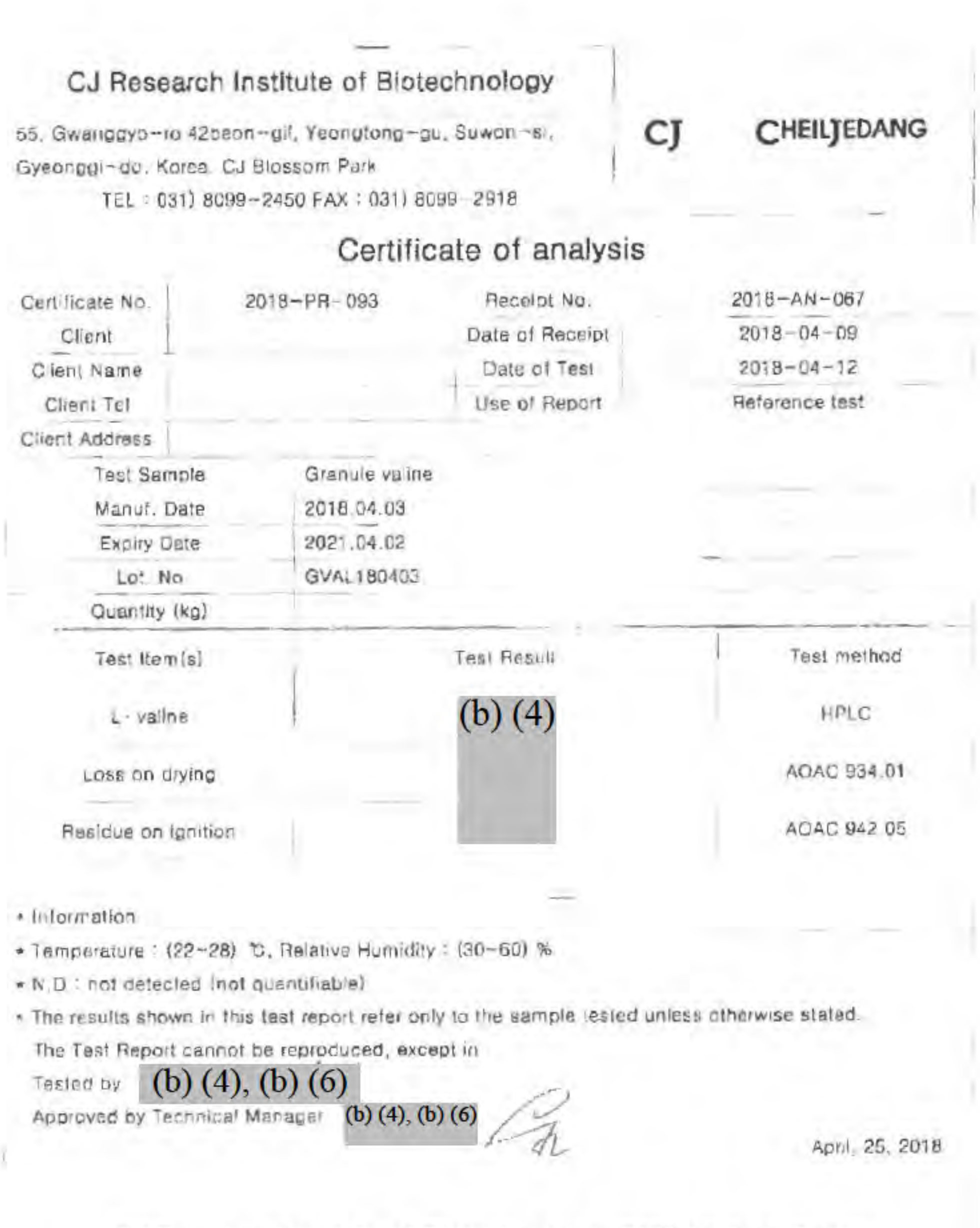
(b) (4)

ORIGINAL
년 월 일
시험책임자

TO-006-S03

(b) (4)

Appendix 5. Certificate of analysis



CJ Research Institute of Biotechnology

55, Gwanggyo-ro 42beon-gil, Yeongtong-gu, Suwon-si, Gyeonggi-do, Korea. CJ Blossom Park
TEL : 031) 8099-2450 FAX : 031) 8099-2918

CJ CHEILJEDANG

Certificate of analysis

Certificate No.	2018-PR-093	Receipt No.	2018-AN-067
Client		Date of Receipt	2018-04-09
Client Name		Date of Test	2018-04-12
Client Tel		Use of Report	Reference test
Client Address			

Test Sample	Granule valine
Manuf. Date	2018.04.03
Expiry Date	2021.04.02
Lot No	GVAL180403
Quantity (kg)	

Test Item(s)	Test Result	Test method
L-valine	(b) (4)	HPLC
Loss on drying		AOAC 934.01
Residue on ignition		AOAC 942.05

- Information
- Temperature : (22~28) ℃, Relative Humidity : (30~60) %
- N.D : not detected (not quantifiable)
- The results shown in this test report refer only to the sample tested unless otherwise stated.
  The Test Report cannot be reproduced, except in

Tested by (b) (4), (b) (6)
Approved by Technical Manager (b) (4), (b) (6)

April. 25. 2018

CJ Research Institute of Biotechnology, BIO) Analysis Team

CJ BIO-AD form 100-01 REV 01

MS Summary(Data)

Acquired by	: System Administrator
Month-Day Acquired	: 3/09/2019
Month-Day Processed	: 4/09/2019
Sample Name	: blank
Vial#	: 1
Injection Volume	: 5
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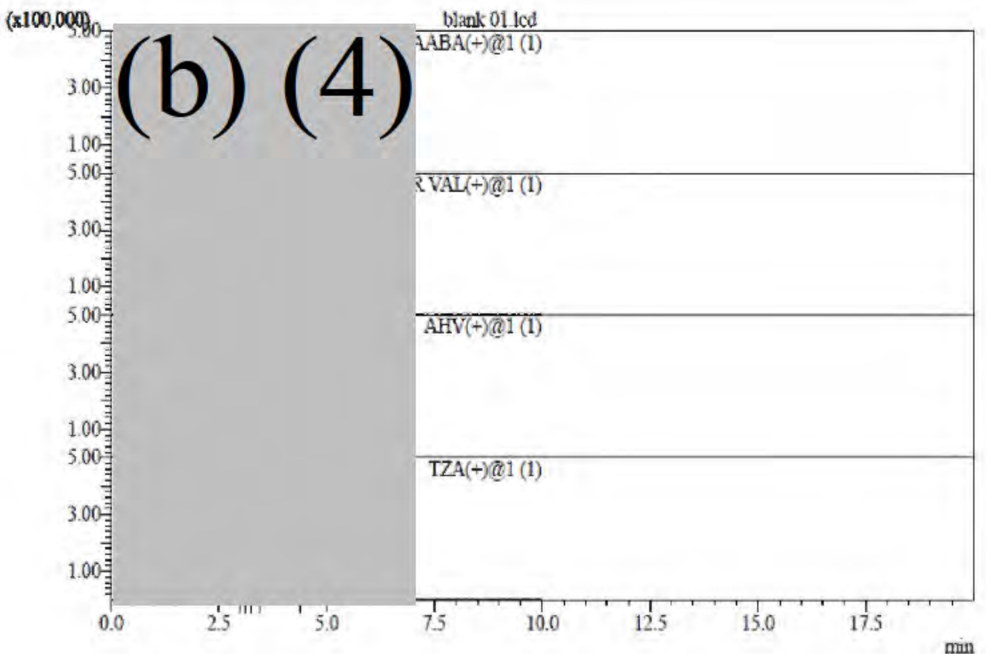

Acquired by	: System Administrator
Month-Day Acquired	: 3/09/2019
Month-Day Processed	: 4/09/2019
Sample Name	: STD
Vial#	: 2
Injection Volume	: 5
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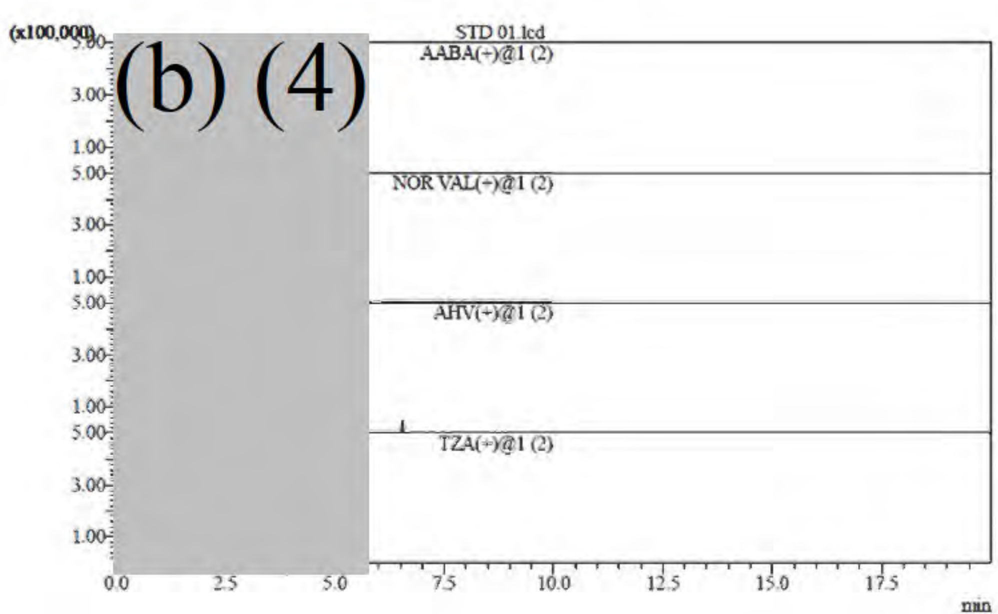

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Month-Day Processed	: 4/09/2019
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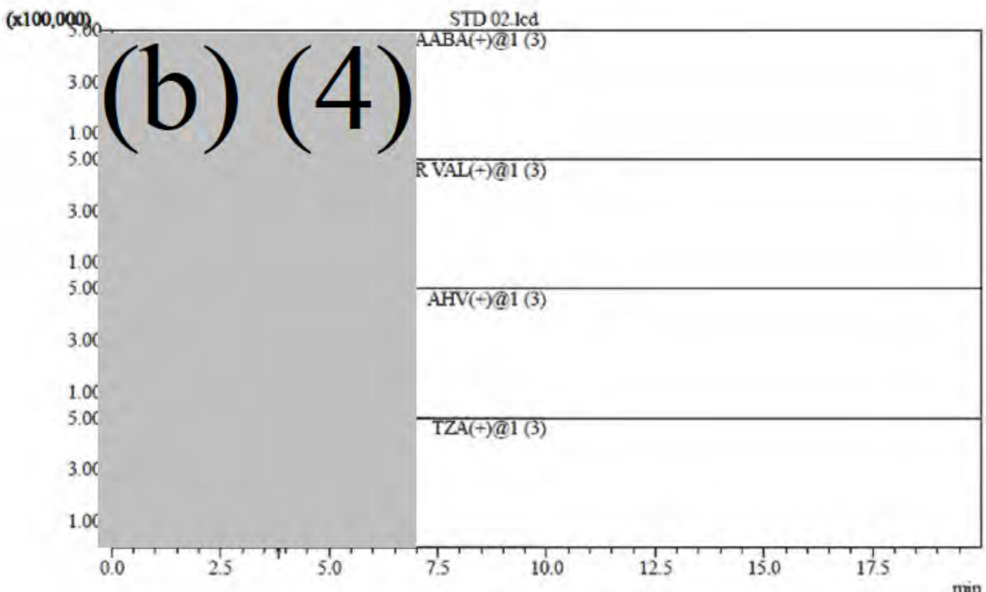
Vial#	: 3
Injection Volume	: 5
Data File	: C:\LC MS\data\val pro 190903\190903\STD 02.lcd



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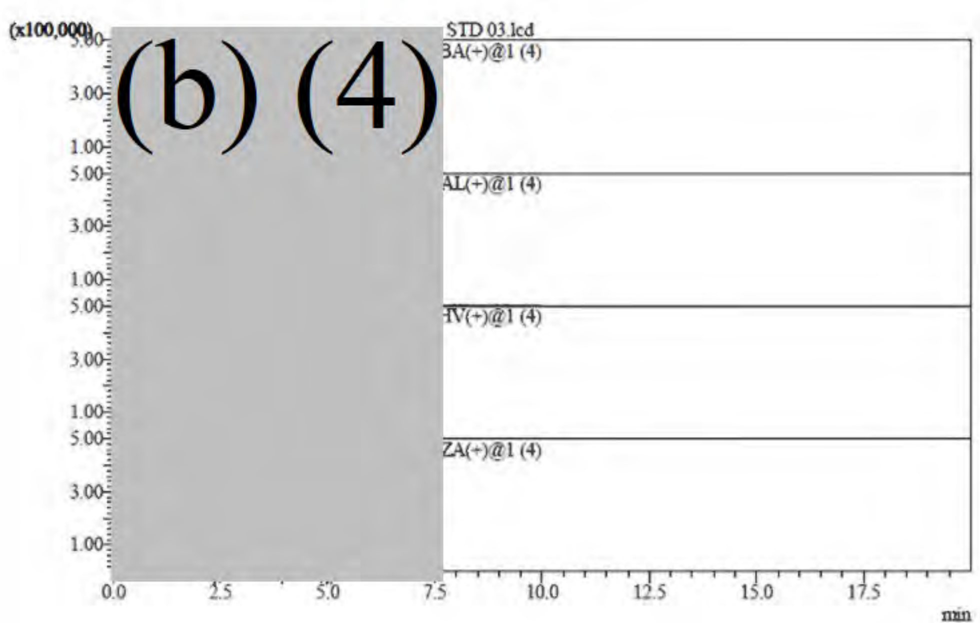
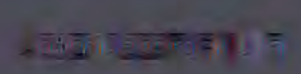
Month-Day Processed	: 4/09/2019
Sample Name	: STD
Vial#	: 4
Injection Volume	: 5
Data File	: C:\LC MS\data\val pro 190903\190903\STD 03.lcd

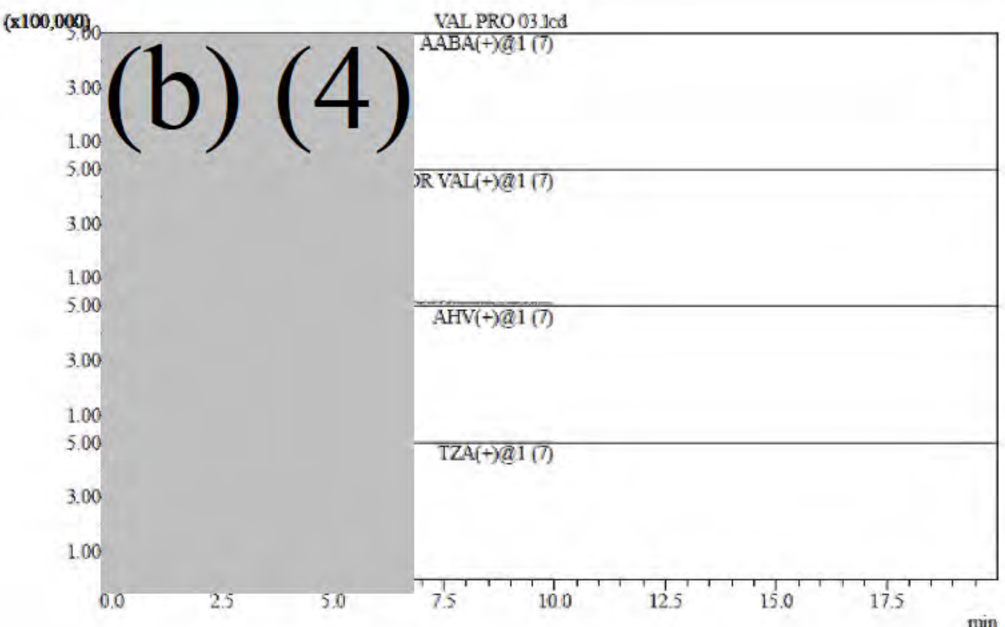




(b) (4)

(b) (4)

Acquired by : System Administrator
Month-Day Acquired : 3/09/2019
Month-Day Processed : 4/09/2019
Sample Name : SAMPLE
Vial# : 7
Injection Volume : 5
Data File : C:\LC MS\data\val pro 190903\190903\VAL PRO 03.lcd

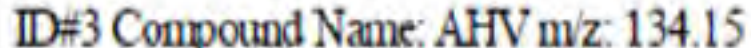


ID#1 Compound Name: AABA m/z: 104.10

Title	Vial#	Ret. Time	Area
blank 01.lcd	1	--	--
STD 01.lcd	2	2.557	(b) (4)
STD 02.lcd	3	2.566	
STD 03.lcd	4	2.538	
VAL PRO 01.lcd	5	--	--
VAL PRO 02.lcd	6	--	--
VAL PRO 03.lcd	7	--	--

ID#2 Compound Name: NOR VAL m/z: 118.15

Title	Vial#	Ret. Time	Area
blank 01.lcd	1	--	--
STD 01.lcd	2	4.074	(b) (4)
STD 02.lcd	3	4.134	
STD 03.lcd	4	4.060	
VAL PRO 01.lcd	5	--	--
VAL PRO 02.lcd	6	--	--
VAL PRO 03.lcd	7	--	--



ID#3 Compound Name: AHV m/z: 134.15

Title	Vial#	Ret. Time	Area
blank 01.lcd	1		
STD 01.lcd	2	4.725	(b) (4)
STD 02.lcd	3	4.616	
STD 03.lcd	4	4.698	
VAL PRO 01.lcd	5		
VAL PRO 02.lcd	6		
VAL PRO 03.lcd	7		

ID#4 Compound Name: TZA m/z: 173.05

Title	Vial#	Ret. Time	Area
blank 01.lcd	1		
STD 01.lcd	2	4.159	(b) (4)
STD 02.lcd	3	4.186	
STD 03.lcd	4	4.182	
VAL PRO 01.lcd	5		
VAL PRO 02.lcd	6		
VAL PRO 03.lcd	7		

REPORT

Valine Derivatives in Dried L-Valine Fermentation Product

Original Final report date: Sep24, 2019

CJ Research Institute of Biotechnology

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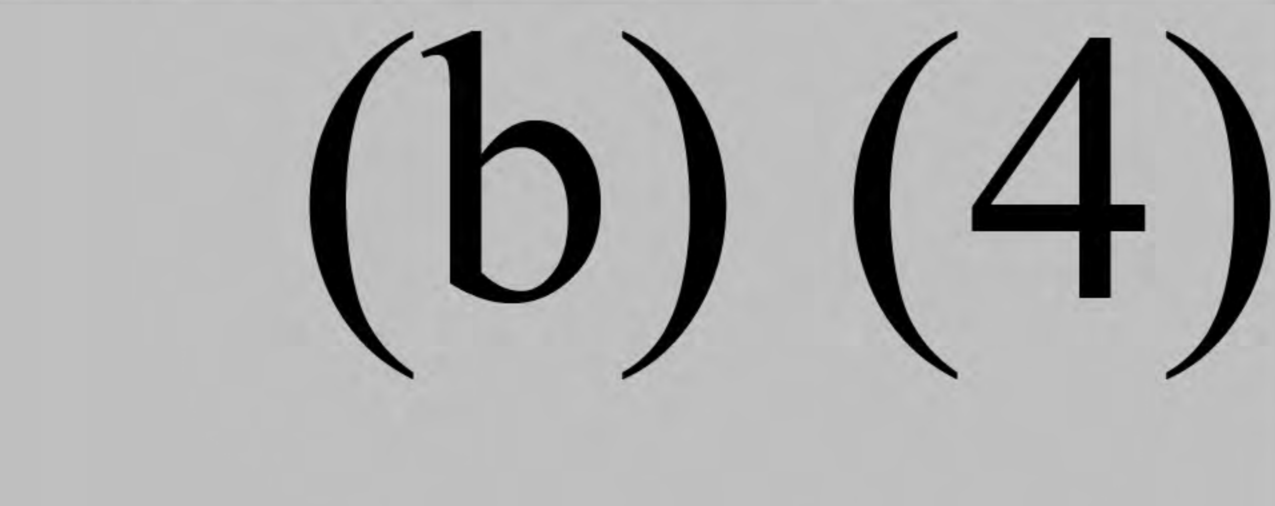
1. Valine Derivatives

The valine producing strain, *Corynebacterium glutamicum* KCCM80058, was

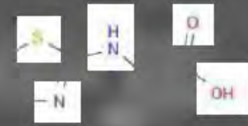


exist in the final product. We demonstrated absence by analyzing L-valine derivatives in the final product.

① α-Aminobutyric acid

② α-Hydroxyvaline

③ Thiazole alanine



④ Norvaline

2. Analysis of valine derivatives

2.1 LC-MS analysis

The following standard reagents were used: α-aminobutyric acid (Sigma-Aldrich, 162663), α-hydroxyvaline (Uorsy), thiazole alanine (Angene chemical), and norvaline (Sigma-Aldrich, N7627). The reagents were analyzed using LC-MS.

For sample preparation, 1 g of each Dried L-Valine fermentation product (Batches GVAL180404, GVAL180405 and GVAL180406) was dissolved in 20 mL of deionized water (which would be 50 g/L).

The Shimadzu LCMS system was used with ODS column (150 x 2.1 mm, 1.8 μm) and temperature of column was 35 °C. For LC conditions, only mobile phase A (0.1 % formic acid in water) was used. The flow rate was 0.2 mL/min and total analysis time was 10 minutes.

For MS conditions, we analyzed using electrospray ionization (ESI) detector at positive mode; speed of nebulizing gas was 1.50 L/min; speed of drying gas was 15 L/min; interface voltage was 4.50 kV; heat block temperature was 200 °C; and detector temperature was −1.10 kV.

There was no official method for four valine derivatives so using LC-MS was the surest way to prove the presence of valine derivatives. The LC-MS test method used was validated for limit of detection and limit of quantification.

Amino acid analyzer condition	
System	Shimadzu LCMS
Column	ODS column (150 x 2.1 mm, 1.8 μm)
Column temperature	35 ℃
Mobile phase	0.1 % formic acid in H2O
Flow rate	0.2 mL/min
Sample temperature	10 ℃
injection column	2 μL

2.2Result

We analyzed four standards and each standard showed good results in the described

LC-MS conditions. In these conditions, the retention time of α-aminobutyric acid was 2.55 min, α-hydroxyvaline was 4.68 min, thiazole alanine was 4.18 min, and norvaline was 4.09 min. These retention times were an average of 3 points calibration curve.

When the Dried L-Valine Fermentation Product was analyzed, we couldn't find any peak in the position of 2.55 min, 4.68 min, 4.18 min and 4.09 min of retention time in chromatogram. The molecular weight of norvaline is same as valine but retention time of valine was 3.07 min.

α-aminobutyric acid, α-hydroxyvaline, thiazole alanine, and norvaline were not present in the Dried L-Valine Fermentation Product at levels above the LOD (limit of detection) level. All chromatograms are shown below (Figure 1).

2.3 Limit of detection (LOD) and limit of quantification (LOQ)

The calibration curve was drawn to express LOD (limit of detection) and LOQ (limit of quantification). In addition, regression analysis was also carried out using this curve to figure out 'Residual standard deviation' to calculate LOD (*Anal. Chem*. 1999, 71, 2672-2677).

LOD may also be calculated based on the standard deviation of the response (σ) of the curve and the slope of the calibration curve (S) at levels approximating the LOD and LOQ according to the formula: LOD = 3.3*(σ/S) and LOQ = 10*(σ/S). The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

In this case, deviation of response would be residual standard deviation. The residual standard deviation is a statistical term used to describe the difference in standard deviations of observed values versus predicted values as shown by points in a regression analysis. Regression analysis is a method used in statistics to show a relationship between two different variables, and to describe how well you can predict the behavior of one variable from the behavior of another.

Residual standard deviation is also referred to as the standard deviation of points around a fitted line or the standard error of estimate. The formula for residual and residual standard deviation is:

$$\text{Residual} = (Y - Y_{est})$$

S_{res}= Residual standard deviation
Y= Observed value
Y_{est}= Estimated or projected value
n= Data points in population

We performed 3 points calibration for three compounds as below and describe the summary output of regression analysis.

2.3.1 α-Aminobutyric acid

1) Calibration curve

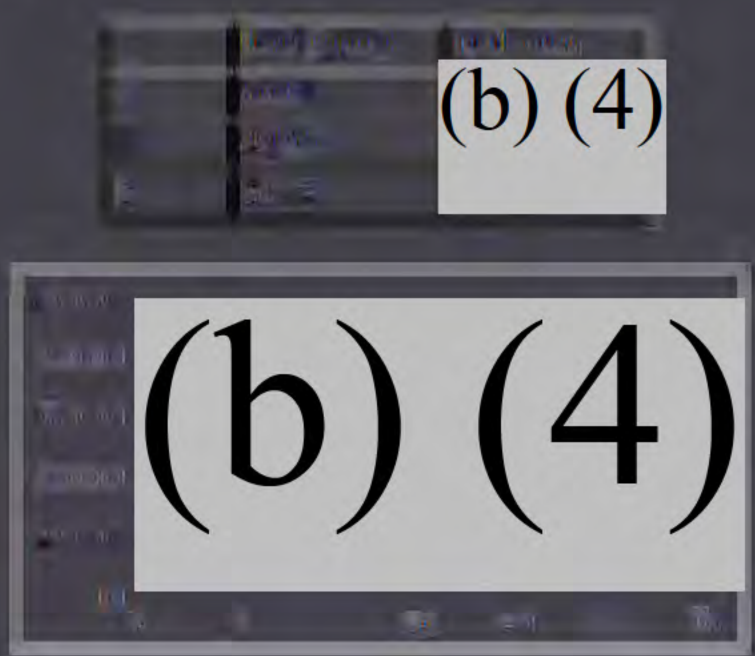
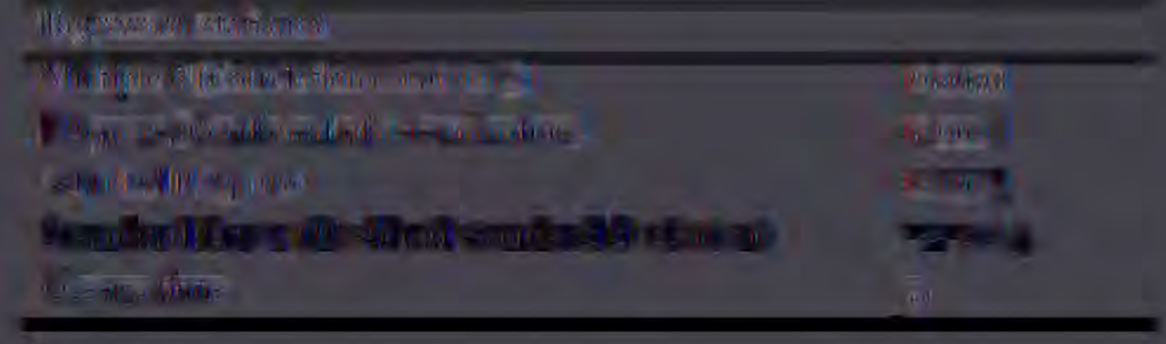


Fig. 1. Calibration curve of α-Aminobutyric acid

2) Summary output for regression analysis study



ANOVA

	degree of freedom	sum of squares	mean square	F stat	critical value of F stat
Regression	1	229,419,251,516	29,419,251,516	4329.22	0.0097
Residual	1	52,993,175	52,993,175		
Total	2	229,472,244,691			

	Coefficients	standard error	t Stat	P-value	Lower 95 %	Upper 95 %
Y intercept	45136.36	8341.15	5.41	0.1163	-60848.04	151120.77
X variable 1	16709.87	253.96	65.80	0.0097	13482.98	19936.76

3) LOD and LOQ of α-Aminobutyric acid

LOD = 3.3 x (residual standard deviation /slope)

= 3.3 x (7279.64 /16709.87) = (b) (4) pm

LOQ = 10 x (residual standard deviation /slope)

= 10 x (7279.64 /16709.87) = (b) (4) ppm

2.3.2 α-hydroxyvaline

1) Calibration curve

	level (ppm)	peak area
1	10.12	(b) (4)
2	24.95	
3	50.06	

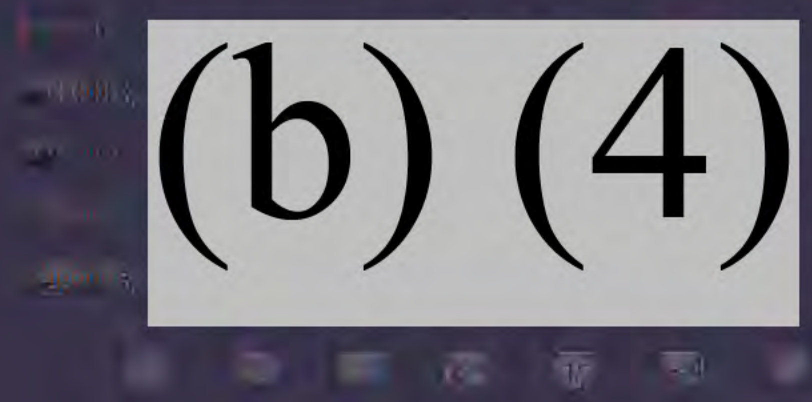
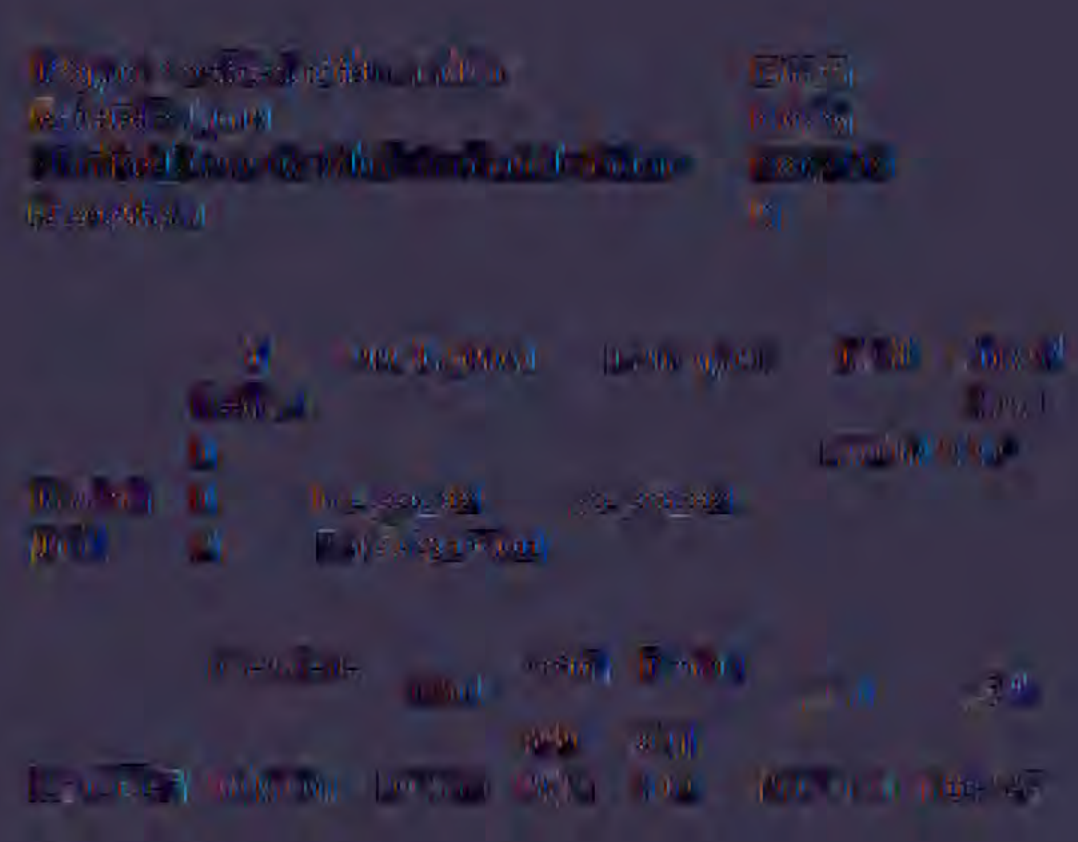


Fig. 2. Calibration curve of α-hydroxyvaline

2) Summary output for regression analysis study



3) LOD and LOQ of α-hydroxyvaline

LOD = 3.3 x (residual standard deviation /slope)

= 3.3 x (30072.18 /38453.56)= (b) (4) ppm

LOQ = 10 x (residual standard deviation /slope)

= 10 x (30072.18 /38453.56) (b) (4) ppm

2.3.3 thiazole alanine

1) Calibration curve

	level (ppm)	peak area
1	10.11	(b) (4)
2	24.97	
3	50.05	

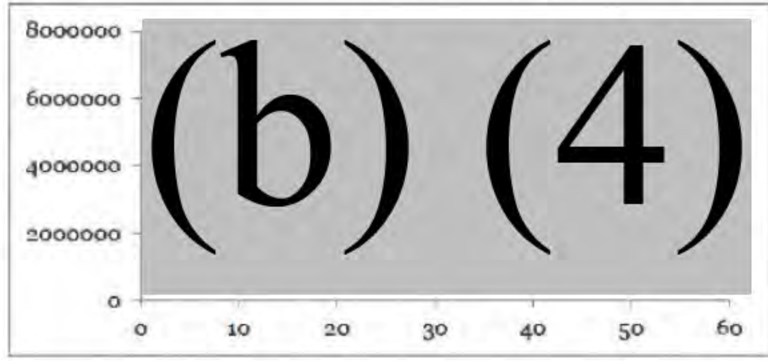


Fig. 3. Calibration curve of thiazole alanine

2) Summary output for regression analysis study

Regression statistics	
Multiple R (Correlation coefficient)	0.9998
R Square (Coefficient of determination)	0.9996
Adjusted R Square	0.9992
Standard Error (Residual standard deviation)	**68671.59**
Observations	3

ANOVA

	degree of freedom	sum of squares	mean square	F stat	critical value of F stat
Regression	1	11,832,835,881,038	11,832,835,881,038	2509.20	0.013
Residual	1	4,715,786,965	4,715,786,965		
Total	2	11,837,551,668,003			

	Coefficients	standard error	t Stat	P-value	Lower 95 %	Upper 95 %
Y intercept	10777.76	78937.69	0.14	0.91	-992220.63	1013776.15
X variable 1	120493.32	2405.45	50.09	0.01	89929.23	151057.42

3) LOD and LOQ of thiazole alanine

LOD = 3.3 x (residual standard deviation /slope)

= 3.3 x (68671.59/120493.32) (b) (4) pm

LOQ = 10 x (residual standard deviation /slope)

= 10 x (68671.59/120493.32) = (b) (4) ppm

2.3.4 norvaline

1)Calibration curve

	level (ppm)	peak area
1	10.11	(b) (4)
2	24.97	
3	50.05	

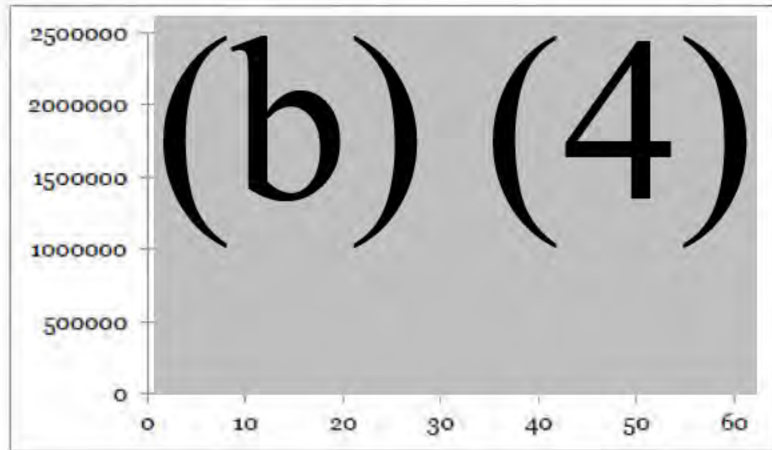


Fig. 4. Calibration curve of norvaline

2) Summary output for regression analysis study

Regression statistics	
Multiple R (Correlation coefficient)	0.9998
R Square (Coefficient of determination)	0.9996
Adjusted R Square	0.9992
Standard Error (Residual standard deviation)	**21883.57**
Observations	3

ANOVA

	degree of freedom	sum of squares	mean square	F stat	critical value of F stat
Regression	1	1,264,032,835,549	1,264,032,835,549	2639.50	0.012
Residual	1	478,890,574	478,890,574		
Total	2	1,264,511,726,123			

	Coefficients	standard error	t Stat	P-value	Lower 95 %	Upper 95 %
Y intercept	14663.51	25155.06	0.58	0.66	-304961.90	334288.91
X variable 1	39382.00	766.54	51.38	0.01	29642.14	49121.85

3) LOD and LOQ of norvaline

LOD = 3.3 x (residual standard deviation /slope)

= 3.3 x (21883.57/39382.00) = (b) (4) ppm

LOQ = 10 x (residual standard deviation /slope)

= 10 x (21883.57/39382.00) = (b) (4) pm

2.4 Summary of LOD

compound	LOD (mg/L)
α-Aminobutyric acid	(b) (4) ng/L
α-hydroxyvaline	ng/L
Thiazole alanine	ng/L
Norvaline	ng/L

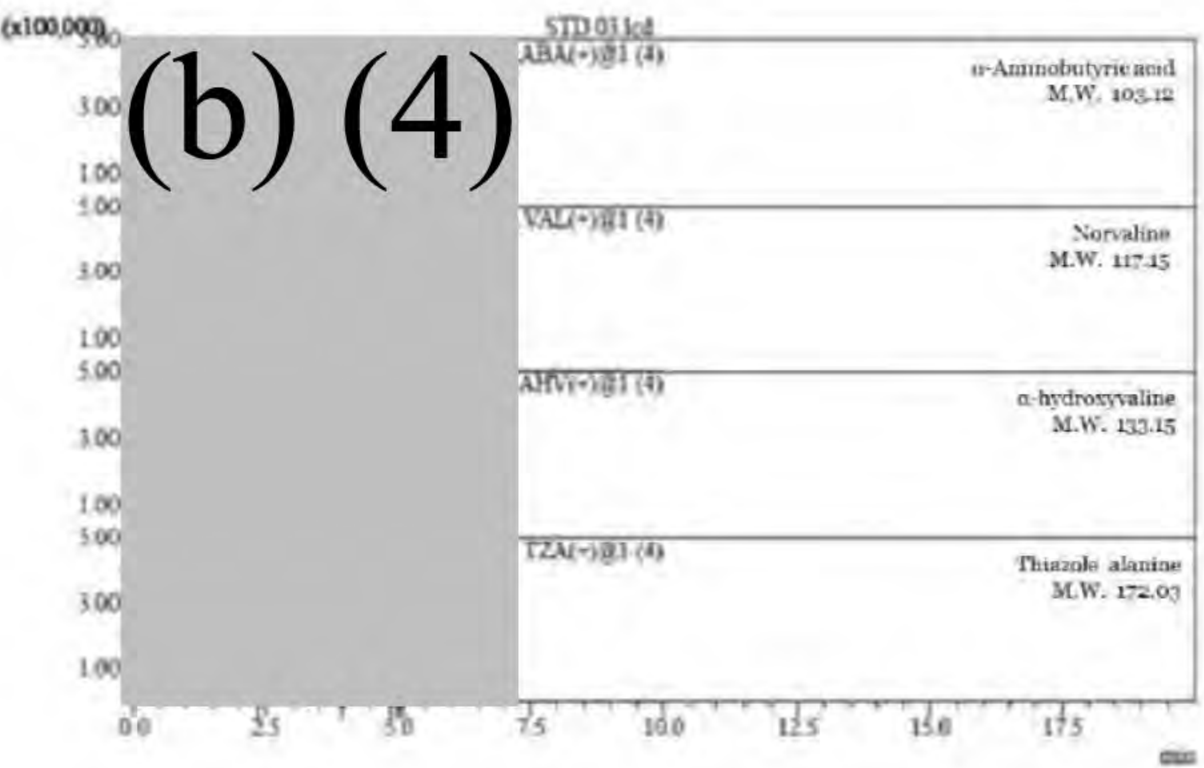


Fig 5. LC-MS chromatogram of valine derivatives

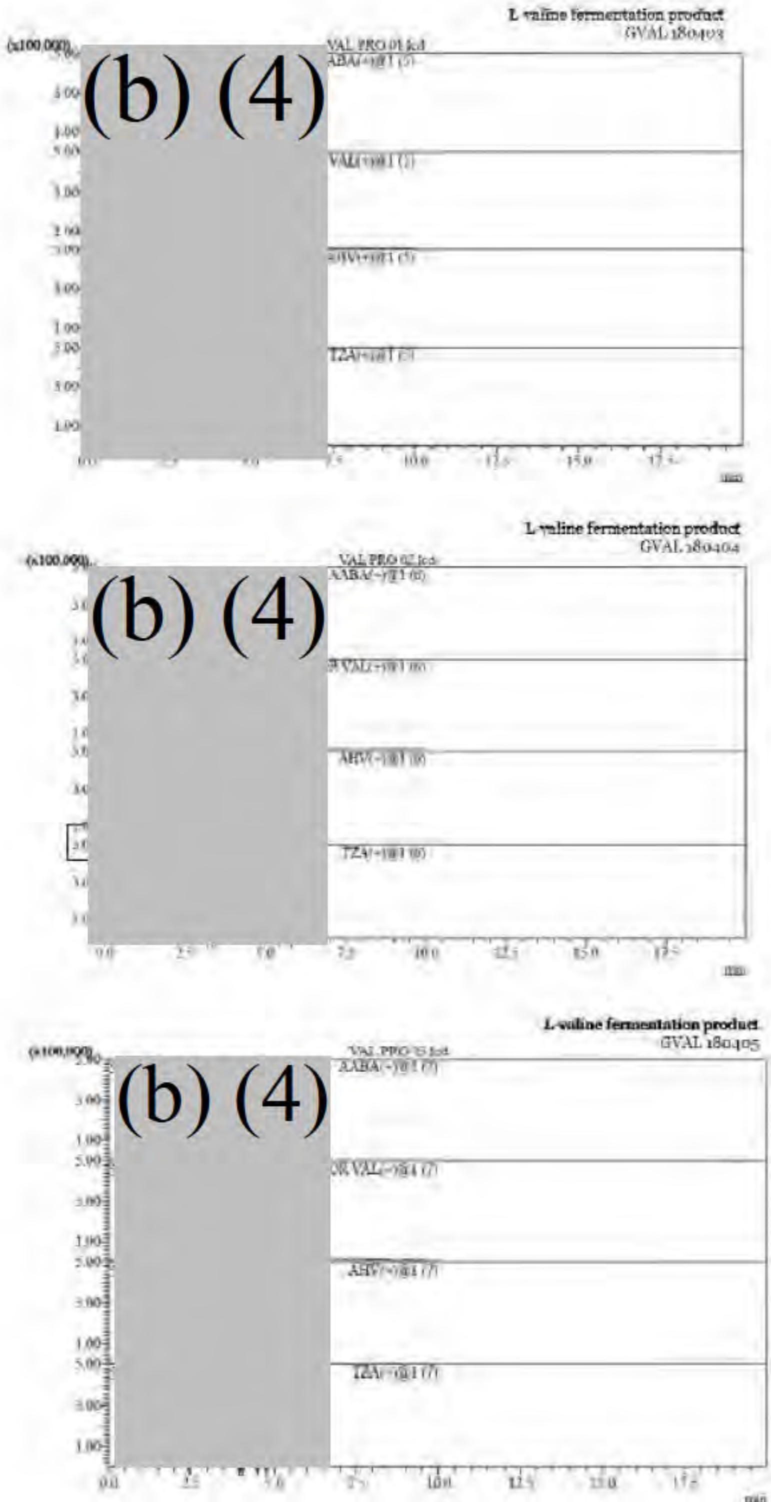


Fig 6. HPLC chromatogram of sample solution

lot No.	Data file name
GVAL180404	VAL PRO 01

GVAL180405	VAL PRO 02
GVAL180406	VAL PRO 03

3. Conclusion

We analyzed four valine derivatives **which areα-aminobutyric acid,** α-hydroxyvaline, thiazole alanine, and norvaline. In Dried L-Valine **Fermentation Product, we couldn't find** any peak in the position of each retention time of valine derivatives in LC-MS chromatogram. Therefore, we concluded that if any valine derivative is present in Dried L-Valine Fermentation Product, it would be less than LOD (1.44 mg/L, 2.58 mg/L, 1.88 mg/L and 1.83 mg/L). In conclusion, there are no valine derivatives in Dried L-Valine Fermentation Product.

4. Attachment

1) Attachment 1. Raw data of L-VAL derivatives analysis report

Appendix 10. Literature Review *Corynebacterium glutamicum* – with references

Review of the safety of *Corynebacterium glutamicum*

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1. INTRODUCTION

This document addresses the safety of the microorganism ***Corynebacterium glutamicum***. It presents scientific data and information gathered from in-depth literature reviews which demonstrate that ***C. glutamicum*** can be used as a microorganism for the industrial production of amino acids and other substances which in turn can be safely added to feed for food-producing animals and poses no risk or health hazards to humans consuming products from food-producing animals consuming the substance. This review, as prescribed by the Division of Animal Feed staff, is intended to refresh the detailed safety review assessment completed in 2003 by the Division with the addition of ***Corynebacterium glutamicum*** and ***Corynebacterium glutamicum*** derived ingredients as an authorized feed ingredient.

2. EVALUATION BY EFSA

2.1 Qualified presumption of safety (QPS)

A wide variety of microorganisms are intentionally added at different stages into the food chain, either directly or as a source of food and feed additives, enzymes or plant protection products. The qualified presumption of safety (QPS) approach was developed by the EFSA Scientific Committee to provide a generic concept to prioritize and to harmonize risk assessment within EFSA of microorganisms intentionally introduced into the food chain (EFSA, 2005, 2007).

The list of QPS microorganisms has been continuously revised and updated since it was established in 2007. The publication of the overall assessment of the taxonomic units (TU) previously recommended for the QPS list is carried out every three years (EFSA, 2007, 2012). The recommendations provided concerning that list of microorganisms are maintained and re-evaluated based on extensive literature reviews and expert knowledge. (EFSA, 2007, 2018).

2.2 Re-evaluation using literature review

The bi-annual re-evaluation of microorganisms begins with a literature review for each TU that is notified to EFSA. QPS recommended TU and those which represent new TU notifications are annually reviewed (EFSA, 2007). The literature review for a new TU is broader to cover the history of use, the potential safety concerns and the ecology. Relevant databases such as Web of Science Core Collection, CAB Abstracts, BIOSIS Citation Index, MEDLINE and Food Science Technology Abstracts are searched using the TU in combination with common keywords (e.g. toxin, disease, antibiotic/antimycotic resistance, safety, syndrome) and respective animal categories. The search terms are broad and cover synonyms or former names of taxonomic units (EFSA, 2012, 2013, 2017). Findings from the literature review are then evaluated, taking into consideration recommendations given in the previous QPS Opinion. A detailed description of the methodology used in carrying out the literature review can be found in EFSA (2013, 2017). A summary of the literature search strategy for the most recent QPS update for ***C. glutamicum*** is given in Table 1.

Table 1.	***Corynebacterium glutamicum***
String for species	
"*Corynebacterium glutamicum*" OR "*C glutamicum*" OR "*Brevibacterium lactofermentum*" OR "*B lactofermentum*"	
Outcome	**String**
1) Antimicrobial/Antibiotic/Antimycotic	"antimicrobial resistan*" OR "antibiotic resistan*" OR "antimicrobial susceptibil*"
2) Infection/Bacteremia/Fungemia/Sepsis	infection* OR abscess* OR sepsis* or septic* OR bacteremia OR bacteraemia OR toxin* OR "pathogen*"
3) Type of disease	Not applied
4) Mortality/Morbidity	clinical* OR death* OR morbidit* OR mortalit* OR disease* OR illness*
5) Disease Risk	opportunistic OR virulen*
Flow records by search strategy resulted in 78 papers being identified using title screening, of which 8 papers were identified using title/abstract screening, of which 1 was identified using article appraisal and was considered relevant for QPS. Following the review of that paper (Yang and Yang, 2017), it was concluded that there were no safety concerns identified in the only article considered relevant for QPS exercise (EFSA, 2019).	
A literature review did not reveal new information about adverse health effects or on safety concerns since the last update (EFSA, 2013). The QPS recommendation has been confirmed.	
Source: EFSA (2018).	

2.3 QPS Classification of *Corynebacterium glutamicum*

The QPS approach is currently used for microorganisms in the three broad categories within which most of the species notified to EFSA fall: bacteria, yeasts and viruses (EFSA, 2005, 2007). Here only information as it relates to the QPS assessment of the bacterium ***C. glutamicum*** is presented.

As noted, each updated QPS Opinion is based on a review of newly available scientific literature and recommendations given in the previous years' opinions. Scientific opinions on the update of the list of QPS-recommended biological agents intentionally added to food or feed that include ***C. glutamicum*** are reported for the years 2007, 2008, 2010, 2011, 2012, 2013, 2016, 2017 and 2019. The recommendations given in each QPS Opinion for these respective years are summarized in Appendix 1. The recommendations unanimously confirm that ***C. glutamicum*** meets the QPS criteria for humans and animals and there are no adverse health effects or on safety concerns.

3. LITERATURE SEARCH (2003-2019)

3.1 Method Used

An electronic literature search (ELS) was conducted by saqual GmbH to collect scientific studies, articles, reports and other documents deemed to be relevant for a review of the

safety/risk assessment of ***C. glutamicum.*** The ELS was carried out in October 30th, 2019 using the Google Scholar database and included information published from 2003 onwards. A detailed description of the ELS strategy employed and a listing of the search "strings" used and "hits" obtained is detailed in Appendix 2. The ELS was based on the search terms or "strings" used by EFSA in the 2017 QPS re-evaluations for ***C. glutamicum*** (Section 2.2, Table 1), but adapted to the Google Scholar and its specific structure. The information collected from the ELS was reviewed and follow-up selective searches were made using the Web of Science Core Collection, CAB Abstracts and Global Health, BIOSIS Citation Index and Current Contents.

3.2 Relevant Records Retrieved

The "hits" or records retrieved in the ELS search were compiled and each publication was reviewed and judged whether it contained information relevant to the safety of C. glutamicum (Appendix 2, Table 2). Some examples of the topics addressing ***C. glutamicum*** in the records retrieved include the role of pathogenic and non-pathogenic ***Corynebacterium spp***., particularly in human clinical trials (Camello et al., 2003; Roux et al., 2004; Bernard, 2005; Eguchi et al., 2008; Olender, 2012; Oliveira et al., 2017), genetic and biochemical characterization of ***C. glutamicum*** and site directed mutagenesis (Zhang et al., 2012), gene identification and sequencing (Ikeda and Nagakawa, 2003; Khamis et al., 2004; Ordonez et al., 2005; Yukawa et al., 2007), gene deletion and the effect on cell morphology and antibiotic resistance (Möker et al., 2004; Oritz-Pérez et al., 2010; Bernard, 2012) and carcass degradation (Kim et al., 2017).

Overall, no studies were retrieved either in the ELS or follow-up selective searches that contained information indicating potential safety issues or hazards associated with ***C. glutamicum***. Those records retrieved from the searches that support the accepted safe use of different strains of C. glutamicum for amino acid production are reviewed in the following narrative.

4. NARRATIVE - CORYNEBACTERIUM GLUTAMICUM

The scientific data and information presented in the following sections demonstrate that ***C. glutamicum*** can be safely used as a microorganism for the industrial production of amino acids under the conditions of intended use for the target animals and humans consuming food derived from food-producing animals consuming the substance.

4.1 Taxonomy and Characteristics

The genus ***Corynebacterium*** belongs to the taxonomic class ***Actinobacteria*** that represents gram-positive bacteria with a high guanine and cytosine content in their DNA (Stackebrandt et al., 1997; Ventura et al., 2007). The genus Corynebacterium which currently has 110 validated species, is highly diversified and includes species that are of medical, veterinary, or biotechnological relevance (Pascual et al., 1995; Khamis et al., 2004; Bernard, 2012; Soares et al., 2013; Oliveira et al., 2017; Dalen et al., 2018).

One of the most prominent members among the genus ***Corynebacterium*** is ***C. glutamicum***, a bacterium isolated in 1956 from an avian-feces-contaminated soil sample collected from Ueno Zoo in Tokyo (Japan) with a natural capacity to accumulate L-glutamate extracellularly in a biotin-limited medium (Kinoshita et al., 1957; Udaka, 1960; Shiio et al., 1962). ***C.***

glutamicum belongs to a broad, diverse group of mycolic acid-containing bacteria that share the property of having an unusual cell envelope composition and architecture, differing from those of other gram-positive bacteria (Peuch et al., 2001).

C. glutamicum is a nonmotile, facultative anaerobic, Gram-positive biotin-auxotrophic soil bacterium, which forms rod-shaped, straight, or slightly curved cells (Becker and Whittman, 2017). The chromosome of the wild-type strain ***C. glutamicum*** ATCC 14067 is 3,273,044 bp in length, with an average GC content of 54.13% (Yangyong Lv et al., 2012). ***C. glutamicum*** can use a variety of carbon sources as growth and energy substrates, including sugars, sugar alcohols, organic acids and aromatic compounds (Becker et al., 2016). For information on taxonomical studies see Abe et al (1967) and Liebl (2005).

Although some ***Corynebacterium spp***. have been detected as components of the bacterial community of cheese surface (Monnet et al., 2006), only ***C. glutamicum*** is considered of relevance for industry feed and food production sectors.

4.2 Amino Acid Production

The global amino acid market is more than $US 7 billion and is forecast to reach $US 11.6 billion by the year 2015 and $US 35 billion by 2022 (Radiant Insights, Inc., 2015). Global volume consumption of feed grade amino acids, estimated at 4.5 million metric tons in 2017, is projected to reach 6.2 million metric tons by 2022. Poultry feed constitutes the largest consumer of feed amino acids globally with 2017 market share of 43.4% (Business Wire, 2017).

C. glutamicum has many fundamental physiological properties that make it an important industrial workhorse. These properties are listed by Lee et al (2016) as follows: (i) not pathogenic and generally recognized as a safe strain (GRAS); (ii) fast growth to high cell densities; (iii) genetically stable owing to the lack of a recombination repair system; (iv) limited restriction-modification system; (v) no autolysis and maintenance of metabolic activity under growth arrested conditions; (vi) low protease activity favoring recombinant protein production; (vii) plasticity of metabolism and strong secondary metabolism properties; and (viii) broad spectrum of carbon utilization (pentoses, hexoses, and alternative carbon sources); stress tolerance to carbon sources.

***C. glutamicum*'s inability to form spores, relatively few growth requirements and natural** capability to produce and secrete glutamate in high amounts makes it one of the most important platform microorganisms used for industrial production of amino acids. The practice of developing amino acid overproducing strains by mutagenesis and selection is a very well-established technique (Rowlands, 1984). Different strains have been utilized for decades by the industry to produce glutamate, lysine, tryptophan, threonine, isoleucine, **valine and leucine as described in the "Handbook of *Corynebacterium glutamicum*"** (Eggeling and Bott, 2005).

Amino acids have a wide variety of characteristics in terms of nutritional value, taste, medicinal action, and chemical properties, and thus have many potential uses, e.g., in food additives, feed supplements, pharmaceuticals, cosmetics, polymer materials, and agricultural chemicals (Ikeda and Takeno, 2013). Industrial amino acids produced by microorganisms are identical to those naturally found in vegetables and animals (Bercovici and Fuller, 1995).

Over the past decades, global competition among leading companies in the field steadily demanded innovation to improve key performance indicators: yield, titer, and productivity (Becker et al., 2016). For this reason, ***C. glutamicum*** has become one of the best characterized microorganisms worldwide with regard to substrate spectrum and nutrient

requirement (Buschke et al., 2013), catabolic and anabolic pathways and their regulation (Kalinowski et al., 2003; Schroder and Tauch, 2010) underlying biochemistry (Blombach and Seibold, 2010) and response to environmental conditions (Ehira et al., 2009).

4.2.1 Production methods

The two microbiological (biotechnology) methods for the industrial production of amino acids are the use of microbial enzymes or immobilized cells (enzymatic method) and fermentation (semi or direct) (Ivanov et al., 2013). The fermentation process is briefly addressed here to illustrate that the purification step within the fermentation process ensures a safe product.

Fermentation processes typically comprise three steps: fermentation, crude isolation and purification (Kusumoto, 2011; Ikeda and Takeno, 2013; Ivanov et al., 2013). In the fermentation process, the desired amino acid is specifically produced by the fermentation microorganism (e.g. ***C. glutamicum*** in the production of L-glutamine, L-lysine, L-valine). During the crude isolation process, most impurities contained in the fermentation broth are removed by combining various technologies. Final purification is performed to ensure the required quality for the intended use. The final product is obtained as a crystalline powder. The product is released only after quality tests have verified that the product meets specific requirements, and the normal functioning of each process step has been verified. All manufacturing processes to produce amino acids must comply with current good manufacturing practice requirements.

4.3 Other Uses

C. glutamicum is also employed in the production of L-phenylalanine (Shu and Liao, 2002), L-serine (Stolz et al. 2007) and for secreted protein production (Kikuchi et al., 2003; Umakoshi et al., 2011). The bacterium can be engineered for production of isobutanol (Blombach et al., 2011) and succinate (Litsanov et al., 2013).

Products for health and nutrition have the longest history in industrial biotechnology, with ***C. glutamicum*** being one of the major producers Meanwhile, processes for other products including non-proteinogenic amino acids, vitamins, flavors and fragrances and other nutrients and health care products are also on the rise (Burnett et al., 2013; Becker et al., 2016).

4.4 Genetic engineering

The past quarter century has seen rapid developments in strain development technology. Metabolic engineering has repeatedly led to successful yield improvements, especially in the field of amino acid production by ***C. glutamicum*** (Kirchner and Tauch, 2003; Eggeling and Bott, 2005; Wendisch, 2006; Becker and Whittmann, 2012; Zahoor et al., 2012; Burkovski, 2013; Buschke et al, 2013; Heider and Wendisch, 2015).

4.5 Safety Concerns

The species, ***C. glutamicum***, which serves as recipient and donor strain is generally considered to be non-pathogenic and no safety concerns are reported for this bacterial species for humans and animals. It is not known to produce toxins or present any other

hazards (Nelson et al., 2000; Kalinowski et al., 2003; Bernard, 2005; Olender, 2012; Oliviera et al., 2017).

As discussed in Section 2, ***C. glutamicum*** meets the EFSA premarket qualified presumption of safety (QPS) assessment criteria when used for fermentation of amino acids.

C. glutamicum is listed as a fermentation organism in several AAFCO feed ingredient definitions (e.g. 36.1, 36.16 and 36.17 (AAFCO 2016). Moreover, amino acids produced by an aerobic fermentation process using ***C. glutamicum*** are generally recognized as a safe (GRAS) for humans and food producing animals.

Due to its importance as an amino acid producer, ***C. glutamicum*** is one of the most-investigated and documented microorganisms (Jetten and Sinskey, 1995; Sahm et al., 1995, 2000; Krömer et al., 2004; Leuchtenberger et al. 2005; Dong et al., 2011; Schneider et al., 2011; Ikeda and Takeno, 2013; Lv et al., 2015; Hirasawa and Shimizu, 2016; Wendisch et al., 2016). Lee et al (2016) reviewed the literature and found that as of 2015 over 2,700 papers and 1,700 patents have been reported relating to ***C. glutamicum.*** The breadth and depth of research carried out on ***C. glutamicum*** substantiates the accepted safety of using this bacterium by the industry.

In addition to being used for the industrial production of amino acids, ***Corynebacterium spp***. have a long history of safe use in food production, including preparation of fermented maize, sorghum, millet, African oil bean seed, rice, soybean and cassava (Caplice and Fitzgerald, 1999; Tateno et al., 2007; Osungbaro, 2009).

4.5.1 Nonpathogenicity

Many of the genes present in the completely sequenced genome of ***C. glutamicum*** are highly conserved in sequence and gene order within the other members of the ***genus Corynebacterium*** (Ikeda and Nakagawa, 2003; Kalinowski ***et al*** 2003). As a non-pathogenic member of the genus, ***C. glutamicum*** is of increasing interest as a model organism for other members of the suborder including important pathogens such as ***C. diphtheriae, Mycobacterium tuberculosis*** and ***M. leprae*** (Camello et al., 2003; Gibson et al., 2003; Moeker et al., 2004; Olender et al., 2012; Tauch and Burkovski, 2015; Cashmore et al., 2017).

5. SUMMARY AND CONCLUSIONS

The data and scientific information presented in this document demonstrate that there are no known safety issues regarding the use of ***C. glutamicum*** in the production of compounds for use in food for humans and for food-producing animals. ***C. glutamicum*** is generally considered to be non-pathogenic and no safety concerns are envisaged. The ELS and follow-up selected literature reviews carried out did not reveal any hazards associated with C. glutamicum when added to food or feed. These findings agree with the EFSA QPS Opinions issued from 2005 onwards.

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7. APPENDIX 1

Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA

Scientific opinions for *C. glutamicum* for each year are extracted from the respective reference cited.

Year 2007

EFSA. 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 2007, 587:1-16.

Corynebacterium glutamicum

C. glutamicum is a soil bacterium widely used for the biotechnological production of amino acids. Amino acid producing strains have been selected and improved by mutagenesis as well as by using recombinant DNA technology. *C. glutamicum* belongs to a genus which also includes significant human pathogenic bacteria. Although some *Corynebacterium* species have been detected as components of the bacterial community of cheese surface, only *C. glutamicum* is considered of relevance for feed and food sectors. Only this species has been considered for the QPS assessment because of its significant role in the industrial production of amino acids.

Taxonomic unit defined

The genus *Corynebacterium* belongs to a branch of the *Actinomycetales* that also includes the genera *Mycobacterium*, *Nocardia* and *Rhodococcus.* Bacterial species belonging to this branch of the Gram-positive bacteria share particular characteristics, such as high G+C content (47– 74%) and a specific cell envelope organization, mainly characterized by the presence of peptidoglycan, arabinogalactan and mycolic acids. The genus currently contains 63 species, which colonize different environments.

Is the body of knowledge sufficient?

The characteristics, the physiology and the genetics of *C. glutamicum* are well known. The genome sequence of this industrial bacterium has been determined (Kalinowski et al., 2003), reflecting the considerable biotechnological importance of these organisms.

Are there safety concerns?

C. glutamicum plays an important role in the amino acid fermentation industry. No safety concerns are reported for this bacterial species for humans and animals, and no information on the presence of acquired antibiotic resistances in this bacterial species is available. However, it should be kept in mind that the direct exposure of consumers to this bacterial species is expected to be very low.

Can the safety concerns be excluded?

C. glutamicum has generally been considered to be non-pathogenic and no safety concerns are envisaged. However, its history of use is as a source of amino acids and has not, to date, involved the direct and deliberate exposure of humans or livestock.

Units proposed for QPS status

There is a long history of safe use of *C. glutamicum* as an amino acid producer; consequently, *C. glutamicum* is proposed for QPS status with the qualification that this status applies only when the species is used for production purposes only.

Year 2008

EFSA. 2008. Scientific Opinion of the Panel on Biological Hazards on a request from EFSA on the maintenance of the QPS list of microorganisms intentionally added to food or feed. EFSA Journal 2008, 923, 1-48.

Corynebacterium glutamicum

QPS status applies only when the species is used for production purposes.**Year 2010**

EFSA. 2010. EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2010 update). EFSA Journal 2010;8(12):1944. 56 pp.

Corynebacterium glutamicum

QPS recommendation only when the species is used for amino acid production.

Year 2011

EFSA. 2011. EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2011 update). EFSA Journal 2011;9(12):2497. 82 pp.

Corynebacteria

A literature review did not reveal new information about adverse health effects or on safety concerns since the last update (EFSA, 2010). The QPS recommendation has been confirmed.

Antimicrobial resistance aspects regarding the qualification

While no actual antibiotic MIC determinations for ***C. glutamicum*** appear to have been done, the antibiotic sensitivity of a strain used for amino acid production, has been tested using a disc method (Costa-Riu et al., 2003). The strain was sensitive to ampicillin, kanamycin, streptomycin, tetracycline, susceptible to gentamicin and resistant to norfloxacin, and chloramphenicol. However, the susceptibility test was not performed according to the methodology recommended by the CLSI guideline (Anonymous, 2007). There is no new information that would require a modification in the qualification of the antimicrobial resistance.

Year 2012

EFSA. 2012. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). EFSA Panel on Biological Hazards. EFSA Journal 2012, 10(12):3020. 84 pp.

Corynebacteria

A literature review did not reveal new information about adverse health effects or safety concerns with regards to the last update (EFSA, 2011). The QPS recommendation has been confirmed.

Antimicrobial resistance aspects regarding the qualification

While no actual antibiotic MIC determinations for ***C. glutamicum*** appear to have been done, the antibiotic sensitivity of a strain used for amino acid production, has been tested using a disc method (Costa-Riu et al., 2003). The strain was sensitive to ampicillin, kanamycin, streptomycin, tetracycline, gentamicin and resistant to norfloxacin, and chloramphenicol. The susceptibility test was not performed according to the methodology recommended by the CLSI guideline (CLSI, 2007). There is no new information that would require a modification in the qualification of the antimicrobial resistance.

Year 2013

EFSA. 2013. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA Panel on Biological Hazards. EFSA Journal 2013;11(11):3449, 107 pp.

Corynebacterium glutamicum

A literature review did not reveal new information about adverse health effects or safety concerns with regards to the last update (EFSA, 2012). The QPS recommendation has been confirmed.

Antimicrobial resistance aspects regarding the qualification

No new relevant information in the last year was published on the antimicrobial susceptibility or resistance of ***C. glutamicum,*** therefore no modifications in the qualification of the antimicrobial resistance are proposed.

Year 2017

EFSA. 2017. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. EFSA Journal 2017, 15(3):4664, 178 pp.

Corynebacterium glutamicum

Taxonomy

Since the last update on the QPS status (EFSA, 2013), no new information on the taxonomy of the *C. glutamicum* has been published.

Update of the body of knowledge on safety concerns

The total number of references found through the ELS was 188; after screening at title/abstract level, 33 passed to the full text phase; of those, two were considered relevant for the QPS assessment. A literature review did not reveal any new information about adverse health effects or safety concerns since the last update (EFSA, 2013).

Revision of antimicrobial resistance aspects

The involvement of class 1 integrons in the AMR towards streptomycin/spectinomycin and tetracycline in *C. glutamicum* isolates has been confirmed and reviewed by Deng et al. (2015). No additional relevant information was published in the last year on the antimicrobial susceptibility or resistance of *C. glutamicum*.

Update on other qualifications

This TU has the following qualification 'QPS only applies when the species is used for amino acid production'. Due to a lack of knowledge in relation to history of use of the viable organisms and because other members of the same genus are pathogenic, the qualification is confirmed.

Other relevant information

No new relevant information was identified.

Conclusion regarding a QPS recommendation

The QPS recommendation is confirmed for *C. glutamicum* as well as the qualification.

Year 2018

EFSA. 2018. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7: suitability of taxonomic units notified to EFSA until September 2017. EFSA Journal 2018, 16(1):5131, 43 pp.

Corynebacterium glutamicum

No safety concerns identified in the only article considered relevant for QPS exercise.

Year 2019

EFSA. 2019. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 10: suitability of taxonomic units notified to EFSA until March 2019. EFSA Journal 2019, 17(7):5753, 79 pp.

Corynebacterium glutamicum

A search for papers potentially relevant for the QPS consideration of Corynebacterium glutamicum provided 45 references. No paper reached the final selection phase, therefore no new safety concerns were identified.

8. APPENDIX 2

Electronic Literature Search for safety / risk assessment of *Corynebacterium glutamicum*

Project: Electronic Literature Search for safety / risk assessment of *Corynebacterium glutamicum*

An electronic literature search (ELS) on *Corynebacterium glutamicum* was conducted to collect studies, articles, reports and reviews that are deemed likely to be relevant for further safety / risk assessment of *Corynebacterium glutamicum*.

The search was conducted with the following information:

1. Name of the database searched: Google Scholar (https://scholar.google.co.in).
2. Dates on which the database searched: October 30-31, 2019.
3. Time period between which the database searched: Publications between 2003 and till date.
4. Other restrictions applied: Search terms present in 'allintitle' and 'anywhere' excluding patents and citations.
5. Languages searched: For pages written in any language.
6. Publications searched: Articles published in any peer reviewed journal; book or book chapters; theses; published reviews; etc.
7. Search strategy applied, and records retrieved: Recorded in Table 1.

Selection of articles: A stepwise exercise was performed to select articles that are deemed likely to be relevant for further safety / risk assessment of *Corynebacterium glutamicum* and the shortlisted articles were made available for the 'full review' at the end of ELS.

1. Step 1: Check if the word "Corynebacterium" is mentioned in title, keywords and/or abstract
2. Step 2: Check if the term "Corynebacterium glutamicum" is described in abstract
3. Step 3: Read the abstract
4. Step 4: Select articles for the 'full review' if abstract describes "Corynebacterium glutamicum" or "Corynebacterium spp" and at least some indicative information that the article covers either safety aspects; hazards / disease events in plant, animals and humans; toxin production; or carry genes for antimicrobial resistance. Further detailed evaluation on deemed likely to be included or excluded for the 'full review' was recorded in Table 2.

Table 1: Electronic Literature Search (ELS) Strategy and Retrieved Hits:

Strategy number	**Terms**	**Hits**	**Notes**
#1	allintitle: "Corynebacterium glutamicum"	2780	First 50 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#2	allintitle: "Corynebacterium"	4550	First 50 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#3	#2 resistance	53	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#4	#2 resistant	52	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#5	#2 antibiotic resistance	4	Both hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#6	#2 antibiotic resistant	4	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#7	#2 antimicrobial susceptibility OR susceptibilities	10	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#8	#2 infection OR infections	252	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#9	#2 abscess OR abscesses	36	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#10	#2 sepsis OR septic	22	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#11	#2 bacteremia OR bacteraemia	27	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#12	#2 toxic OR toxin OR toxins	42	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.

Strategy number	Terms	Hits	Notes
#13	#2 pathogen OR pathogenic OR pathogenicity	91	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#14	#2 opportunistic OR virulence OR virulent	50	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#15	#2 safety OR risk	28	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#16	#2 mutagenic OR mutagenicity	00	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#17	#2 toxicity OR toxicology	5	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#18	#2 clinical OR clinically	96	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#19	#2 death OR deaths	2	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#20	#2 morbidity OR morbidities	00	
#21	#2 mortality OR mortalities	2	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#22	#2 disease OR diseases	24	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#23	#2 illness OR illnesses	5	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#24	anywhere: "Corynebacterium glutamicum"	611	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.

Strategy number	Terms	Hits	Notes
#25	#24 resistance	453	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#26	#24 resistant	494	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#27	#24 antibiotic resistance	436	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#28	#24 antibiotic resistant	353	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#29	#24 antimicrobial susceptibility OR susceptibilities	269	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#30	#24 infection OR infections	271	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#31	#24 abscess OR abscesses	15	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#32	#24 sepsis OR septic	32	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#33	#24 bacteremia OR bacteraemia	18	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#34	#24 toxic OR toxin OR toxins	300	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#35	#24 pathogen OR pathogenic OR pathogenicity	296	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#36	#24 opportunistic OR virulence OR virulent	217	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.

Strategy number	Terms	Hits	Notes
#37	#24 safety OR risk	223	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#38	#24 mutagenic OR mutagenicity	39	First 10 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#39	#24 toxicity OR toxicology	205	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#40	#24 clinical OR clinically	252	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#41	#24 death OR deaths	219	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#42	#24 morbidity OR morbidities	28	First 10 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#43	#24 mortality OR mortalities	235	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#44	#24 disease OR diseases	355	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#45	#24 illness OR illnesses	43	First 10 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.

Table 2: Relevant References / Articles:

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
#1 / 2780	allintitle: "Corynebacterium glutamicum"	**Handbook of Corynebacterium glutamicum** Eggeling L, Bott M. CRC Press, 2005. ISBN: 9781420039696	Review / Exclude Not relevant to safety of C. glutamicum
		The Corynebacterium glutamicum genome: features and impacts on biotechnological processes agawa S. Applied Microbiology and Biotechnology, 2003. Vol. 62(2 – 3), pp 99 – 109.	Review / Exclude Not relevant to safety of C. glutamicum
		Comparative analysis of the Corynebacterium glutamicum group and complete genome sequence of strain R Yukawa H, et al. Microbiology, 2007. Vol. 153, pp. 1042 – 1058. doi: 10.1099/mic.0.2006/003657-0	Review / Exclude Not relevant to safety of C. glutamicum
		Deletion of the genes encoding the MtrA–MtrB two-component system of Corynebacterium glutamicum has a strong influence on cell morphology, antibiotics susceptibility and expression of genes involved in osmoprotection Möker N, et al. Molecular Microbiology, 2004. Vol. 54 (2), pp. 420 – 438.	Review / Exclude Not relevant to safety of C. glutamicum
#2 / 4550	allintitle: "Corynebacterium"	**The Corynebacterium glutamicum genome: features and impacts on biotechnological processes** M.Ikeda et al. Applied Microbiology and Biotechnology., 2003. Vol.62 (2-3), pp. 99 – 109.	Review / Exclude Not relevant to safety of C. glutamicum
		Several results repeated	
#3 / 53	allintitle: Corynebacterium resistance	**Analysis of Genes Involved in Arsenic Resistance in Corynebacterium glutamicum**	Review / Exclude Not relevant to safety

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		ATCC 13032 Efrén Ordóñez et al. Applied of Genes Involved in Arsenic Resistance in Corynebacterium glutamicum ATCC13032, 2005. Vol. 71(10), pp. 6206 – 6215.	of C. glutamicum
		A Corynebacterium glutamicum gene conferring multidrug resistance in the heterologous host Escherichia coli.W Jäger, et al. Journal of Biotechnology 1997. Vol. 179(7), pp. 2449 – 2451.	Review / Exclude Not relevant to C. glutamicum
		The alanine racemase gene alr is an alternative to antibiotic resistance genes in cloning systems for industrial Corynebacterium glutamicum strains Andreas Tauch, et al Journal of Biotechnology, 2002. Vol. 99(1), pp. 79 – 91.	Review / Exclude Not relevant to safety of C. glutamicum
		Mechanisms of Antibiotic Resistance in Corynebacterium spp. Causing Infections in People Olender A. 2012 https://www.intechopen.com/ https://cdn.intechopen.com/pdfs-wm/34699.pdf	Review / Exclude Not relevant to safety of C. glutamicum
		The identification and resistance analysis to 66 strains of corynebacterium clinical isolates Zhang LWZ. Chinese Journal of Laboratory Diagnosis, 2007. Vol. 7. http://en.cnki.com.cn/Article_en/CJFDTOTAL-ZSZD200707029.htm	Exclude (based on abstract; no translation of full paper)) Not relevant to safety of C. glutamicum
		Antimicrobial Resistance in Corynebacterium spp., Arcanobacterium spp., and Trueperella pyogenes. Feßler AT, Schwarz S. Microbiology Spectrum, 2017. Vol. 5(6). DOI: 10.1128/microbiolspec.ARBA-	Review / Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		0021-2017	
		Extracytoplasmic function sigma factor σD confers resistance to environmental stress by enhancing mycolate synthesis and modifying peptidoglycan structures in Corynebacterium glutamicum Koichi Toyoda, Toyoda K, Masayuki I. Molecular Microbiology, 2018. Vol. 107 (3), pp. 312 – 329.	Review / Exclude Not relevant to safety of C. glutamicum
		Phenotypic and genotypic characterization of high-level macrolide and lincosamide resistance in Corynebacterium species in Canada and the distribution of the ermX resistance determinant among Corynebacterium species Singh, Cathleen. Theses, 2010.	Review / Exclude Not relevant to safety of C. glutamicum
		A National Survey of Multi-Drug Resistance in Ophthalmic Clinical Isolates of Corynebacterium in Japan Eguchi H, et al., Investigative Ophthalmology and Visual Science, 2008. Vol.49, pp. 5530	Review / Exclude Not relevant to safety of C. glutamicum
		Several results repeated	
#4 / 52	allintitle: Corynebacterium resistant	**Feedback-resistant acetohydroxy acid synthase increases valine production in Corynebacterium glutamicum**Veronika Elišáková, et al. Genetics and Molecular Biology, 2005.,pp 207 – 213.	Review / Exclude Not relevant to safety of C. glutamicum
		Co-expression of feedback-resistant threonine dehydratase and acetohydroxy acid synthase increase l-isoleucine production in Corynebacterium glutamicum Author links open overlay panelLianghongYin. et al.	Review / Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		Metabolic Engineering, 2012. Vol. 14 (5), pp.542 – 550.	
		Corynebacterium resistens sp. nov., a New Multidrug-Resistant Coryneform Bacterium Isolated from Human Infections Yoshihito Otsuka, et al. Journal of Clinical Microbiology, 2005. Vol. 43 (8), pp 3713 – 3717.	Review / Exclude Not relevant to C. glutamicum
		Adaptive evolution of Corynebacterium glutamicum resistant to oxidative stress and its global gene expression profilingJY Lee, et al. Biotechnology Letters, 2013. Vol. 35 (5), pp 709 – 717.	Review / Exclude Not relevant to safety of C. glutamicum
		Genetic and biochemical characterization of Corynebacterium glutamicum ATP phosphoribosyltransferase and its three mutants resistant to feedback inhibition by histidine Yun Zhang, et al. Japanese Journal of Infectious, 2012. Vol. 94(3). Pp 829-838	Review / Exclude Not relevant to safety of C. glutamicum
		Characteristics of Multidrug-resistant Corynebacterium spp. Isolated from Blood Cultures from Hospitalized Patients in Japan Liang Qin, et al. Japanese Journal of Infectious Diseases, 2017. Vol.70(2), pp.152-157	Review / Exclude Not relevant to safety of C. glutamicum
		Generation of branched-chain amino acids resistant Corynebacterium glutamicum acetohydroxy acid synthase by site-directed mutagenesis Guo Y, et al. Biotechnology and Bioprocess Engineering, 2014. Vol. 19(3), pp. 456 – 467.	Review / Include Article discusses antibiotic resistance.
		Few results repeated	

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
#5 / 4	allintitle: Corynebacterium antibiotic resistance	Results repeated	
#6 / 4	allintitle: Corynebacterium antibiotic resistant	none	
#7 / 10	allintitle: Corynebacterium antimicrobial susceptibility OR susceptibilities	**Antimicrobial Susceptibility and Species Identification of Corynebacterium spp. Strains Collected in Europe and USA Medical Centers (2006-2010)** Sader HS, et al. Sentry Antimicrobial Surveillance, 2012. P1092 ECCMID 2012 JMI Laboratories North Liberty, IA, USA	Review / Exclude Not relevant to safety of C. glutamicum
		Few results repeated	
#8 / 252	allintitle: Corynebacterium infection OR infections	**Idiopathic Granulomatous Mastitis Associated with Corynebacterium Sp. Infection** Creed Michael Stary, et al. Hawai'I Medical Journal, 2011. Vol.70 (5), pp. 99 –101.	Review / Exclude Not relevant to safety of C. glutamicum
		Corynebacterium-associated skin infections Blaise G, et al. International Journal of Dermatology, 2008. Vol. 47 (9), pp. 884 – 890.	Review / Exclude Not relevant to safety of C. glutamicum
		Corynebacterium Species Isolated from Bone and Joint Infections Identified by 16S rRNA Gene Sequence Analysis Raoult D, et al. J. Clin. Microbiol., 2004. Vol. 42 (5), pp. 2231 – 2233.	Review / Exclude Not relevant to safety of C. glutamicum
		Case of erythema nodosum associated with granulomatous mastitis probably due to Corynebacterium infection Kubo Y, et al. The Journal of Dermatology, 2014. Vol. 41(9), pp. 821 – 823.	Review / Exclude Not relevant to safety of C. glutamicum
		[Wound infections due to	Review / Exclude

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		opportunistic corynebacterium species] Olender A, Łetowska I. Medycyna Doswiadczalna i Mikrobiologia, 2010. Vol. 62 (2), pp. 135 – 140.	(based on abstract; no translation of full paper)) Not relevant to safety of C. glutamicum
		Identification of Corynebacterium spp. isolated from bovine intramammary infections by matrix-assisted laser desorption ionization-time of flight mass spectrometry dos Santos MV, et al. Veterinary Microbiology, 2014. Vol. 173 (1 – 2), pp. 147 – 151.	Review / Exclude Not relevant to safety of C. glutamicum
		Ocular Infections Caused by Corynebacterium Species Eguchi H. Infection Control, 2013. Dr. Silpi Basak (Ed.), In Tech, DOI: 10.5772/56214.	Review / Exclude Not relevant to safety of C. glutamicum
		Hardware Infection with Corynebacterium spp.: a Case Report and Review of the Literature Clarridge III JE, et al. Clinical Microbiology Newsletter, 2014. Vol. 36(2), pp. 9 – 13.	Review / Exclude Not relevant to safety of C. glutamicum
		Cerebrospinal fluid shunt infection caused by Corynebacterium sp: Case report and review Randi BA, et al. Brain Injury, 2014. Vol. 28(9), pp. 1223 – 1225.	Review / Exclude Not relevant to safety of C. glutamicum
		Transmission dynamics of intramammary infections caused by Corynebacterium species Delen G, et al. Journal of Dairy Science, 2018. Vol. 101 (1), pp. 472 – 479.	Review / Exclude Not relevant to safety of C. glutamicum
		Modelling and dynamics of intramammary infections caused by Corynebacterium species	Review / Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		Rachah A, et al. 7th International Conference on Modeling, Simulation, and Applied Optimization (ICMSAO), 2017. Conference proceedings.	
		Few results repeated	
#9 / 36	allintitle: Corynebacterium abscess OR abscesses	none	
#10 / 22	allintitle: Corynebacterium sepsis OR septic	none	
#11 / 27	allintitle: Corynebacterium bacteremia OR bacteraemia	none	
#12 / 42	allintitle: Corynebacterium toxic OR toxin OR toxins	none	
#13 / 91	allintitle: Corynebacterium pathogen OR pathogenic OR pathogenicity	**Corynebacterium occurance and pathogenicity for humans and animals** Banaszkiewicz T, Krukowski H. Medycyna Weterynaryjna, 2011. Vol.67 No.4 pp.229-232	Exclude (based on abstract; no translation of full paper)) Not relevant to safety of C. glutamicum
		Insight of Genus Corynebacterium: Ascertaining the Role of Pathogenic and Non-pathogenic Species Oliveira A, et al. Front. Microbiol., 2017. https://doi.org/10.3389/fmicb.2017.01937	Review / Exclude Not relevant to safety of C. glutamicum
		Few results repeated	
#14 / 50	allintitle: Corynebacterium opportunistic OR virulence OR virulent	**Molecular armory or niche factors: virulence determinants of Corynebacterium species** Olender A, Łetowska I Microbiology Letters, 2010. Vol. 62(2), pp.135-140	Review / Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		Few results repeated	
#15 / 28	allintitle: Corynebacterium safety OR risk	**Safety and efficacy of L arginine produced by Corynebacterium glutamicum KCTC 10423BP for all animal species** EFSA. EFSA Journal, 2016. DOI: 10.2903/j.efsa.2016.4345	Review / Include Assessment reviews safety, efficacy and toxicity
		Scientific Opinion on the safety and efficacy of L-valine produced by Corynebacterium glutamicum (KCCM 80058) for all animal species, based on a dossier submitted by CJ Europe GmbH EFSA. EFSA Journal, 2013. DOI: 10.2903/j.efsa.2013.3429	Review / Include Assessment reviews safety, efficacy and toxicity
		Safety and efficacy of l-arginine produced by Corynebacterium glutamicum KCCM 80099 for all animal species EFSA. EFSA Journal, 2017. DOI: 10.2903/j.efsa.2017.4858	Review / Include Assessment reviews safety, efficacy and toxicity
		Opinion of the Panel on additives and products or substances used in animal feed (FEEDAP) on the safety and efficacy of the product containing L-arginine produced by fermentation from Corynebacterium glutamicum (ATCC-13870) for all animal species EFSA. EFSA Journal, 2007. DOI: 10.2903/j.efsa.2007.473	Review / Include Assessment reviews safety, efficacy and toxicity
		Scientific Opinion on the safety and efficacy of L-valine (ValAMINO®) produced by Corynebacterium glutamicum (DSM 25202) for all animal species, based on a dossier submitted by Evonik Industries AG EFSA. EFSA Journal, 2014. DOI:	Review / Include Assessment reviews safety, efficacy and toxicity

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		10.2903/j.efsa.2014.3795	
		Scientific Opinion on the safety and efficacy of L-lysine monohydrochloride, technically pure, produced with Escherichia coli CGMCC 3705 and L-lysine sulphate produced with Corynebacterium glutamicum CGMCC 3704 for all animal species, based on a dossier submitted by HELM AG EFSA. EFSA Journal, 2015. DOI: 10.2903/j.efsa.2015.4156	Review / Include Assessment reviews safety, efficacy and toxicity
		Safety of concentrated l-lysine (base), l-lysine monohydrochloride and l-lysine sulfate produced using different strains of Corynebacterium glutamicum for all animal species based on a dossier submitted by FEFANA asbl EFSA. EFSA Journal, 2019. DOI: 10.2903/j.efsa.2019.5532	Review / Include Assessment reviews safety, efficacy and toxicity
		Safety and efficacy of l-lysine monohydrochloride and concentrated liquid l-lysine (base) produced by fermentation using Corynebacterium glutamicum strain NRRL B-50775 for all animal species based on a dossier submitted by ADM EFSA. EFSA Journal, 2019. DOI: 10.2903/j.efsa.2019.5537	Review / Include Assessment reviews safety, efficacy and toxicity
		Safety and efficacy of l-arginine produced by fermentation using Corynebacterium glutamicum KCCM 10741P for all animal species EFSA. EFSA Journal, 2018. DOI: 10.2903/j.efsa.2018.5277	Review / Include Assessment reviews safety, efficacy and toxicity

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		Safety and efficacy of l-arginine produced by fermentation with Corynebacterium glutamicum KCCM 80182 for all animal species EFSA. EFSA Journal, 2019. DOI: 10.2903/j.efsa.2019.5696	Review / Include Assessment reviews safety, efficacy and toxicity
		Safety and efficacy of l-histidine monohydrochloride monohydrate produced using Corynebacterium glutamicum KCCM 80172 for all animal species EFSA. EFSA Journal, 2019. DOI: 10.2903/j.efsa.2019.5783	Review / Include Assessment reviews safety, efficacy and toxicity
		Few results repeated	
#16/ 0	allintitle: Corynebacterium mutagenic OR mutagenicity		
#17 / 5	allintitle: Corynebacterium toxicity OR toxicology	**Transcriptomic analysis of Corynebacterium glutamicum in the response to the toxicity of furfural present in lignocellulosic hydrolysates** Park HS, et al. Process Biochemistry, 2015. Vol. 50(3), pp. 347 – 356.	Review / Exclude Not relevant to safety of C. glutamicum
#18 / 96	allintitle: Corynebacterium clinical OR clinically	**The clinical course of peritoneal dialysis-related peritonitis caused by Corynebacterium species** Szeto CC, et al. Nephrology Dialysis Transplantation, 2005. Vol. 20 (12), pp. 2793 – 2796. https://doi.org/10.1093/ndt/gfi123	Review / Exclude Not relevant to safety of C. glutamicum
		Nondiphtherial Corynebacterium species isolated from clinical specimens of patients in a university hospital, Rio de Janeiro, Brazil Camello TCF, et al. Braz. J.	Review / Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		Microbiol., 2003. Vol. 34 (1).	
		Antibiotic susceptibility of Corynebacterium isolated from clinical specimens Chen D, et al. Chinese Journal of Clinical Laboratory Science, 2011. Vol. 3	Review / Exclude Not relevant to safety of C. glutamicum
		Relationship Between Susceptibility to Quinolones in Corynebacterium Ophthalmic Clinical Isolates and the GyrA Gene Mutations Katome T, et al. Investigative Ophthalmology & Visual Science, 2008. Vol. 49 (13).	Review / Exclude Not relevant to safety of C. glutamicum
		Relationship Between Mutations in the gyrA Gene and Quinolone Resistance in Ophthalmic Clinical Isolates of Corynebacterium Species Eguchi H, et al., Investigative Ophthalmology & Visual Science, 2006. Vol. 47 (13), pp. 3566.	Review / Exclude Not relevant to safety of C. glutamicum
		Endophthalmitis Caused by Corynebacterium Species: Clinical Features, Antibiotic Susceptibility, and Treatment Outcomes Kuriyan AE, et al. Ophthalmology retina, 2017. Vol. 1 (3), pp. 200 – 205.	Review / Exclude Not relevant to safety of C. glutamicum
#19 /2	allintitle: Corynebacterium death OR deaths	none	
#20/ 0	allintitle: Corynebacterium morbidity OR morbidities	none	
#21 / 2	allintitle: Corynebacterium mortality OR mortalities	**Biodegradation of Contaminated Environments Using Corynebacterium glutamicum and Its Application to Livestock Mortalities Burials** [rest of the details are in Chinese]	Exclude (based on abstract; no translation of full paper)) Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
#22 / 24	allintitle: Corynebacterium disease OR diseases	**Corynebacterium species and coryneforms: An update on taxonomy and diseases attributed to these taxa** Bernard K. Clinical Microbiology Newsletter, 2005. Vol. 27(2), pp 9 – 18. DOI: https://doi.org/10.1016/j.clinmicnews.2005.01.002.	Exclude Not relevant to safety of C. glutamicum
#23 / 5	allintitle: Corynebacterium illness OR illnesses	none	
#24 / 611	anywhere: "Corynebacterium glutamicum"	Few results repeated	
#25 / 453	anywhere: "Corynebacterium glutamicum" resistance	none	
#26 / 494	anywhere: "Corynebacterium glutamicum" resistant	none	
#27 / 436	anywhere: "Corynebacterium glutamicum" antibiotic resistance	none	
#28 / 353	anywhere: "Corynebacterium glutamicum" antibiotic resistant	**Drivers of bacterial genomes plasticity and roles they play in pathogen virulence, persistence and drug resistance** Patel S. Infection, Genetics and Evolution, 2016. Vol. 45, pp. 151 – 164.	Exclude Not relevant to safety of C. glutamicum
#29 / 269	anywhere: "Corynebacterium glutamicum" antimicrobial susceptibility OR susceptibilities	none	
#30 / 271	anywhere: "Corynebacteriu	none	

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
	m glutamicum" infection OR infections		
#31 / 15	anywhere: "Corynebacterium glutamicum" abscess OR abscesses	Corynebacterium ulcerans, an emerging human pathogen Hacker E, et al. Future Microbiology, 2016. Vol. 11 (9). https://doi.org/10.2217/fmb-2016-0085	Exclude Not relevant to C. glutamicum
#32 / 32	anywhere: "Corynebacterium glutamicum" sepsis OR septic	none	
#33 / 18	anywhere: "Corynebacterium glutamicum" bacteremia OR bacteraemia	none	
#34 / 300	anywhere: "Corynebacterium glutamicum" toxic OR toxin OR toxins	none	
#35 / 296	anywhere: "Corynebacterium glutamicum" pathogen OR pathogenic OR pathogenicity	none	
#36 / 217	anywhere: "Corynebacterium glutamicum" opportunistic OR virulence OR virulent	none	
#37 / 223	anywhere: "Corynebacterium glutamicum" safety OR risk	none	
#38 / 39	anywhere: "Corynebacterium glutamicum" mutagenic OR mutagenicity	none	
#39 /	anywhere:	none	

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
205	"Corynebacterium glutamicum" toxicity OR toxicology		
#40 / 252	anywhere: "Corynebacterium glutamicum" clinical OR clinically	none	
#41 / 219	anywhere: "Corynebacterium glutamicum" death OR deaths	none	
#42 / 28	anywhere: "Corynebacterium glutamicum" morbidity OR morbidities	none	
#43 / 235	anywhere: "Corynebacterium glutamicum" mortality OR mortalities	none	
#44 /355	anywhere: "Corynebacterium glutamicum" disease OR diseases	none	
#45 / 43	anywhere: "Corynebacterium glutamicum"	none	

Cerrito, Chelsea

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Monday, July 27, 2020 3:00 PM
To: Tang, Lei; Wong, Geoffrey K; Animalfood-premarket
Cc: '강민경님 [Min Kang]'; Keith D. Haydon; Biesiada,Thomas님
Subject: GRAS AGRN 35 --AMENDMENT --Email 1
Attachments: CJ-FDA AMENDMENT GRN 35 July 27 2020.pdf; ICH Guidelines Q2(R1) Validation.pdf; iso 17043 ref En value.pdf; Kong and Adeola AAJAS 27-917.pdf; Parsons AFST 59-147.pdf

Dr. Tang:

On behalf of CJ, I am providing the amendment of AGRN 35, L-Valine fermentation product , as requested. This email provides a part of the supporting material for this amendment.

You will be receiving a series of emails, to assure that all supplements (attachments) are received. I will number them in the subject line of the email, as well as provide a brief description in the body of each email. You should receive 4 reference files and 15 supplements (attachments).

This email will include the signed amendment letter as well as all reference publications.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

RECEIVED DATE
JUL 28, 2020

Ph. 703-590-7337
Cel (b) (6)
Fax 703-580-8637

From: Tang, Lei [mailto:Lei.Tang@fda.hhs.gov]
Sent: Wednesday, July 08, 2020 12:02 PM
To: Kristi Smedley
Cc: Wong, Geoffrey K
Subject: RE: GRAS AGRN 35

Dear Dr. Smedley,

This letter is in response to your email dated July 3, 2020 requesting for meeting minutes from the July 1, 2020 meeting between the Center for Veterinary Medicine and CJ CheilJedang Corporation.

Please find enclosed a copy of our meeting minutes for the referenced meeting.

If you have any questions concerning this letter, please contact me via email at Lei.Tang@fda.hhs.gov or by phone at 240-402-5922. Please refer to AGRN #35 in any future correspondences.

Sincerely,

Lei Tang, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal feeds
U.S. Food and Drug Administration

Tel: 240-402-5922
lei.tang@fda.hhs.gov

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From: Kristi Smedley <smedley@cfr-services.com>
Sent: Friday, July 3, 2020 9:32 AM
To: Tang, Lei <Lei.Tang@fda.hhs.gov>
Cc: Wong, Geoffrey K <Geoffrey.Wong@fda.hhs.gov>; Keith D. Haydon <keith.haydon@cj.net>; '강민경님 [Min Kang]' <mg.kang@cj.net>
Subject: RE: GRAS AGRN 35

Dr. Tang

This is a request for the notes of the FDA teleconference (July 1, 2020) specific to issues raised during the AGRN 35 review.

We will accept these notes by email.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

Centre for Regulatory Services, Inc.
5200 Wolf Run Shoals Road Woodbridge, VA 22192-575.5
703 590 7337 (Fax 703 580 8637)
Smedley@cfr-scrviccs.com

July 27, 2020

Dr. David Edwards Director
Division of Animal Feeds (HFV- 230)
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Pl.,
Rockville, MD 20855

Subject: Amendment AGRN 35
L-Valine Fermentation Product

Notifier: CheilJedang Corporation (CJ)
330, Dongho-Ro,
Jung-Gu, SEOUL,04560, KOREA

Dear Dr. Edwards:

On behalf of CheilJedang Corporation, I am providing an amendment to the AGRN 35, as discussed in our teleconference on July 1, 2020. In that teleconference, (which was summarized in a memo dated July 8, 2020 and received by email on July 8, 2020) we discussed the listed items, below. We were requested to respond in a two-week period after receiving the notes of meeting. However, by email (July 15, 2020) CVM agreed to extend that deadline 3 working days to July 27, 2020.

1. Identity, method of manufacture and specification

a. Identity of the notified substance

The notified substance is dried L-valine fermentation product containing minimum 72% of L-valine. However, CVM pointed out that in Appendix 4, the notifier states multiple times that the finished product contains a minimum of 72% L-valine Fermentation Product. CVM asked the notifier to clarify this discrepancy. The notifier confirmed that the notified substance is dried L-valine fermentation product containing at least 72% of L-valine. The notifier agreed to revise the Appendix 4 and ensure the description of the notified substance is consistent throughout the notice.

▶ The notified substance is dried L-Valine Fermentation Product (L-VFP) containing a minimum 72% of L-valine. However, CVM pointed out that in Appendix 4 there were a number of misrepresentation of the product. CJ has reviewed Appendix 4, and made the appropriate revisions such that it is clear that the notified substances is dried L-Valine Fermentation Product containing a minimum 72% of L-valine. The REVISED Appendix 4 is found in [Supplement #1].

b. Starting materials

The notice contains a statement that the quality of starting raw materials is based on feed grade specifications which are suitable for use in the manufacture of feed. However, the identity and specifications of the starting raw materials are not provided in the notice. CVM asked the notifier to provide a list of starting raw materials (including antifoam substance) the regulatory status and specifications for all starting raw materials. The notifier agreed to provide requested information.

▶ CVM requested a listing of all starting materials and the specifications. CVM indicated that certifying the feed grade status was not sufficient. We have provided a listing of raw materials and the purchasing specifications. We are reiterating our certification that starting materials are suitable for the manufacture of a feed grade substance. See [Supplement #2] for the raw material list and the purchasing specifications.

c. Composition

CVM pointed out that the footnote to the Table 2-1 (Chemical composition) indicates that the carrier can be any one of the following: Starch, Dextrin, Corn gluten meal, Soybean mill run, or Corncob. Because the reported composition is determined for the finished L-valine fermentation product including the carrier, it is not clear that the constituent contents of the carrier do not contribute to the quantified constituent contents. For example, if soybean mill run is used, then the reported amino acids content of L-valine product includes the contribution from the protein content in the carrier soybean mill run. When the carrier content is added as 6% by manufacture calculation in the final composition table, the contribution of carrier, soybean mill run, is counted twice in the final composition table. CVM asked the notifier to provide the identity, specifications, and composition of the carrier used in the manufacture of the batches that were analyzed to demonstrate the composition of the L-valine fermentation product, and to clarify whether the constituent contents of the carrier contribute to any quantified contents of the constituents of the L-valine fermentation product. The notifier agreed to clarify the identity of the carrier and account for any constituents in the carrier that are in common with the quantified constituents. CVM also suggested that in the future submission, the notifier should consider performing the compositional analyses on the fermentation product before it is formulated into the final product to be marketed.

▶ We apologize for the fact that the Table 2.1 Chemical Composition was not clear. The data found in this table was a summary of data provided in Appendix 1 of the notice. The analysis of the L-Valine Fermentation Product (Appendix 1) included a (b) (4) of the L-Valine Fermentation Product. (The certificate of analysis for the corn starch is provided in [Supplement #3] of AGRN 35 amendment). (b) (4)

(valine) levels of L-Valine Fermentation Product. The line "carrier" should not have been included in the Table 2.1 Chemical Composition. We have revised Table 2.1 as found in [Supplement #4].

d. Specifications

CVM pointed out that the reported ash contents from five batches are in a tight range of 2.87% - 2.94%. CVM explained that the product specifications should be established based on the batch analyses and asked the notifier to justify the ash specification as 5%. CVM also pointed out that higher ash content may indicate higher amount of mineral salts in the final product. In the justification, CVM asked the notifier to also include an explanation why heavy metals from the higher amounts of mineral salts are not a safety concern. The notifier agreed to provide the requested justification for the ash specification and include an explanation for why heavy metals

will not be a safety concern.

▶ CVM questioned the ash specification for the L-Valine Fermentation Product (L-VFP). The notifier has modified Table-2-4 ash specification to 4%

REVISED Table-2-4: L-Valine Fermentation Product

Component	Amount	Method
Valine, minimum	72%	HPLC (Appendix 1)
Moisture, maximum	5%	AOAC 934.01
Ash, maximum	4%	AOAC 942.05

As requested we decreased the ash specification. CVM was interested in understanding the impact of the specification on heavy metals. As the production of L-Valine Fermentation Product is a closed system and the starting materials are tightly controlled we are able to assure that the product has minimal (and safe) level of heavy metals. See [Supplement #5] for the assessment of 3 batches.

e. Potential impurities/contaminants

CVM pointed out that for the heavy metal analyses, the Limit of Detection (LOD) for mercury was reported as 0.000 mg/kg. CVM asked the notifier to clarify why the LOD for mercury is 0.000 mg/kg. The notifier agreed to provide an explanation.

▶ CVM questioned the Limit of Detection (LOD) for mercury. The certificate of analysis reported the LOD units as mg/kg, but the actual validation unit for mercury was µg/kg. The report was reissued with the corrected test result. (The report function rounded the 0.150 µg/kg to "0.000mg/kg".) Thank you for bringing this error to our attention. The REVISED Appendix 2 is found in [Supplement #5].

f. The HPLC method to determine L-Valine content in the finished fermentation product – CJ BIO-06:2018

CVM appreciated that a method validation report is included in the notice. However, the method procedures are not provided. The notifier agreed to include the method procedures in the amendment.

▶ When CJ was reviewing the validation of the L-Valine method CJ BIO-06:2018, we noted a few corrections were needed due to typos and calculations error. The revised validation report [Supplement #7] is correct. We have provided a second report that describes each change to the validation report in [Supplement #9].

CVM explained the following issues in the provided method validation report:

• Identity

CVM asked the notifier to clarify how the L-valine product sample was prepared for the identity test using LC-MS/MS and FT-IR.

▶ For LC-MS/MS identification, to prepare 0.1 g/L (on the basis of L-valine concentration) sample solution, 0.14 g of Dried L-Valine Fermentation Product was added to a 1 L volumetric flask and make up to volume with ultra-pure water. ([Supplement #6], pages 5~7)

For FT-IR identification, the test substance, Dried L-Valine Fermentation Product includes L-valine and residual biomass. In order to conduct the identification analysis, a sample was taken prior to adding carrier to the fermentation broth (identification test ONLY). Other impurities in

the test substance were removed by column separation with SCX column (Ionosphere 5C). The eluent for column separation was 10 mM KH2PO4 for mobile phase (A) and 10 mM KH2PO4 with 500 mM KCl in 5% acetonitrile for mobile phase (B). The gradient was applied as following: maintained only mobile phase (A) for 5 min; 40% of mobile phase (B) in mobile phase (A) until 10 min. Eluted sample fraction at the second gradient phase was collected. For sample preparation of ATR-FT-IR, 20 g/L standard solution was prepared and 28 g/L sample solution was prepared then 100 µL sample was loaded onto the column.
After this step, salt from the mobile phase was removed by using OASIS C18 cartridge column (WATERS) with 50% acetonitrile as an elution buffer.
Same purification process was also carried out for standard solution. Both standard and sample solution were dried using SpeedVac then FT-IR analysis was performed. A detailed description can be found in [Supplement #6], page 6.

• **Stability of the mobile phase**
CVM asked the notifier to clarify whether this test was conducted to demonstrate the stability of the HPLC mobile phase or the stability of the prepared sample solutions ready to be analyzed by HPLC. CVM noted that samples were tested at 0, 6, 12, 18, 24, 30, 36, 42, and 48 hours, but the sample storage conditions were not described. CVM asked the notifier to clarify that sample storage condition.

▶ This test was conducted to observe the stability of the prepared sample solution. Samples were stored in the refrigerator (4°C). ([Supplement #7], page 7)

• **Accuracy**
CVM explained that finished L-Valine Fermentation Product (L-VFP) should be used to demonstrate the method accuracy. CVM asked the notifier to clarify what is the Certified Reference Material PHR1172 used in the accuracy test. In addition, CVM asked the notifier to provide reference(s) for the data analysis approach used in the accuracy test so the reviewer can fully understand the calculations described on pages 17-20 of the Appendix 1_ Attachment 2 (Method validation report). Or the notifier can refer to CVM's Guidance for Industry #64 on how to test for method accuracy.

▶ Certified Reference Material PR1172 is a guaranteed L-valine by Sigma Corporation and the Certificate of Analysis can be found in [Supplement #11].

In accordance with 'ICH guideline, Validation of Analytical Procedures: Text and Methodology Q2 (R1)', an accuracy test was conducted. The ICH guidance referenced is nearly identical to the VICH GFI 64, and provides under the heading of Accuracy (4.1.1(a) "application of an analytical procedure to an analyte of known purity (e.g., reference material). This is the option CJ used. The quantitative results for accuracy used the method as found in ISO 17043 (General requirements for proficiency testing) ,The details of the calculations are described in [Supplement #7], pages 12~23.

• **Robustness**
CVM asked the notifier to clarify what samples were used in the robustness test.

▶ The samples of robustness test used Dried L-Valine Fermentation Product (Lot.VAL180116).

• **L-valine retention time shift**
CVM asked the notifier to provide a justification on observed L-valine retention time variations between 5.4 min and 6.2 min on different testing days.

▶ Accuracy test was the last test in this validation and the retention time of this test was little shifted forward. This phenomenon could have occurred due to column age, pH, or acetonitrile ratio of the mobile phase. Because the CRM arrived later than expected, we performed the

accuracy testing almost 1 month later.

However, robustness of this method was confirmed and it showed favorable recovery rate for all factors. The time shift of retention time in the accuracy test was shorter than in the robustness test. ([Supplement #7], page12~13)

• **Extra peaks**

CVM pointed out that there are two extra peaks at about 3.9 min and 7.3min in some of the chromatograms provided in the notice. CVM asked what the sources for these two peaks are.

▶ A peak at retention time 3.9 min came from the eluent of a second previously injected sample. When the analysis time was extended, this peak appeared around at 20.1 min. was confirmed as phenylalanine. The other peak at 7.3min was confirmed as tyrosine. The detail description is found in [Supplement #10].

2. *Intended effect/Utility*

a. Clarification of intended use of the substance

CVM pointed out that the descriptions of the target animal species are not consistent throughout the notice. CVM asked the notifier to clarify if the target animal species is poultry and swine, livestock and poultry, or just poultry. The notifier confirmed that the target animal species is livestock and poultry.

▶ As stated in the signed certification (section 1.8) and the header for section 1 of the GRAS notice, "CJ CheilJedang Corporation (hereinafter referred to as "CJ") is submitting a GRAS notice for the substance Dried L-Valine Fermentation Product as a source of L-Valine in livestock and poultry diets". We apologize for using the common term "animal" to describe the intended use.

b. Extrapolation argument

CVM explained that if the target animal species is different than poultry, the notifier should provide an extrapolation argument discussing why data from poultry can be extrapolated to other animal species. The notifier agreed to provide an extrapolation argument.

▶ The intent of demonstrating bioavailability of essential nutrients is to provide data that the manufacture of the essential nutrient and its composition does not impact the bioavailability of the essential nutrient for the animal. Some have expressed a concern that residual biomass when left in final product may impact the essential nutrient bioavailability. Because of this issue, CJ provided an *in vivo* test to demonstrate that the limited biomass in the GRAS substance did not impact the L-Valine bioavailability. The model chosen (growing poultry) has been demonstrated to be an effective model to discern the limitation of nutrient availability.

In their review, Kong and Adeola (2014) stated that bioavailability studies (which cover digestion, absorption, and utilization) is considered the absolute standard for estimating bioavailability of amino acid compared to other methods. CJ completed and published a 28-day study using a broilers model (Wensley et.al, 2019). The study demonstrates that there was no impact of the biomass (28%) on L-Valine bioavailability of the GRAS substance. This model suggests that the *C. glutamicum* biomass did not impact the bioavailability of L-valine in the L-Valine Fermentation Product as it provided similar (P>.05) biological response (growth and feed utilization) as the 98.5% L-Valine control diet. Swine bioavailability of L-valine of a L-Valine Fermentation Product containing approximately 35% *C. glutamicum* biomass was confirmed by in a recent report by Oliveira et.al. (2019), as provided in AGRN 35. Also, Parsons (1996)

review of digestible amino acids in poultry and swine reported positive correlation between cecetomized roosters and ileal-cannulated pigs. However, as Kong and Adeola (2014) noted digestibility is only one factor when assessing bioavailability. Biological responses as provided in AGRN 35 would be the best indicator of biomass interference with L-valine bioavailbility in the GRAS substance. As the AGRN 35 pointed out, other *C. glutamicum* amino acids sources (specifically lysine) has been assessed for bioavailability (AAFCO definition 36.15). There is no concern for this lysine source as a suitable additive for use in livestock, poultry and aquaculture. When feeding ruminants amino acids; the bacteria rich rumen typically consumes the amino acids and building microbial proteins that are digested and absorbed later down the gastrointestinal tract.

The data presented in AGRN 35, positively demonstrates the L-Valine Fermentation Product is a bioavailable source of L-Valine for the intended use in livestock and poultry.

3. Target animal safety

CVM pointed out that the notice did not include information on the analysis of biogenic amines. CVM asked the notifier to analyze potential biogenic amines in the fermentation media of the Page **4** of **6,** GRAS Notice M000087Z0002, Dried L-Valine Fermentation Product (>72% L-valine), teleconference, minutes final production strain and the parent strain under the production conditions. The notifier asked whether the analysis should be specific to the production strain that produces the notified L-valine fermentation product. CVM explained that generation of the biogenic amines could be impacted by the genetic modifications in the production strain, therefore, the analysis should be conducted specifically for the notified production strain. The notifier agreed to provide the requested analysis.

▶ CVM requested the analysis of the biogenic amines in the fermentation media of the final production strain. The division also requested the biogenic amines from the parent strain.
CJ analyzed for eight typical biogenic amines including tyramine, phenethylamine, putrescine, cadaverine, histamine, tryptamine, spermidine, and spermine in fermentation media of L-Valine Fermentation Product ([Supplement #12]). The seven of biogenic amines were below LOD. However, tyramine was detected at 7~8 ppm in all of the analyzed strains including wild type, parental strain and production strain.

Table.1 Biogenic amine analysis in the fermentation media of wild type (ATCC14067), parental strain (KCCM 11201P) and production strain (KCCM 80058)

Biogenic Amine	Concentration (mg/L)			LOD (mg/L)	LOQ (mg/L)
	ATCC 14067	KCCM 11201P	KCCM 80058		
Tryptamine	N.D.	N.D.	N.D.	0.3	1.0
2-Phenylethylamine	N.D.	N.D.	N.D.	0.3	1.0
Putrescine	N.D.	N.D.	N.D.	0.3	1.0
Cadaverine	N.D.	N.D.	N.D.	0.3	1.0
Histamine	N.D.	N.D.	N.D.	0.3	1.0
Tyramine	8.88	7.77	8.33	0.3	1.0
Spermidine	N.D.	N.D.	N.D.	0.3	1.0
Spermine	N.D.	N.D.	N.D.	0.3	1.0

The genetic modification in the product strain has no impact on the generation of biogenic amines.

This analysis was conducted by using HPLC in a reverse-phase column with pre-column derivatization with dansyl chloride and UV detection.

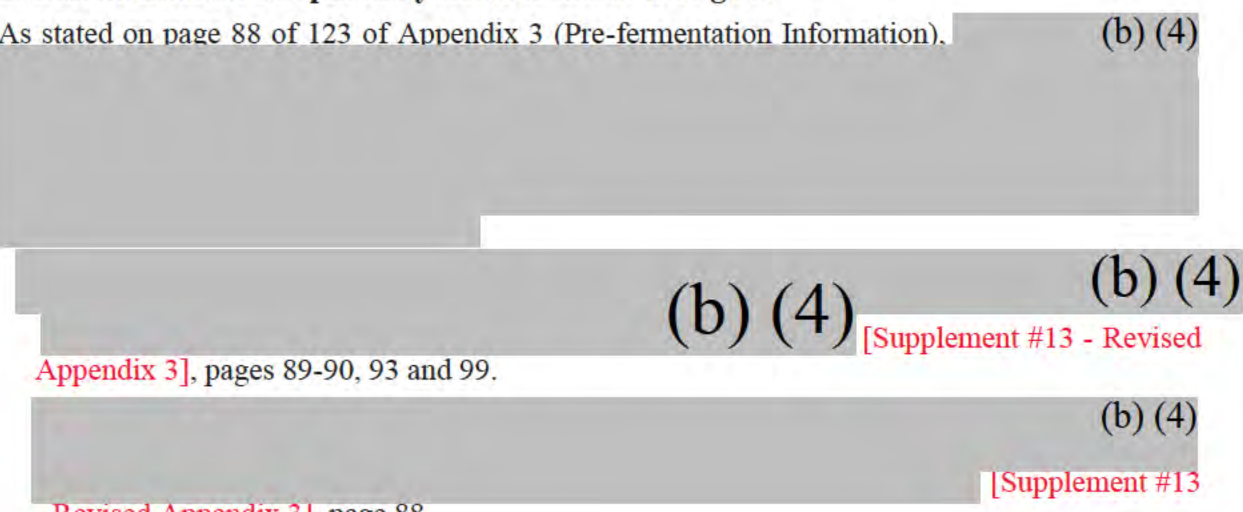
This analysis was conducted by third party laboratory, Korea Research Institute of Analytical Technology (KRIAT) in accordance with the method of KFDA Food Code 6.14.6 The test report is provided in [Supplement #12].

4. Molecular techniques used to develop and characterize C. glutamicum KCCM 80058

▶ CVM pointed out that there are numerous inconsistencies in Appendix 3 of the notice and requested the notifier to explain the following concerns listed below (All explanations are provided in [Supplement #13- Revised Appendix 3])

a. Clarification of the partially deleted size of *ilvA* gene

As stated on page 88 of 123 of Appendix 3 (Pre-fermentation Information), (b) (4)



(b) (4) (b) (4) [Supplement #13 - Revised Appendix 3], pages 89-90, 93 and 99.

(b) (4) [Supplement #13 - Revised Appendix 3], page 88.

b. Terminology of genetic modification

On pages 79 and 88 of 123 of Appendix 3 (Pre-fermentation Information), the notifier states that (b) (4) CVM suggested that the terminology used to describe the genetic modification should be consistent throughout the notice.

▶ The terminology used to describe the genetic modification was revised to "partially deleted", which is reflected in the [Supplement #13 - REVISED Appendix 3], pages 66, 79-81, 88-97, 99, 111 and 113.

c. Clarification of modified gene sequence

The nucleotide sequence of the "original *ilv*A ORF" was provided in Table B-7-2 an (b) (4) ee below). (b) (4)

CVM pointed out that primer 2, which binds to this region and a downstream region, does not

contain the guanine residue (see below).

(b) (4)

Further, the results of the whole genome analysis show that the gene fragments are (b) (4)

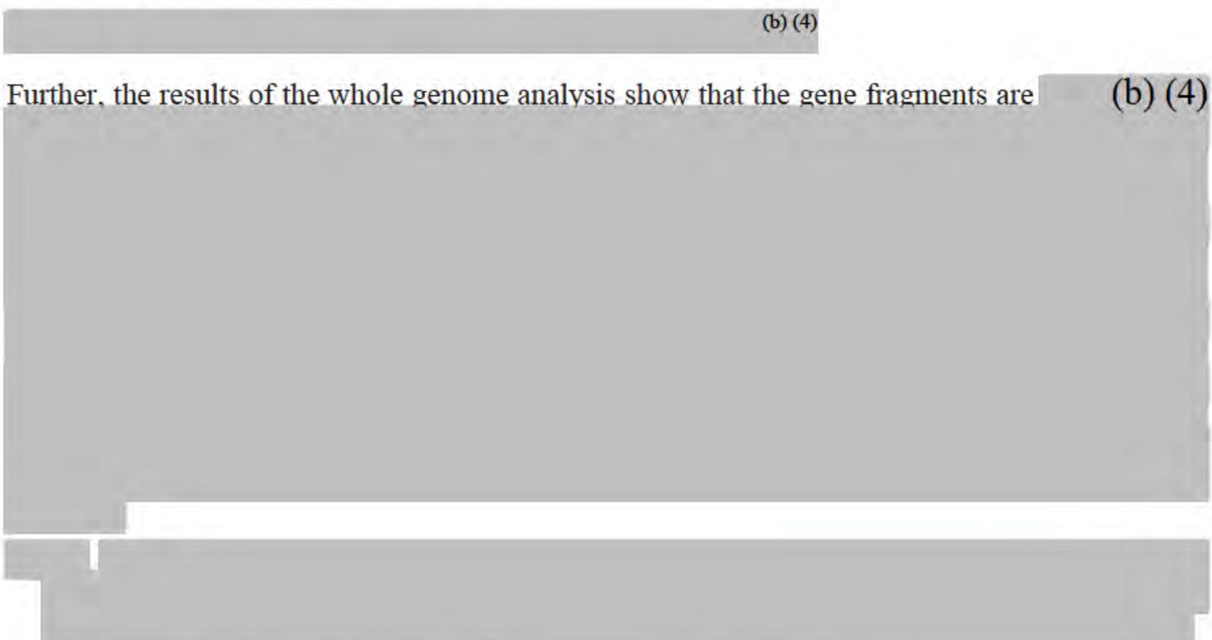
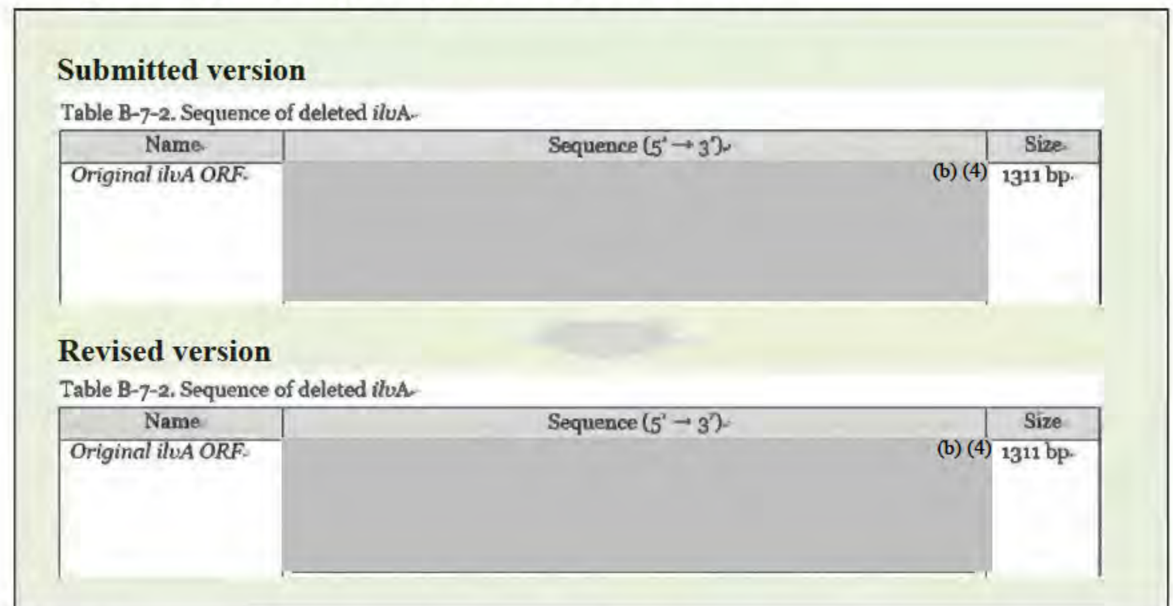


Figure 1. Sequence of partially deleted *ilv*A after amendment



Submitted version

Table B-7-2. Sequence of deleted *ilvA*

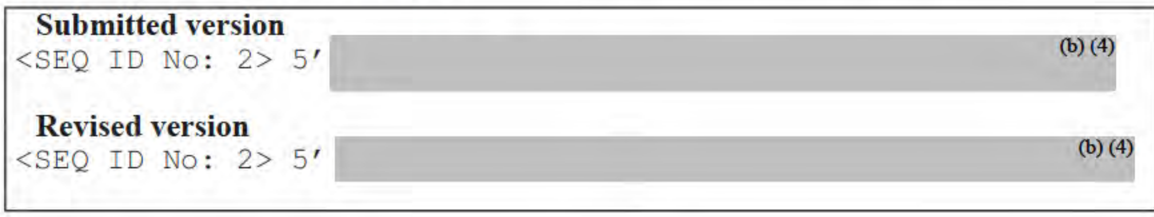
Name	Sequence (5' → 3')	Size
Original ilvA ORF	(b) (4)	1311 bp

Revised version

Table B-7-2. Sequence of deleted *ilvA*

Name	Sequence (5' → 3')	Size
Original ilvA ORF	(b) (4)	1311 bp

As CVM mentioned, the mismarked the guanine nucleotide sequence was not included in the primer 2. It is corrected as follows:



Submitted version

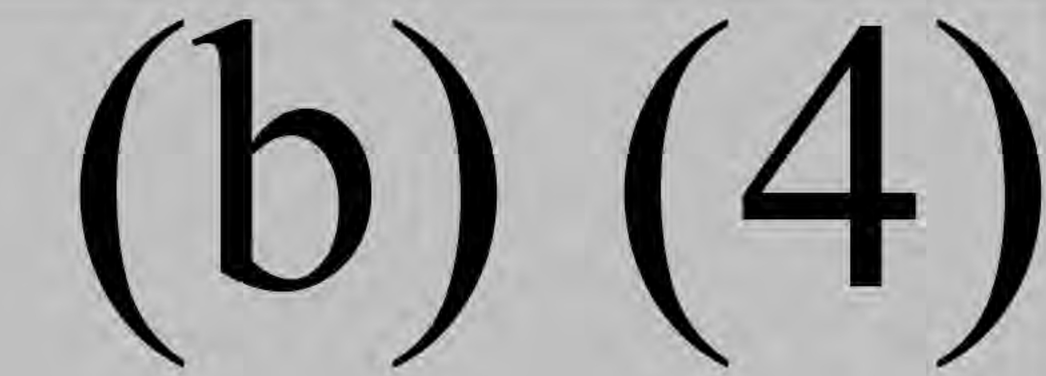
<SEQ ID No: 2> 5′ (b) (4)

Revised version

<SEQ ID No: 2> 5′ (b) (4)

(b) (4)

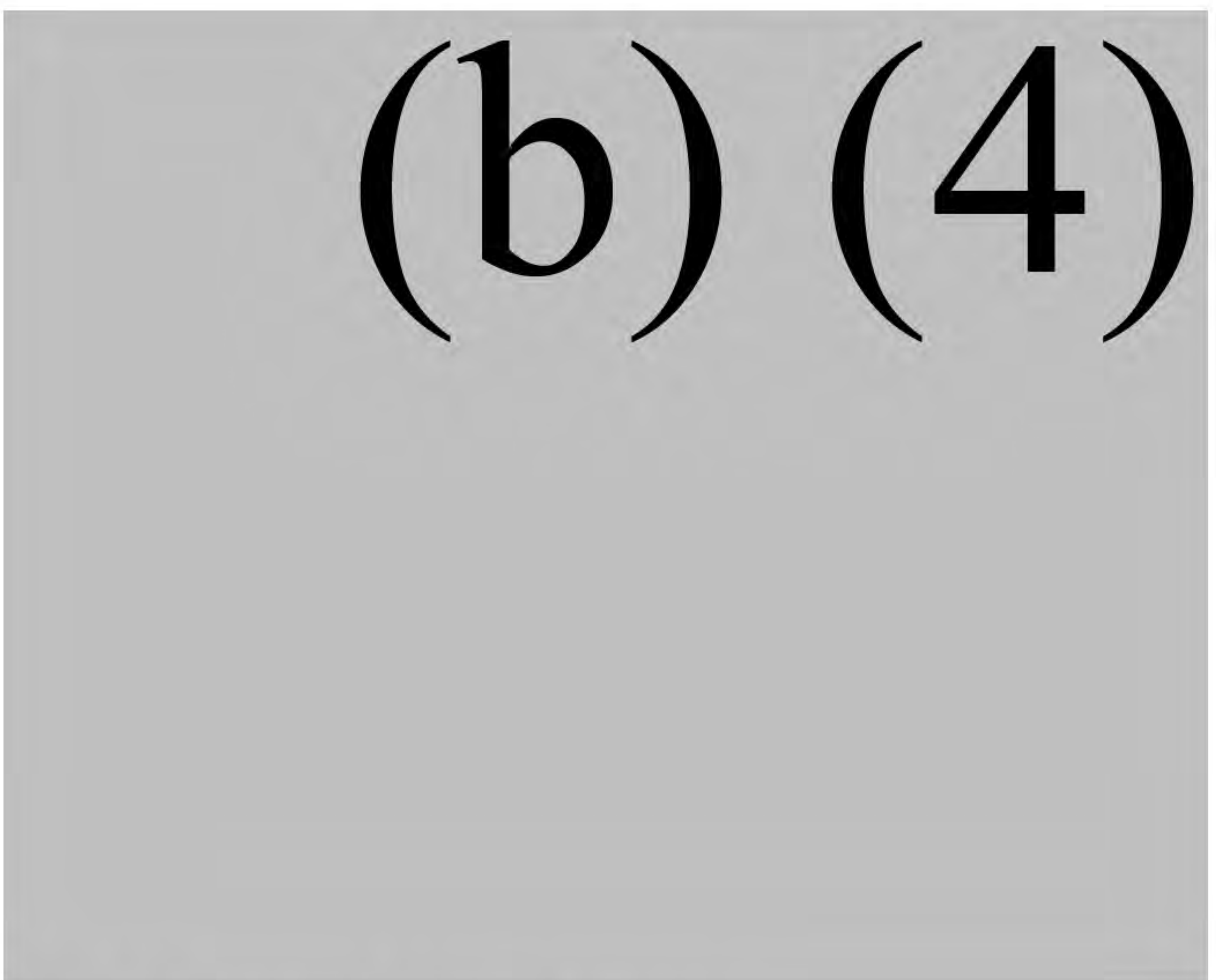
(b) (4)



(b) (4)

(b) (4)

(b) (4)



g. Confirmation of no unintended modification

The notifier states in Part 6.2 of the notice, "As shown in Appendix 3 of this notice, the assessment of the genetic engineering process demonstrates that there is no hazard imparted due to the engineering process." This section will need to be revised. CVM explained that this section should discuss whether the genome of the host organism has been modified by unintended insertions or deletions and, in this particular case, whether the frameshift within the partially deleted *ilvA* gene would result in the production of a protein that raises safety concerns. The notifier agreed to provide a response to address all above-mentioned issues related to the molecular techniques used to develop and characterize *C. glutamicum* KCCM 80058 strain.

▶ Please refer to the answer of comments c, d and f as well as the revised Appendix3 [Supplment #13]. We confirm that unintended modification (i.e. insertion, deletion or frame-shift) on the genome of *C. glutamicum* KCCM80058 did not occurr during the genetic engineering.

Should you have any questions on this amendment, please contact me directly.

Sincerely,
Kristi Smedley Digitally signed by Kristi Smedley DN: cn=Kristi Smedley o=Center for Regulatory Services Inc ou email=smedley@cfr services.com c=US Date: 2020.07.27 14:48:51 -04'00'
Kristi O. Smedley
Consultant to CheilJedang Corporation

Cc: Keith Hayden, CJ
Min Kang, CJ
Thomas Biesiada, CJ

Supplement:

1. REVISED Appendix 4_Manufacturing process LVFP
2. Starting Materials for the L-Valine Fermentation Batch
3. Certificate of Analysis for Corn Starch
4. REVISED Table 2.1 Chemical Composition
5. REVISED Appendix2_Heavy Metal CoA
   5-1. Raw data_ Heavy Metal CoA_GVAL180404
   5-2. Raw data_ Heavy Metal CoA_GVAL180405
   5-2. Raw data_ Heavy Metal CoA_GVAL180406
6. Valine Analytical Method (SOP)
7. REVISED Appendix 1_Method Validation
8. REVISED Appendix 1_Method Validation-raw data
9. REVISED Appendix 1_Method Validation_Amendment Report
10. The Detailed Description of Extra Peaks
11. REVISED Appendix 1_Method Validation-CRM1172 VAL COA
12. Test report_Biogenic amine analysis_Parental strain vs Production strain
    12-1. Raw Data_Biogenic amine analysis_ATCC14067
    12-2. Raw Data_Biogenic amine analysis_KCCM11201P
    12-3. Raw Data_Biogenic amine analysis_KCCM80058
13. REVISED Appendix 3_Pre-fermentation_LVFP
14. Open Reading Frame Analysis for the Full Genome Sequence of *Corynebacterium glutamicum* KCCM80058
15. Full-Genome Sequence Analysis of *Corynebacterium glutamicum* KCCM80058

References:

ISO/IEC 17043 (General requirements for proficiency testing) , 2010, B.3 Calculation of performance statistics, pp30~33

Kong. C and O. Adeola. 2014. Invited Review: Evaluation of amino acids and energy utilization in feedstuff for swine and poultry diets. Asian Australia J. Animal Science 27: 917.

Parsons, C. 1996. Digestible amino acids for poultry and swine. Animal Feed Science Technology, 59:147.

INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY
Q2(R1)

Current *Step 4* version
Parent Guideline dated 27 October 1994
(Complementary Guideline on Methodology dated 6 November 1996
incorporated in November 2005)

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

Q2(R1)
Document History

First Codification	History	Date	New Codification **November 2005**
Parent Guideline: Text on Validation of Analytical Procedures			
Q2	Approval by the Steering Committee under *Step 2* and release for public consultation.	26 October 1993	Q2
Q2A	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	27 October 1994	Q2
Guideline on Validation of Analytical Procedures: Methodology developed to complement the Parent Guideline			
Q2B	Approval by the Steering Committee under *Step 2* and release for public consultation.	29 November 1995	in Q2(R1)
Q2B	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	6 November 1996	in Q2(R1)
Current *Step 4* version			
Q2A and Q2B	The parent guideline is now renamed Q2(R1) as the guideline Q2B on methology has been incorporated to the parent guideline. The new title is "Validation of Analytical Procedures: Text and Methodology".	November 2005	Q2(R1)

VALIDATION OF ANALYTICAL PROCEDURES:
TEXT AND METHODOLOGY

ICH Harmonised Tripartite Guideline

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PART I:

TEXT ON VALIDATION OF ANALYTICAL PROCEDURES

ICH Harmonised Tripartite Guideline

Having reached Step 4 of the ICH Process at the ICH Steering Committee meeting on 27 October 1994, this guideline is recommended for adoption to the three regulatory parties to ICH

1. Introduction

This document presents a discussion of the characteristics for consideration during the validation of the analytical procedures included as part of registration applications submitted within the EC, Japan and USA. This document does not necessarily seek to cover the testing that may be required for registration in, or export to, other areas of the world. Furthermore, this text presentation serves as a collection of terms, and their definitions, and is not intended to provide direction on how to accomplish validation. These terms and definitions are meant to bridge the differences that often exist between various compendia and regulators of the EC, Japan and USA.

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included. Other analytical procedures may be considered in future additions to this document.

2. Types of Analytical Procedures to be Validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

- Identification tests;
- Quantitative tests for impurities' content;
- Limit tests for the control of impurities;
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Although there are many other analytical procedures, such as dissolution testing for drug products or particle size determination for drug substance, these have not been addressed in the initial text on validation of analytical procedures. Validation of these additional analytical procedures is equally important to those listed herein and may be addressed in subsequent documents.

A brief description of the types of tests considered in this document is provided below.

- Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc) to that of a reference standard;

- Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than for a limit test;

- Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures (e.g., dissolution).

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below:

Accuracy

Precision

Repeatability

Intermediate Precision

Specificity

Detection Limit

Quantitation Limit

Linearity

Range

Each of these validation characteristics is defined in the attached Glossary. The table lists those validation characteristics regarded as the most important for the validation of different types of analytical procedures. This list should be considered typical for the analytical procedures cited but occasional exceptions should be dealt with on a case-by-case basis. It should be noted that robustness is not listed in the table but should be considered at an appropriate stage in the development of the analytical procedure.

Furthermore revalidation may be necessary in the following circumstances:

- changes in the synthesis of the drug substance;
- changes in the composition of the finished product;
- changes in the analytical procedure.

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

TABLE

Type of analytical procedure characteristics	IDENTIFICATION	TESTING FOR IMPURITIES quantitat.	 limit	ASSAY - dissolution (measurement only) - content/potency
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm.Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated

\+ signifies that this characteristic is normally evaluated

(1) in cases where reproducibility (see glossary) has been performed, intermediate precision is not needed

(2) lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)

(3) may be needed in some cases

GLOSSARY

1. ANALYTICAL PROCEDURE

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc.

2. SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications:

Identification: to ensure the identity of an analyte.

Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency):
to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

3. ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

This is sometimes termed trueness.

4. PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

4.1. Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision .

4.2. Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

4.3. Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

5. DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

6. QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

7. LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

8. RANGE

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

9. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

PART II:

VALIDATION OF ANALYTICAL PROCEDURES: METHODOLOGY

ICH Harmonised Tripartite Guideline

Having reached Step 4 of the ICH Process at the ICH Steering Committee meeting on 6 November 1996, and incorporated into the core guideline in November 2005, this guideline is recommended for adoption to the three regulatory parties to ICH

INTRODUCTION

This document is complementary to the parent document which presents a discussion of the characteristics that should be considered during the validation of analytical procedures. Its purpose is to provide some guidance and recommendations on how to consider the various validation characteristics for each analytical procedure. In some cases (for example, demonstration of specificity), the overall capabilities of a number of analytical procedures in combination may be investigated in order to ensure the quality of the drug substance or drug product. In addition, the document provides an indication of the data which should be presented in a registration application .

All relevant data collected during validation and formulae used for calculating validation characteristics should be submitted and discussed as appropriate.

Approaches other than those set forth in this guideline may be applicable and acceptable. It is the responsibility of the applicant to choose the validation procedure and protocol most suitable for their product. However it is important to remember that the main objective of validation of an analytical procedure is to demonstrate that the procedure is suitable for its intended purpose. Due to their complex nature, analytical procedures for biological and biotechnological products in some cases may be approached differently than in this document.

Well-characterized reference materials, with documented purity, should be used throughout the validation study. The degree of purity necessary depends on the intended use.

In accordance with the parent document, and for the sake of clarity, this document considers the various validation characteristics in distinct sections. The arrangement of these sections reflects the process by which an analytical procedure may be developed and evaluated.

In practice, it is usually possible to design the experimental work such that the appropriate validation characteristics can be considered simultaneously to provide a sound, overall knowledge of the capabilities of the analytical procedure, for instance: specificity, linearity, range, accuracy and precision.

1. SPECIFICITY

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure.

It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination). In this case a combination of two or

more analytical procedures is recommended to achieve the necessary level of discrimination.

1.1. Identification

Suitable identification tests should be able to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (perhaps by comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgement with a consideration of the interferences that could occur.

1.2. Assay and Impurity Test(s)

For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labelled. Similar considerations should be given to other separation techniques.

Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be demonstrated by the resolution of the two components which elute closest to each other.

In cases where a non-specific assay is used, other supporting analytical procedures should be used to demonstrate overall specificity. For example, where a titration is adopted to assay the drug substance for release, the combination of the assay and a suitable test for impurities can be used.

The approach is similar for both assay and impurity tests:

1.2.1 Impurities are available

For the assay , this should involve demonstration of the discrimination of the analyte in the presence of impurities and/or excipients; practically, this can be done by spiking pure substances (drug substance or drug product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials (by comparison with the assay result obtained on unspiked samples).

For the impurity test, the discrimination may be established by spiking drug substance or drug product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

1.2.2 Impurities are not available

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g.: pharmacopoeial method or other validated analytical procedure (independent procedure). As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation.

- for the assay, the two results should be compared;

- for the impurity tests, the impurity profiles should be compared.

Peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry).

2. LINEARITY

A linear relationship should be evaluated across the range (see section 3) of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighings of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample.

For the establishment of linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified.

3. RANGE

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

The following minimum specified ranges should be considered:

- for the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration;
- for content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;
- for dissolution testing: +/-20 % over the specified range;

e.g., if the specifications for a controlled released product cover a region from 20%, after 1 hour, up to 90%, after 24 hours, the validated range would be 0-110% of the label claim.

- for the determination of an impurity: from the reporting level of an impurity[1] to 120% of the specification;
- for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be commensurate with the level at which the impurities must be controlled;

  Note: for validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit.

- if assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities[1] to 120% of the assay specification.

4. ACCURACY

Accuracy should be established across the specified range of the analytical procedure.

4.1. Assay

4.1.1 Drug Substance

Several methods of determining accuracy are available:

a) application of an analytical procedure to an analyte of known purity (e.g. reference material);

b) comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.);

c) accuracy may be inferred once precision, linearity and specificity have been established.

4.1.2 Drug Product

Several methods for determining accuracy are available:

a) application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analysed have been added;

b) in cases where it is impossible to obtain samples of all drug product components , it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.);

c) accuracy may be inferred once precision, linearity and specificity have been established.

---

[1] see chapters "Reporting Impurity Content of Batches" of the corresponding ICH-Guidelines: "Impurities in New Drug Substances" and "Impurities in New Drug Products"

4.2. Impurities (Quantitation)

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities.

In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure (see 1.2.). The response factor of the drug substance can be used.

It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte.

4.3. Recommended Data

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

5. PRECISION

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

5.1. Repeatability

Repeatability should be assessed using:

a) a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each);

or

b) a minimum of 6 determinations at 100% of the test concentration.

5.2. Intermediate Precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

5.3. Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

5.4. Recommended Data

The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

6. DETECTION LIMIT

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

6.1. Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

6.2. Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

6.3 Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\,\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example:

6.3.1 Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

6.3.2 Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

6.4 Recommended Data

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

7. QUANTITATION LIMIT

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

7.1. Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

7.2. Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

7.3. Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$$QL = \frac{10\,\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways for example:

7.3.1 Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

7.3.2 Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

7.4 Recommended Data

The quantitation limit and the method used for determining the quantitation limit should be presented.

The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.

8. ROBUSTNESS

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are:

- stability of analytical solutions;
- extraction time.

In the case of liquid chromatography, examples of typical variations are:

- influence of variations of pH in a mobile phase;
- influence of variations in mobile phase composition;
- different columns (different lots and/or suppliers);
- temperature;
- flow rate.

In the case of gas-chromatography, examples of typical variations are:

- different columns (different lots and/or suppliers);
- temperature;
- flow rate.

9. SYSTEM SUITABILITY TESTING

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. See Pharmacopoeias for additional information.

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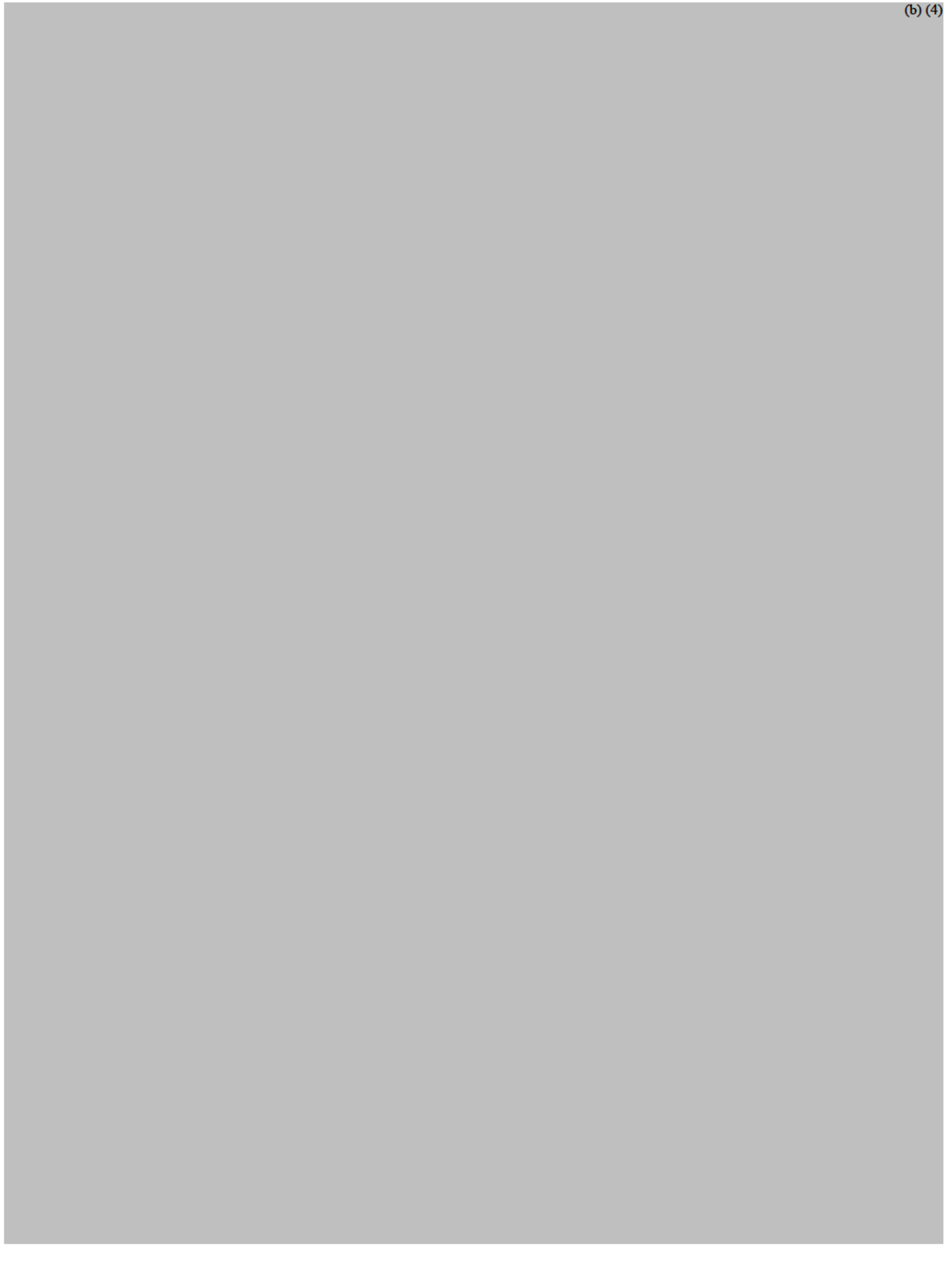

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- Invited Review -

Evaluation of Amino Acid and Energy Utilization in Feedstuff for Swine and Poultry Diets

C. Kong and O. Adeola*
Department of Animal Sciences, Purdue University, West Lafayette, IN 47907-2054, USA

ABSTRACT: An accurate feed formulation is essential for optimizing feed efficiency and minimizing feed cost for swine and poultry production. Because energy and amino acid (AA) account for the major cost of swine and poultry diets, a precise determination of the availability of energy and AA in feedstuffs is essential for accurate diet formulations. Therefore, the methodology for determining the availability of energy and AA should be carefully selected. The total collection and index methods are 2 major procedures for estimating the availability of energy and AA in feedstuffs for swine and poultry diets. The total collection method is based on the laborious production of quantitative records of feed intake and output, whereas the index method can avoid the laborious work, but greatly relies on accurate chemical analysis of index compound. The direct method, in which the test feedstuff in a diet is the sole source of the component of interest, is widely used to determine the digestibility of nutritional components in feedstuffs. In some cases, however, it may be necessary to formulate a basal diet and a test diet in which a portion of the basal diet is replaced by the feed ingredient to be tested because of poor palatability and low level of the interested component in the test ingredients. For the digestibility of AA, due to the confounding effect on AA composition of protein in feces by microorganisms in the hind gut, ileal digestibility rather than fecal digestibility has been preferred as the reliable method for estimating AA digestibility. Depending on the contribution of ileal endogenous AA losses in the ileal digestibility calculation, ileal digestibility estimates can be expressed as apparent, standardized, and true ileal digestibility, and are usually determined using the ileal cannulation method for pigs and the slaughter method for poultry. Among these digestibility estimates, the standardized ileal AA digestibility that corrects apparent ileal digestibility for basal endogenous AA losses, provides appropriate information for the formulation of swine and poultry diets. The total quantity of energy in feedstuffs can be partitioned into different components including gross energy (GE), digestible energy (DE), metabolizable energy (ME), and net energy based on the consideration of sequential energy losses during digestion and metabolism from GE in feeds. For swine, the total collection method is suggested for determining DE and ME in feedstuffs whereas for poultry the classical ME assay and the precision-fed method are applicable. Further investigation for the utilization of ME may be conducted by measuring either heat production or energy retention using indirect calorimetry or comparative slaughter method, respectively. This review provides information on the methodology used to determine accurate estimates of AA and energy availability for formulating swine and poultry diets. (**Key Words:** Chickens, Digestibility, Methodology, Pigs)

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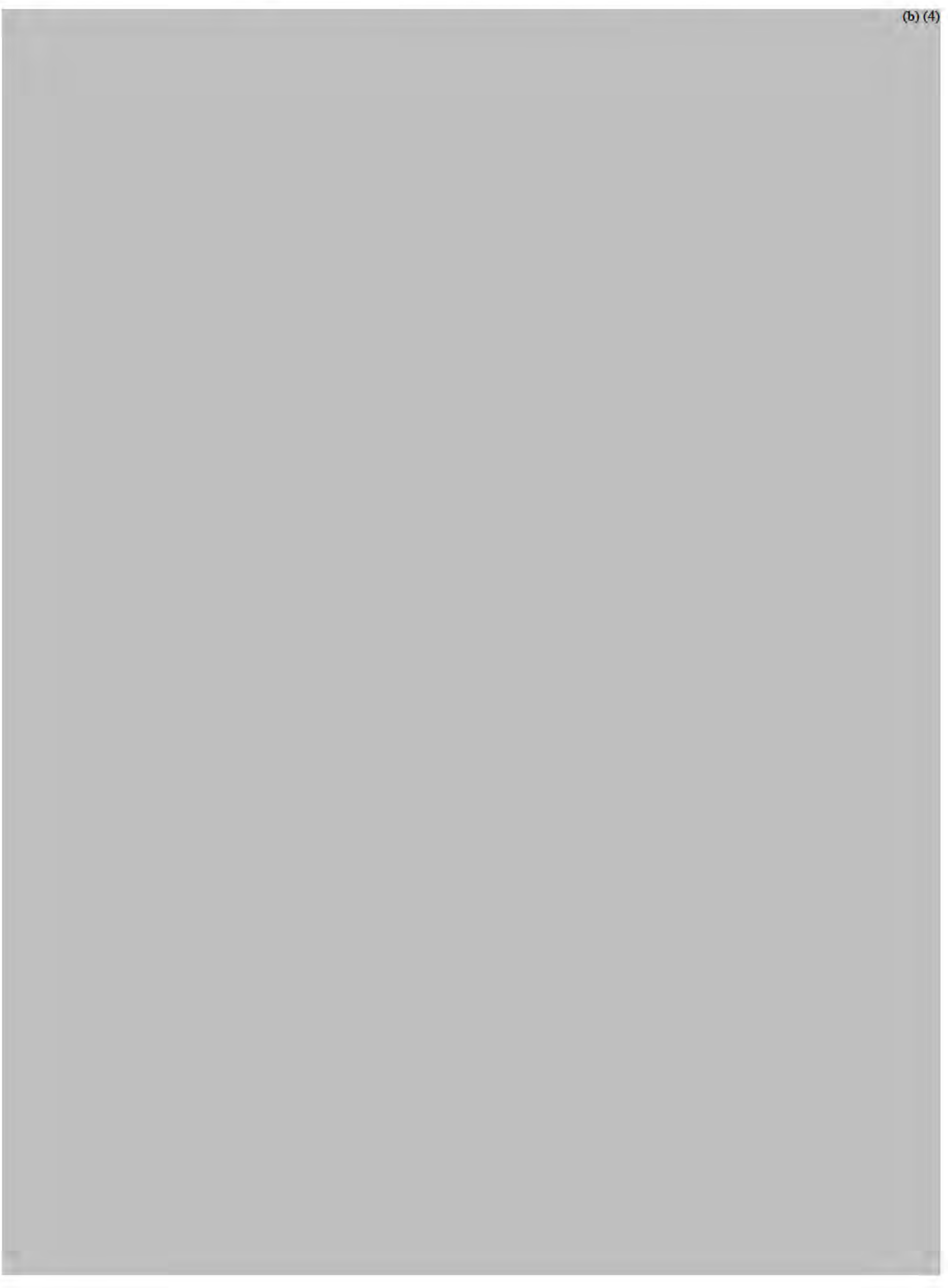
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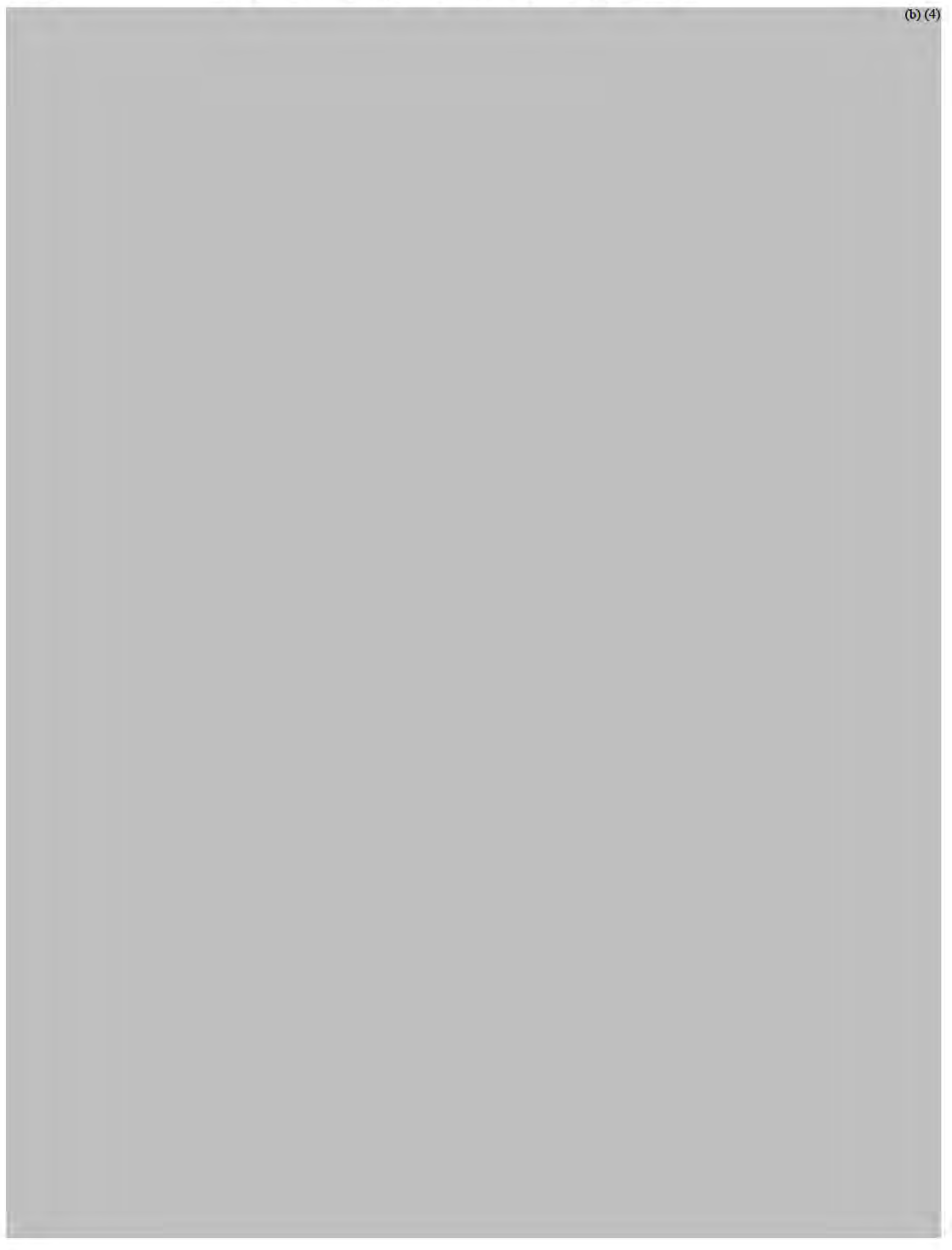
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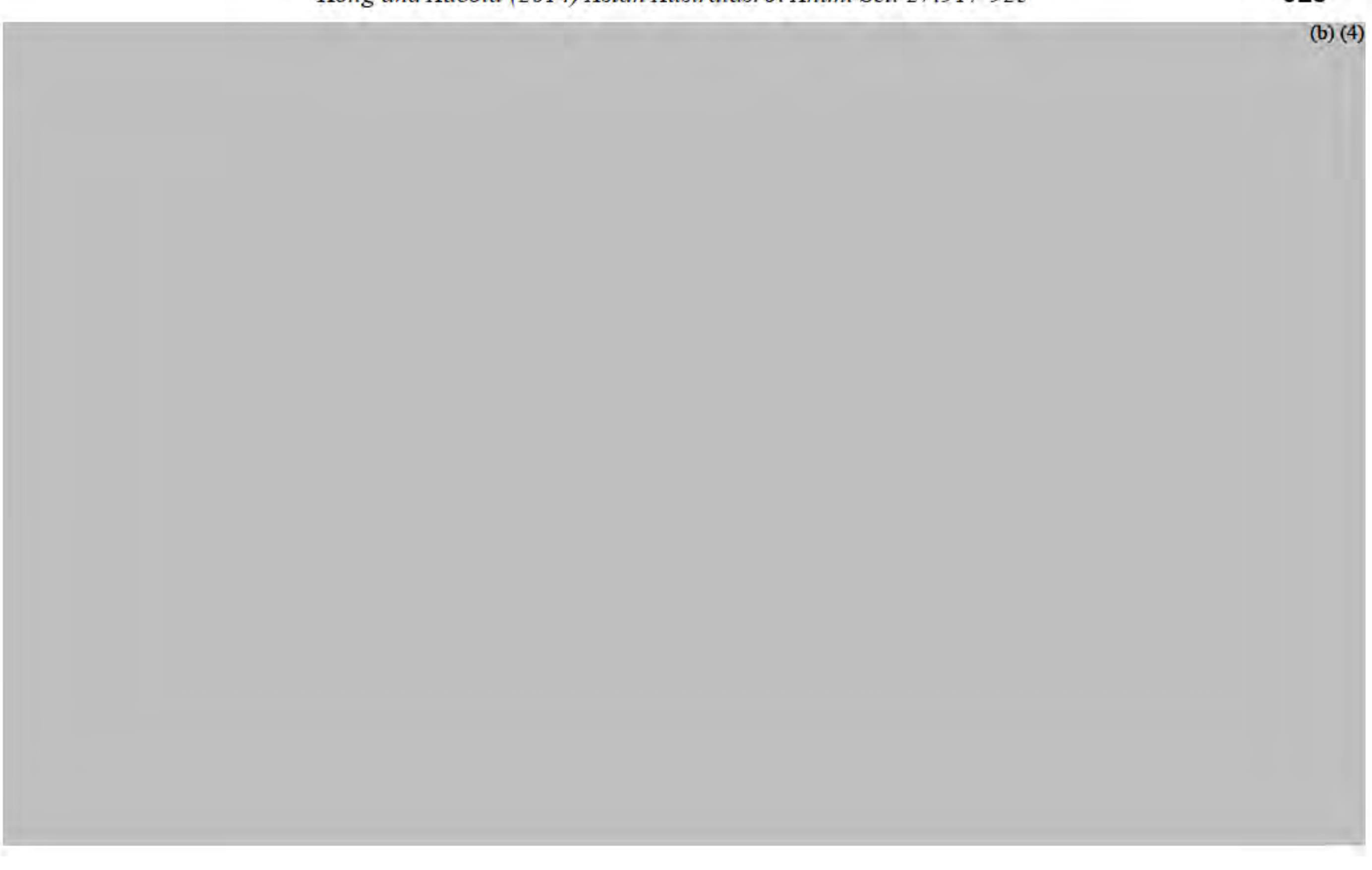
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ELSEVIER Animal Feed Science Technology 59 (1996) 147–153

ANIMAL FEED SCIENCE AND TECHNOLOGY

Digestible amino acids for poultry and swine

Carl M. Parsons

Department of Animal Sciences, University of Illinois, Urbana, IL 61801, USA

Abstract

A review and comparison of feedstuff amino acid digestibility values for poultry and swine is presented and the use of amino acid digestibility and availability is discussed. The effect of overprocessing on amino acid digestibility of oilseed meals is also reviewed. In general, true digestible amino acid values determined in cecectomized roosters are 5–10% higher than apparent digestibility values determined in ileal-cannulated pigs. Several studies have shown beneficial responses to formulating diets based digestible amino acid values vs. total amino acid values. However, feedstuff amino acid digestibility values determined via balance assays are often higher than amino acid availability values determined by slope-ratio growth assays. In addition, recent work with pigs suggests that the ileal digestibility assay overestimates the amount of amino acids available or utilizable for protein synthesis. Finally, overprocessing of oilseed meals greatly reduces the amount of digestible or available lysine, whereas other amino acids are usually much less affected. Protein solubility in KOH is a good in vitro assay for detecting decreased protein quality due to overprocessing and the use of Coomassie Blue dye simplifies and reduces the time required to conduct the assay.

Keywords: Poultry; Swine; Amino acid digestibility

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Cerrito, Chelsea

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Monday, July 27, 2020 3:02 PM
To: Tang, Lei; Wong, Geoffrey K; Animalfood-premarket
Cc: '강민경님 [Min Kang]'; Keith D. Haydon; Biesiada,Thomas님
Subject: RE: GRAS AGRN 35 --AMENDMENT --Email 2---supplements 1-6
Attachments: Supplement 1. REVISED Appendix 4_Manufacturing process_LVFP.pdf; Supplement 2 Starting Materials.pdf; Supplement 3. Corn Starch CoA.pdf; Supplement 4 Revised Table 2.1.pdf; Supplement 5. Heavy metal COA.pdf; Supplement 5-1. Raw Data-Heavy metal COA-GVAL180404.pdf; Supplement 5-2. Raw Data-Heavy metal COA-GVAL180405.pdf; Supplement 5-3. Raw Data-Heavy metal COA-GVAL180406.pdf; Supplement 6. Valine Analytical Method (SOP)_MK EDIT.pdf

Dr. Tang:

This email contains Supplements (attachments 1-6) to support AGRN 35 amendment.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cel (b) (6)
Fax 703-580-8637

From: Kristi Smedley [mailto:smedley@cfr-services.com]
Sent: Monday, July 27, 2020 3:00 PM
To: 'Tang, Lei'; 'Wong, Geoffrey K'; Animalfood-premarket (Animalfood-premarket@fda.hhs.gov)
Cc: '강민경님 [Min Kang]' (mg.kang@cj.net); Keith D. Haydon (keith.haydon@cj.net); Biesiada,Thomas님 (thomas.biesiada@cj.net)
Subject: GRAS AGRN 35 --AMENDMENT --Email 1

Dr. Tang:

On behalf of CJ, I am providing the amendment of AGRN 35, L-Valine fermentation product , as requested. This email provides a part of the supporting material for this amendment.

You will be receiving a series of emails, to assure that all supplements (attachments) are received. I will number them in the subject line of the email, as well as provide a brief description in the body of each email. You should receive 4 reference files and 15 supplements (attachments).

This email will include the signed amendment letter as well as all reference publications.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.

Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

From: Tang, Lei [mailto:Lei.Tang@fda.hhs.gov]
Sent: Wednesday, July 08, 2020 12:02 PM
To: Kristi Smedley
Cc: Wong, Geoffrey K
Subject: RE: GRAS AGRN 35

Dear Dr. Smedley,

This letter is in response to your email dated July 3, 2020 requesting for meeting minutes from the July 1, 2020 meeting between the Center for Veterinary Medicine and CJ CheilJedang Corporation.

Please find enclosed a copy of our meeting minutes for the referenced meeting.

If you have any questions concerning this letter, please contact me via email at Lei.Tang@fda.hhs.gov or by phone at 240-402-5922. Please refer to AGRN #35 in any future correspondences.

Sincerely,

Lei Tang, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal feeds
U.S. Food and Drug Administration

Tel: 240-402-5922
lei.tang@fda.hhs.gov

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From: Kristi Smedley <smedley@cfr-services.com>
Sent: Friday, July 3, 2020 9:32 AM

To: Tang, Lei <Lei.Tang@fda.hhs.gov>
Cc: Wong, Geoffrey K <Geoffrey.Wong@fda.hhs.gov>; Keith D. Haydon <keith.haydon@cj.net>; '강민경님 [Min Kang]' <mg.kang@cj.net>
Subject: RE: GRAS AGRN 35

Dr. Tang

This is a request for the notes of the FDA teleconference (July 1, 2020) specific to issues raised during the AGRN 35 review.

We will accept these notes by email.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cel (b) (6)
Fax 703-580-8637

APPENDIX 4 - Manufacturing Process (CONFIDENTIAL)

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A. Manufacturing Process

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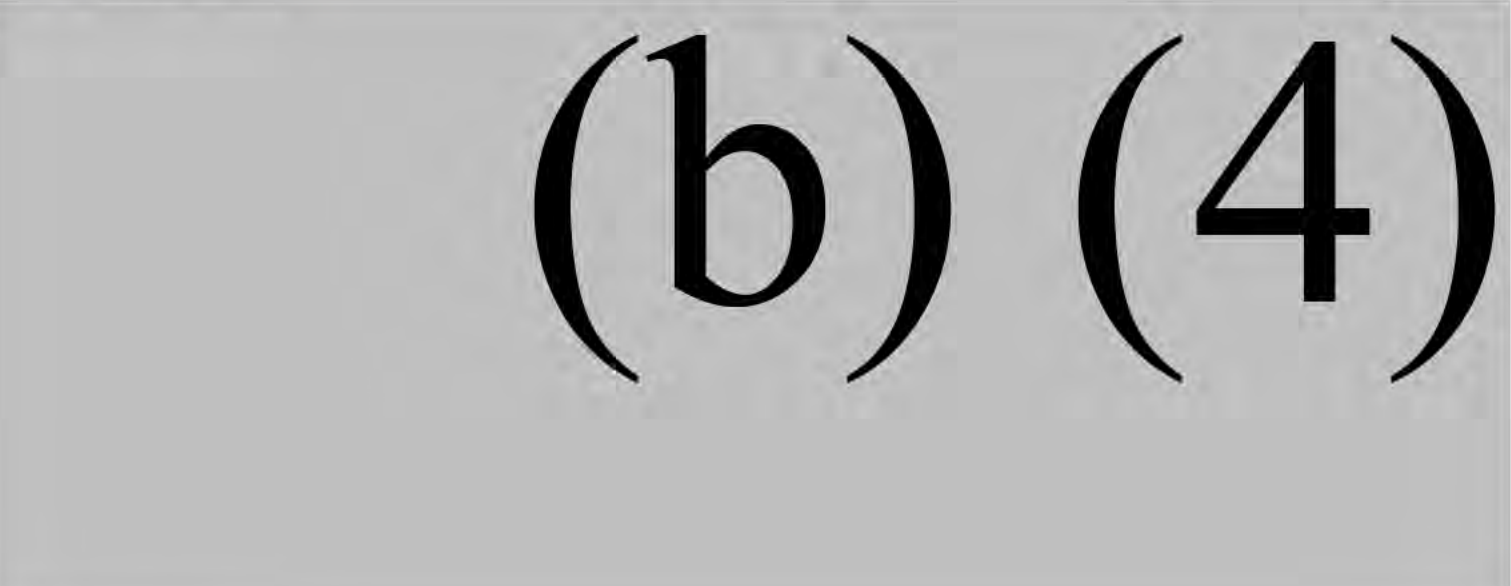
1. Raw Materials



2. Fermentation

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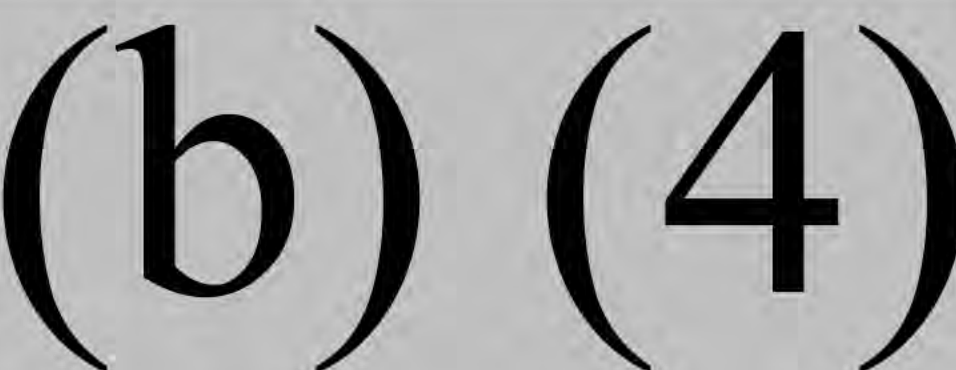
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(b) (4)

[Supplement #3] Corn Starch Certificate of Analysis

CJ CHEILJEDANG CORPORATION

CERTIFICATE OF ANALYSIS

Product Name	CORN STARCH (NGMO)		
Manufactured Date	2018.02.19	Delivery Date	
Quantity	20kg		

Analysis Data

No	ITEM	SPECIFICATION	RESULT	REMARK
1	(b) (4)			
2				
3				
4				
5				
6				
7				
8				
9				
10				

We here certify that above figures are true and correct.

Analyzed . Jihye. Lee

Q.C Manager . Jaeyoun. Im

ADD : 141. Yongdam-ro, Sangnok-gu.
Ansan-si, Gyeonggi-do, Korea
TEL (031) 400 – 3099
FAX (031) 438 – 1603

[Supplement #4] REVISED Table 2.1 Chemical Composition of L-Valine Fermentation Product formulated with Carrier (Corn Starch)$^{+}$

Test	Units	Method	Batch 01	Batch 02	Batch 03	Batch 04	Batch 05	Average
L-valine	%	HPLC (Appendix 1, Attachment2)						
Hydrolyzed amino acids (in insoluble Biomass part) (Total)	%							
Aspartic acid		AOAC 994.12						
Lysine								
Serine								
Glutamic acid								
Glycine								
Alanine								
Valine								
Cystine		AOAC 985.28						
Isoleucine		AOAC 994.12						
Leucine								
Phenylalanine								
Tryptophan		AOAC 988.15						
Methionine		AOAC 985.28						
Threonine		AOAC 994.12						
Arginine								
Histidine								
Proline								
Free amino acids (Total, other than valine)	%	AOAC 999.13						
Lysine								
Glycine								
Alanine								
Threonine								
Isoleucine								
Leucine								
Phenylalanine								
Histidine								
Moisture	%	AOAC 934.01						
Ammonium	%	ASTM D4327-03						
Sugars (Total)	%	AOAC 995.13						
Glucose								
Trehalose								
Organic acids	%	Korean Feed						

(b) (4)

(Total)		Standards Codex, 1 of chapter 14						
Malic Acid			(b) (4)					
Succinic Acid								
Lactic Acid								
Inorganic anions/cations (Total)	%	ASTM D4327-03						
Sodium								
Potassium								
Magnesium								
Calcium								
Chloride								
Phosphate								
Sulfate								
Ash	%	AOAC 942.05						

+Note that this table does not include complex carbohydrates or fats

CJ Research Institute of Biotechnology

55, Gwanggyo-ro 42beon-gil, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16495, Korea, CJ BLOSSOM PARK

www.cj.co.kr

TEL : 031) 8099-2450 FAX : 031) 8099-2918

Certificate of analysis

(b) (4), (b) (6)

CJ Research Institute of Biotechnology

CJ Research Institute of Biotechnology

55, Gwanggyo-ro 42beon-gil, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16495, Korea, CJ BLOSSOM PARK

www.cj.co.kr

TEL : 031) 8099-2450 FAX : 031) 8099-2918

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(b) (4), (b) (6)

July, 07, 2020

CJ Research Institute of Biotechnology

CJ Research Institute of Biotechnology

55, Gwanggyo-ro 42beon-gil, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16495, Korea, CJ BLOSSOM PARK

www.cj.co.kr

TEL : 031) 8099-2450 FAX : 031) 8099-2918

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(b) (4), (b) (6)

July, 07, 2020

CJ Research Institute of Biotechnology

Quantitation Report

Data File Name	023SMPL.d
Acq/Data Batch	C:\Agilent\ICPMH\1\DATA\190829.b
Acq Time	2019-08-28 16:47:32
Sample Name	KIS_4
Sample Type	Sample
Comment	---
Prep Dilution	100.0000
Auto Dilution	1.0000
Total Dilution	100.0000
Operator Name	admin
Acq Mode	Spectrum
Cal Title	---
Cal Type	External Calibration
Last Calib	2020-07-02 13:47:57
Bkg File	---
Bkg Mode	Count Subtraction for All
FQ BlankFile	007QBLK.d
VIS Fit	Point to Point

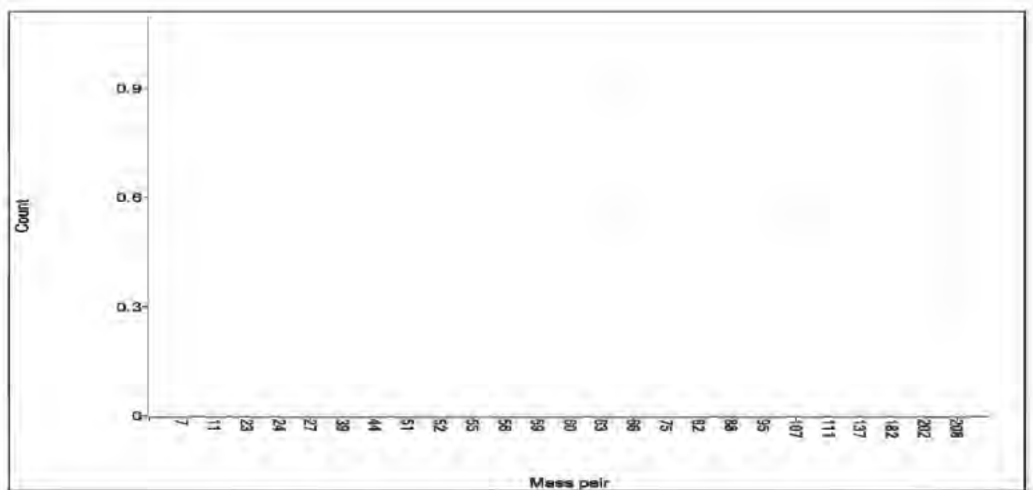
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time(sec)	Rep
As	75		He	<0.003	mg/kg	N/A	19.86		Pulse	0.9900	3
Pb	208		He	<0.003	mg/kg	N/A	112.22		Pulse	0.3000	3
Cd	111		H2	<0.001	mg/kg	N/A	6.67		Pulse	0.1000	3
Hg	202		H2	<0.150	ug/kg	N/A	46.67		Pulse	0.1000	3

ISTD Table:

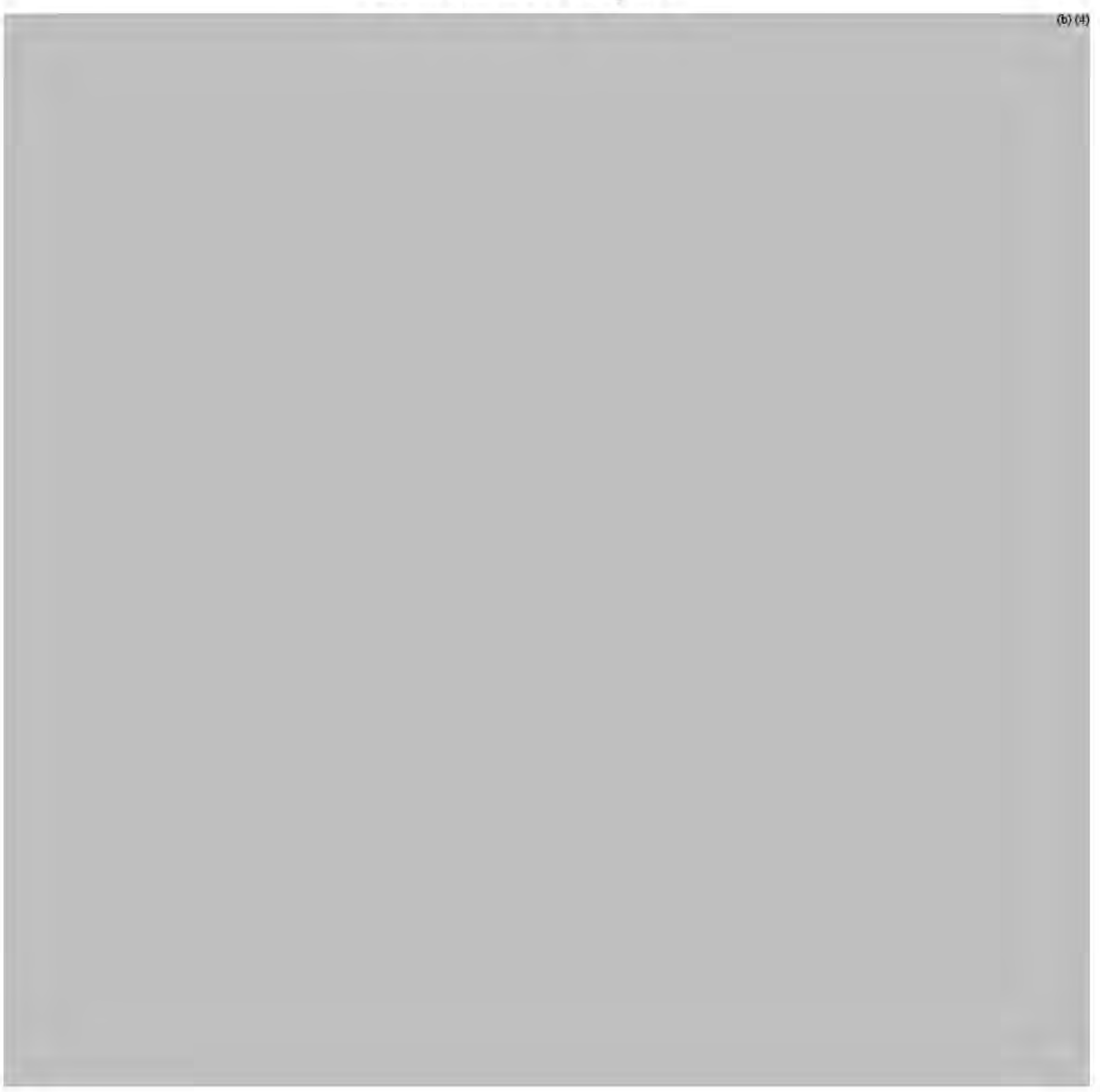
Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery %	Det.	Time(seq)	Rep

He



(b) (4)

Quantitation Report



(b) (4)

Quantitation Report

Data File Name	024SMPL.d
Acq/Data Batch	C:\Agilent\ICPMH\1\DATA\190829.b
Acq Time	2019-08-28 16:52:14
Sample Name	KIS_5
Sample Type	Sample
Comment	—
Prep Dilution	100.0000
Auto Dilution	1.0000
Total Dilution	100.0000
Operator Name	admin
Acq Mode	Spectrum
Cal Title	—
Cal Type	External Calibration
Last Calib	2020-07-02 13:47:57
Bkg File	—
Bkg Mode	Count Subtraction for All
FQ BlankFile	007QBLK.d
VIS Fit	Point to Point

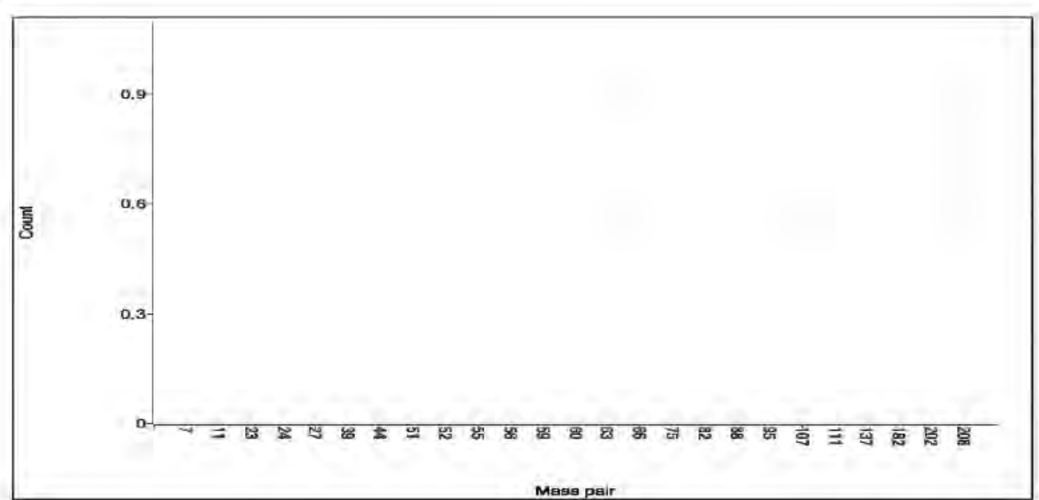
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time(sec)	Rep
As	75		He	<0.003	mg/kg	N/A	14.81		Pulse	0.9900	3
Pb	208		He	<0.003	mg/kg	N/A	88.89		Pulse	0.3000	3
Cd	111		H2	<0.001	mg/kg	N/A	0.00		Pulse	0.1000	3
Hg	202		H2	<0.150	ug/kg	N/A	46.67		Pulse	0.1000	3

ISTD Table:

Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery %	Det.	Time(seq)	Rep

He



(b) (4)

Quantitation Report

(b) (4)

(b) (4)

Quantitation Report

Data File Name	025SMPL.d
Acq/Data Batch	C:\Agilent\ICPMH\1\DATA\190829.b
Acq Time	2019-08-28 16:56:54
Sample Name	KIS_6
Sample Type	Sample
Comment	---
Prep Dilution	100.0000
Auto Dilution	1.0000
Total Dilution	100.0000
Operator Name	admin
Acq Mode	Spectrum
Cal Title	---
Cal Type	External Calibration
Last Calib	2020-07-02 13:47:57
Bkg File	---
Bkg Mode	Count Subtraction for All
FQ BlankFile	007QBLK.d
VIS Fit	Point to Point

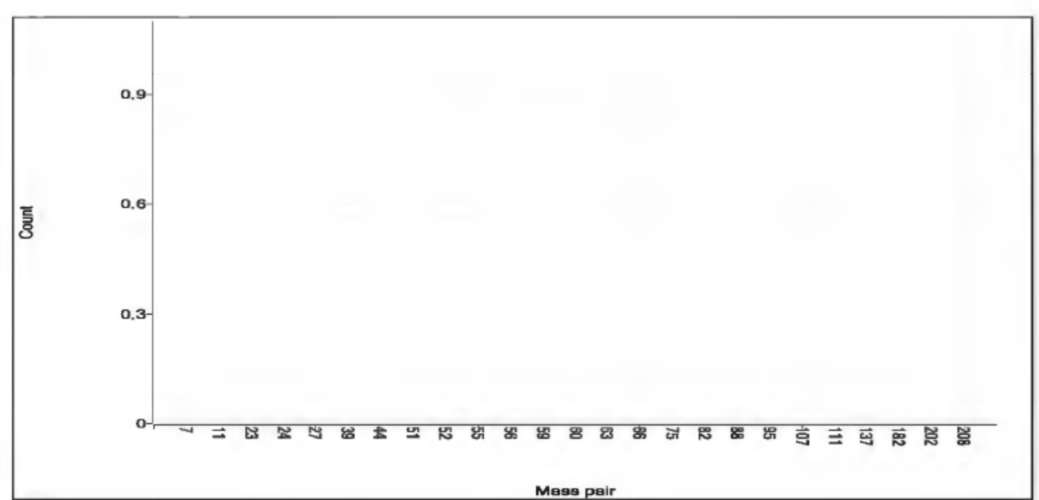
FullQuant Table

Element	Mass	ISTD	Tune Mode	Conc.	Units	RSD(%)	CPS	Ratio	Det.	Time(sec)	Rep
As	75		He	<0.003	mg/kg	N/A	22.89		Pulse	0.9900	3
Pb	208		He	<0.003	mg/kg	N/A	162.22		Pulse	0.3000	3
Cd	111		H2	<0.001	mg/kg	N/A	0.00		Pulse	0.1000	3
Hg	202		H2	<0.150	ug/kg	N/A	50.00		Pulse	0.1000	3

ISTD Table:

Tune Mode	Element	Mass	CPS	RSD(%)	ISTD Recovery %	Det.	Time(seq)	Rep

He



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Quantitation Report

(b) (4)

REPORT

Procedure of HPLC method validation of Dried L-Valine Fermentation Product

(Confidential)

CJ Research Institute of Biotechnology

I. ANALYTICAL PROCEDURE

1. Application Scope

Quantitative analysis of Dried L-Valine Fermentation Product.

2. Method summary

Perform quantitative analysis of Dried L-Valine Fermentation Product using HPLC and dilute each samples with ultra-purified water before analysis.

3. High Performance Liquid Chromatography (HPLC) analysis method

3.1. Reference Standard

1) Identity: L-Valine
2) Product No.: V0500
3) Purity: > 98.0 %
4) Storage Conditions: Room temperature
5) Supplier: SIGMA-ALDRICH

3.2. Apparatus & Instrument

3.2.1. Apparatus
1) Balance (XS-205, METTLER TOLEDO)
2) Stirrer (with magnetic bar)
3) pH meter
4) Aspirator (or decompression Pump)
5) Ultrasonic cleaner

3.2.2. Others
1) Graduate Cylinder
2) Volumetric flask (250 mL)
3) Filter (0.45μm)
4) Weighing paper

3.3. Mobile phase preparation reagent

Reagent Name	Molecular formula	MW	Purity	Manufacturer
Potassium Dihydrogenphosphate	KH_2PO_4	(b) (4)		
1-Octanfonic acid sodium salt(OSA)	$C_8H_{17}NaO_3S$			
Acetonitrile	CH_3CN			
Phosphoric acid	H_3PO_4			
Distilled water	H_2O			

3.4. Analytical Instrument

Parts	Model	Manufacturer
System Controller	CBM-20A	Shimadzu
Degasser	DGU-20A	Shimadzu
Pump	LC-20AD	Shimadzu
Auto Sampler	SIL-20AD	Shimadzu
Column Oven	CTO-20AD	Shimadzu
Detector	SPD-M20A	Shimadzu
Software	Labsolution	Shimadzu

3.5. HPLC Analytical Condition

	Condition
System	HPLC (Shimadzu)
Detector	Photodiode array detector (Shimadzu) UV 210 nm
Column	ODS C18, 150 × 4.6 mm, particle size 3 μm
Column Temp.	40 °C
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, pH 2.5 (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Sample Temp	10 °C
Injection volume	5 μl
Concentration of sample and standard solution	2 g/L (L-valine concentration basis)

3.5.1 Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			

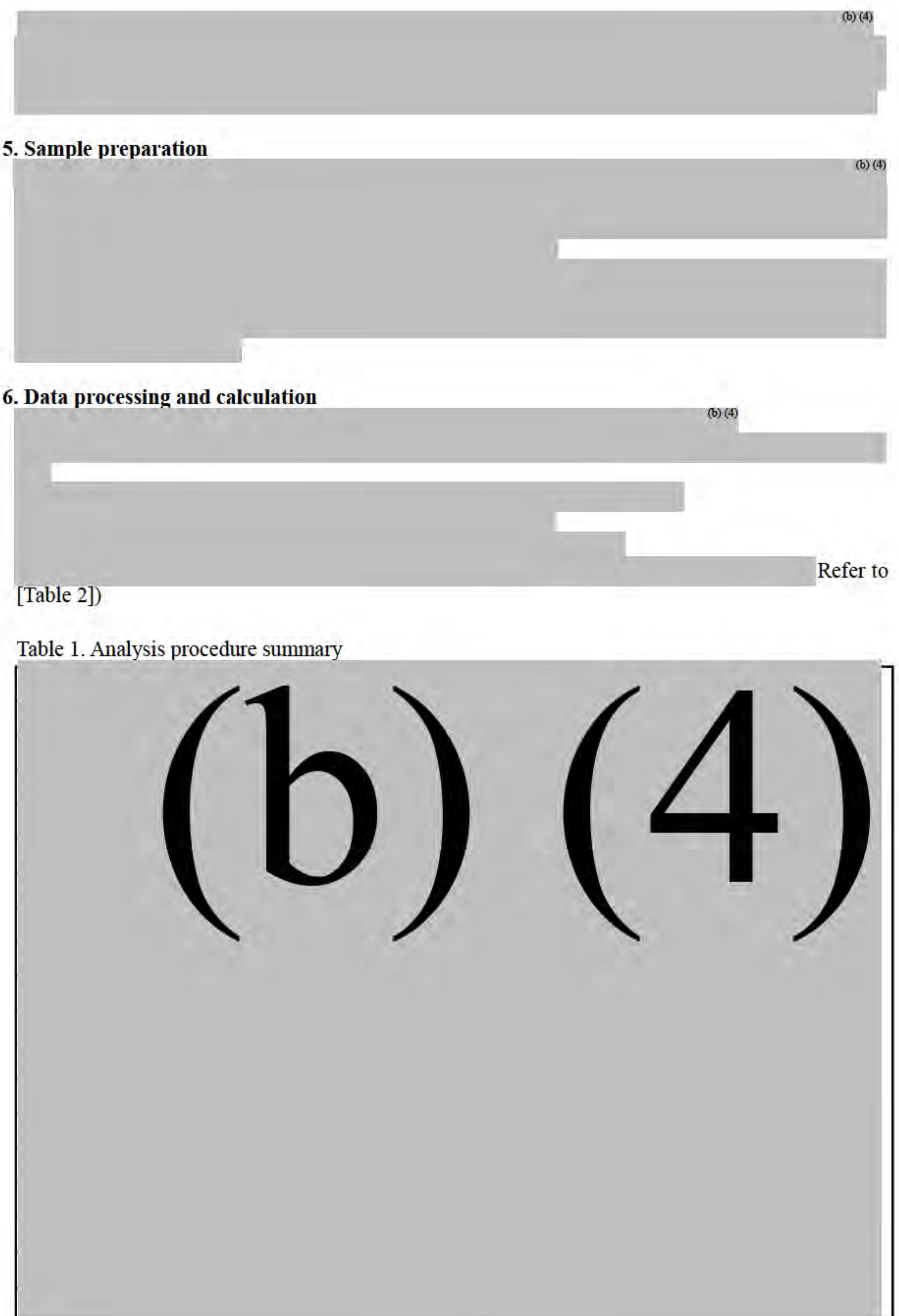
1) (b) (4)

2) (b) (4)

3) (b) (4) (b) (4)

4) (b) (4)

4. Standard preparation



(b) (4)

5. Sample preparation

(b) (4)

6. Data processing and calculation

(b) (4)

Refer to [Table 2])

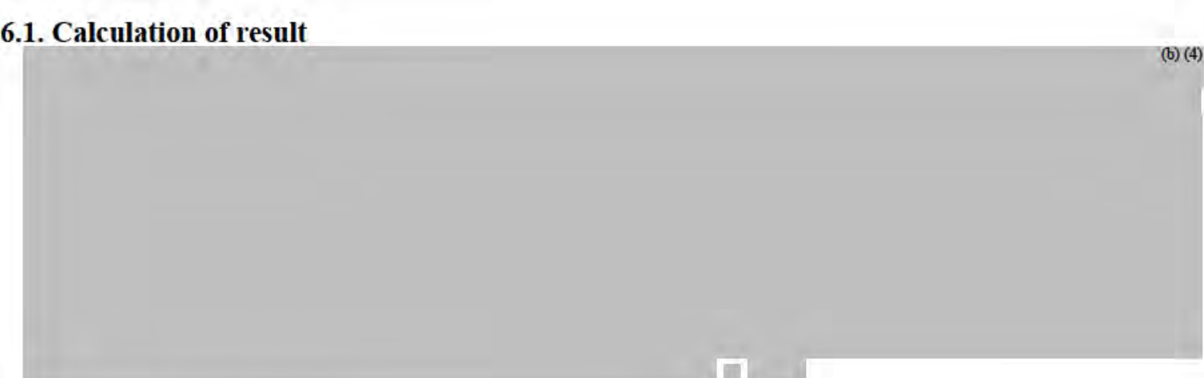
Table 1. Analysis procedure summary

(b) (4)

Table 2. Data calculation

	Standard solution	Sample solution
Weight		(b) (4)
Preparation concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

6.1. Calculation of result



(b) (4)

7. Data management

(b) (4)

II. METHOD VALIDATION

1. Identification test

(b) (4)

1.1. LC-MS/MS identification

To prepare 0.1 g/L (on the basis of L-valine concentration) sample solution, 0.14 g of Dried L-Valine

(b) (4)

Table 3. Gradient program of mobile phase of LC-MS/MS

Time (min)	Mobile phase A (%)	Mobile phase B (%)
Initial	80	20
5.0	(b) (6)	
14.0		
16.0		
16.1		
20.0		

1.2. FT-IR identification

(b) (4)

(b) (4)

2. Specificity (Blank test)
Blank test was evaluated by checking the existence of interfering peaks at retention time of the analytic when diluent (ultra-pure water) injected.

3. System suitability

of
on

4. Precision

(b) (4)

4.1. Homogeneity

(b) (4)

Table 6. Injection of homogeneity test

injection No.	file name	information	preparation
1	STD 1	standard solution 1	0.5 g/250 mL
2	H1_1	sample solution 1_1	0.7 g/250 mL
3	H2_1	sample solution 2_1	
4	H3_1	sample solution 3_1	
5	H4_1	sample solution 4_1	
6	H5_1	sample solution 5_1	
7	STD 2	standard solution 2	0.5 g/250 mL
8	H1_2	sample solution 1_2	0.7 g/250 mL
9	H2_2	sample solution 2_2	
10	H3_2	sample solution 3_2	

11	H4_2	sample solution 4_2	
12	H5_2	sample solution 5_2	
13	STD 3	standard solution 3	0.5 g/250 mL
14	H1_3	sample solution 1_3	
15	H2_3	sample solution 2_3	
16	H3_3	sample solution 3_3	0.7 g/250 mL
17	H4_3	sample solution 4_3	
18	H5_3	sample solution 5_3	
19	STD 4	standard solution 4	0.5 g/250 mL

Injection should according to the order as table above. It should start with standard solution and end with standard solution.

Table 7. Calculation the result of sample solution 1

	Standard solution	Sample solution
Weight		(b) (4)
prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of sample solution 1

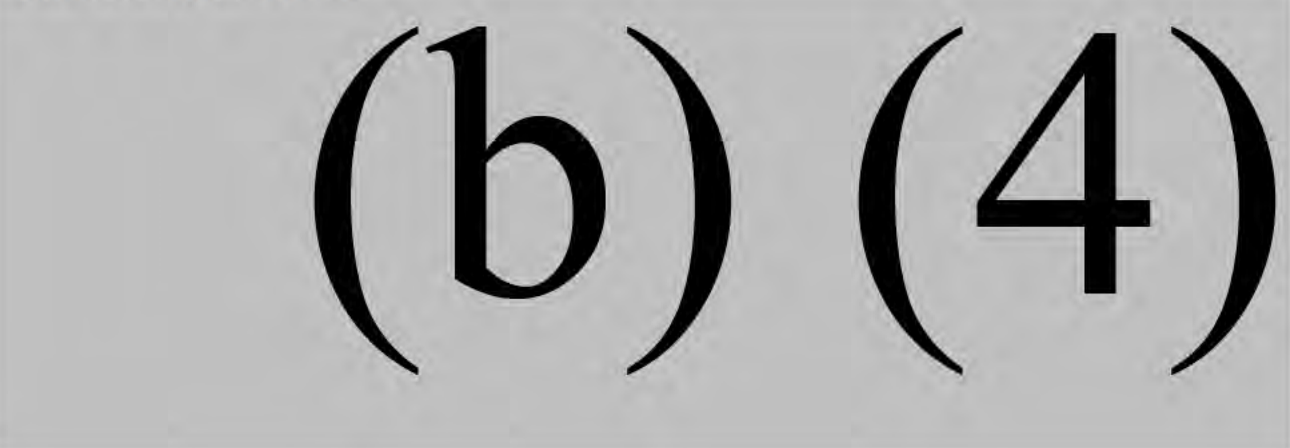


Table 8. Calculation the result of sample solution 2

	Standard solution	Sample solution
Weight		(b) (4)
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of sample solution 2

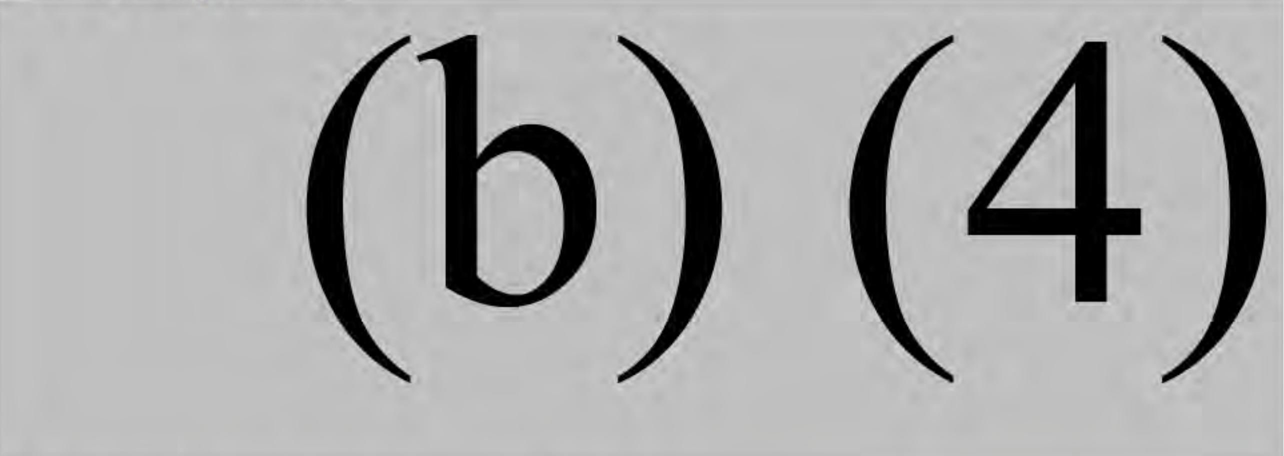
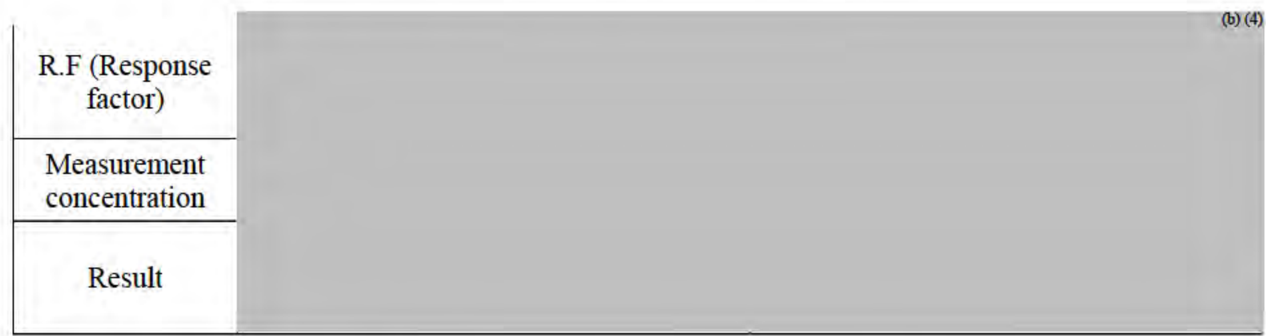


Table 9. Calculation the result of sample solution 3

	Standard solution	Sample solution
Weight		(b) (4)
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		

R.F (Response factor)	
Measurement concentration	
Result	

Result of sample solution 3

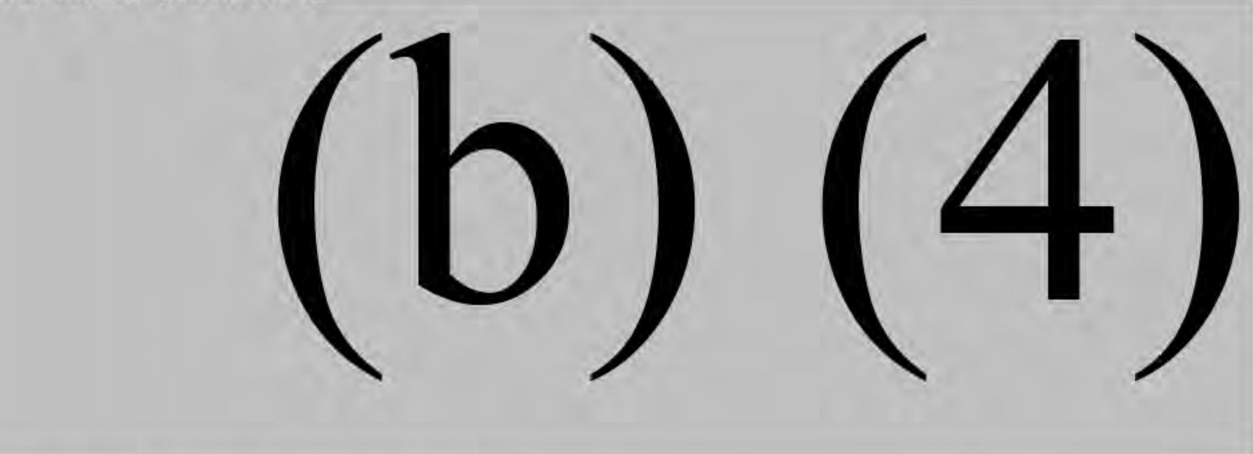


Table 10. Calculation the result of sample solution 4

	Standard solution	Sample solution
Weight		(b) (4)
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of sample solution 4

(b) (4)

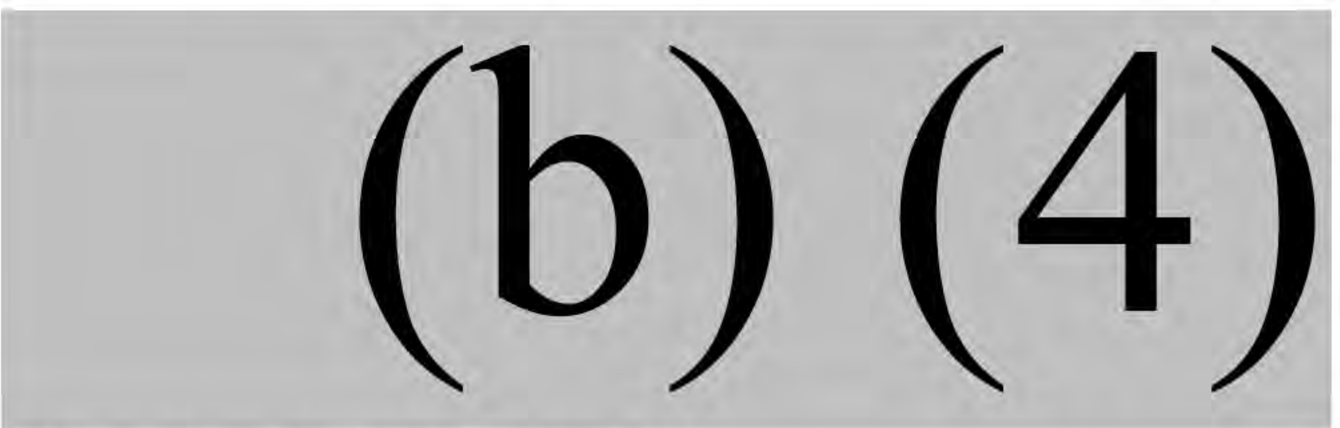
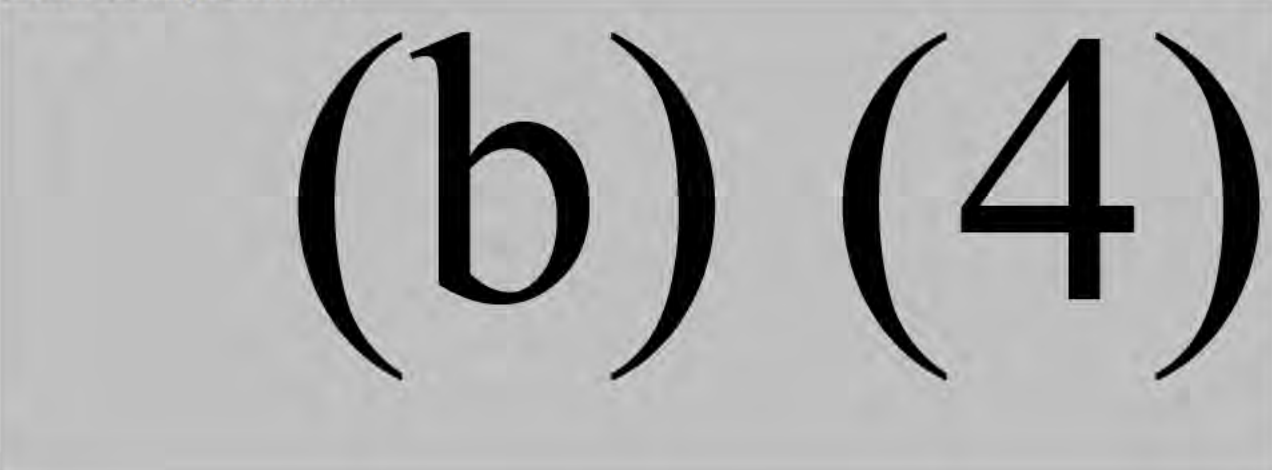


Table 11. Calculation the result of sample solution 5

	Standard solution	Sample solution
Weight		
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

(b) (4)

Result of sample solution 5



4.2. Stability

It was demonstrated by injecting the test solution at different time intervals of 0, 6, 12, 18, 24, 30, 36, 42, and 48 h. Test solution was prepared just before the analysis and it was stored in the refrigerator. The recovery of sample was satisfied with the acceptance criteria of 99%-101% and %RSD criteria of < 1%. Recovery was calculated compare to the results of 0hour. All test should be start if only 1 time point of results are not in acceptance criteria. Injection should according to the order as table. It should start with standard solution and end with standard solution.

Table 12. Injection of 0 hour

0 hour		
injection No.	file name	information
1	MSSTD0_1	standard solution 1
2	MSSAM0_1	sample solution 1
3	MSSTD0_2	standard solution 2
4	MSSAM0_2	sample solution 2
5	MSSTD0_3	standard solution 3
6	MSSAM0_3	sample solution 3
7	MSSTD0_4	standard solution 4

Table 13. Calculation the result of 0 hour

	Standard solution	Sample solution
Weight		
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

(b) (4)

Result of 0 hour of stability test
First, check the %RSD of peak area of standard solution and sample solution and it should less than 1%. After check the precision of peak area and calculate the R.F. of standard. The calculation equation would be

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

Table 14. Injection of 6 hour

6 hour		
injection No.	file name	information
8	MSSTD6_1	standard solution 1
9	MSSAM6_1	sample solution 1
10	MSSTD6_2	standard solution 2
11	MSSAM6_2	sample solution 2
12	MSSTD6_3	standard solution 3
13	MSSAM6_3	sample solution 3
14	MSSTD6_4	standard solution 4

Table 15. Calculation the result of 6 hour

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of 6 hour of stability test

(b) (4)

be

(b) (4)

The next step is calculate measurement concentration using R.F. value. The equation of measurement concentration of sample solution would be

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

Table 16. Injection of 12 hour

12 hour		
injection No.	file name	information
15	MSSTD12_1	standard solution 1
16	MSSAM12_1	sample solution 1
17	MSSTD12_2	standard solution 2
18	MSSAM12_2	sample solution 2
19	MSSTD12_3	standard solution 3
20	MSSAM12_3	sample solution 3
21	MSSTD12_4	standard solution 4

Table 17. Calculation the result of 12 hour

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of 12 hour of stability test

The next step is calculate measurement concentration using R.F. value. The equation of measurement concentration of sample solution would be

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

Table 18. Injection of 18 hour

18 hour		
injection No.	file name	information
22	MSSTD18_1	standard solution 1
23	MSSAM18_1	sample solution 1
24	MSSTD18_2	standard solution 2
25	MSSAM18_2	sample solution 2
26	MSSTD18_3	standard solution 3
27	MSSAM18_3	sample solution 3
28	MSSTD18_4	standard solution 4

Table 19. Calculation the result of 18 hour

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of 18 hour of stability test

(b) (4)

(b) (4)

The next step is calculate measurement concentration using R.F. value. The equation of measurement concentration of sample solution would be

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

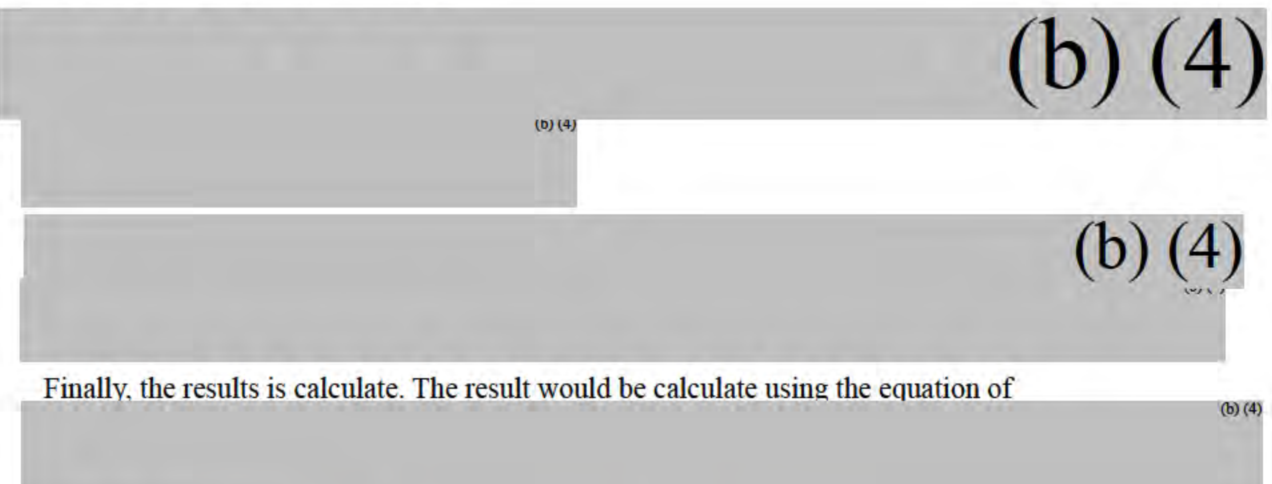
Table 20. Injection of 24 hour

24 hour		
injection No.	file name	information
29	MSSTD24_1	standard solution 1
30	MSSAM24_1	sample solution 1
31	MSSTD24_2	standard solution 2
32	MSSAM24_2	sample solution 2
33	MSSTD24_3	standard solution 3
34	MSSAM24_3	sample solution 3
35	MSSTD24_4	standard solution 4

Table 21. Calculation the result of 24 hour

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of 24 hour of stability test



Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

Table 22. Injection of 30 hour

30 hour		
injection No.	file name	information
36	MSSTD30_1	standard solution 1
37	MSSAM30_1	sample solution 1
38	MSSTD30_2	standard solution 2
39	MSSAM30_2	sample solution 2
40	MSSTD30_3	standard solution 3
41	MSSAM30_3	sample solution 3
42	MSSTD30_4	standard solution 4

Table 23. Calculation the result of 30 hour

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of 30 hour of stability test

(b) (4)

(b) (4)

The next step is calculate measurement concentration using R.F. value. The equation of measurement concentration of sample solution would be

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

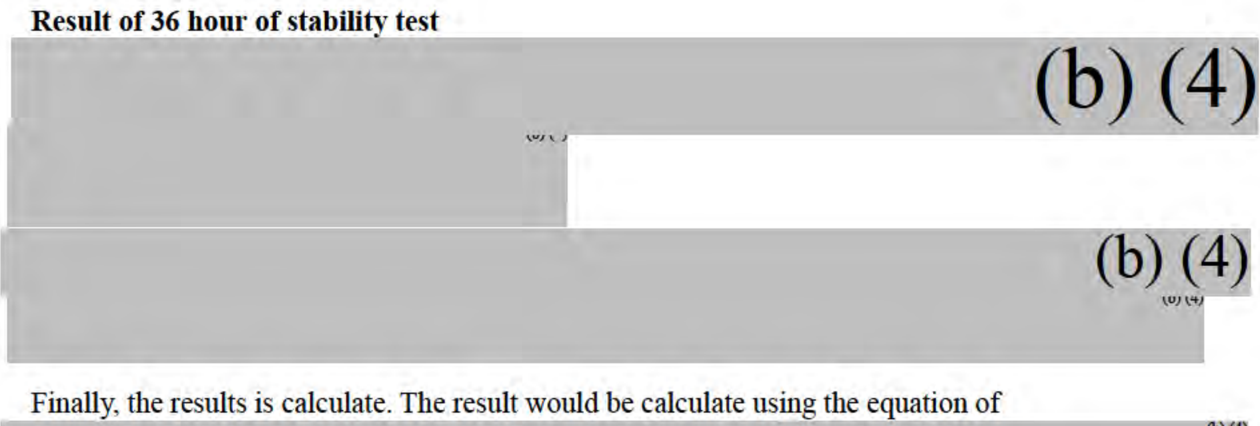
Table 24. Injection of 36 hour

36 hour		
injection No.	file name	information
43	MSSTD36_1	standard solution 1
44	MSSAM36_1	sample solution 1
45	MSSTD36_2	standard solution 2
46	MSSAM36_2	sample solution 2
47	MSSTD36_3	standard solution 3
48	MSSAM36_3	sample solution 3
49	MSSTD36_4	standard solution 4

Table 25. Calculation the result of 36 hour

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of 36 hour of stability test



Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

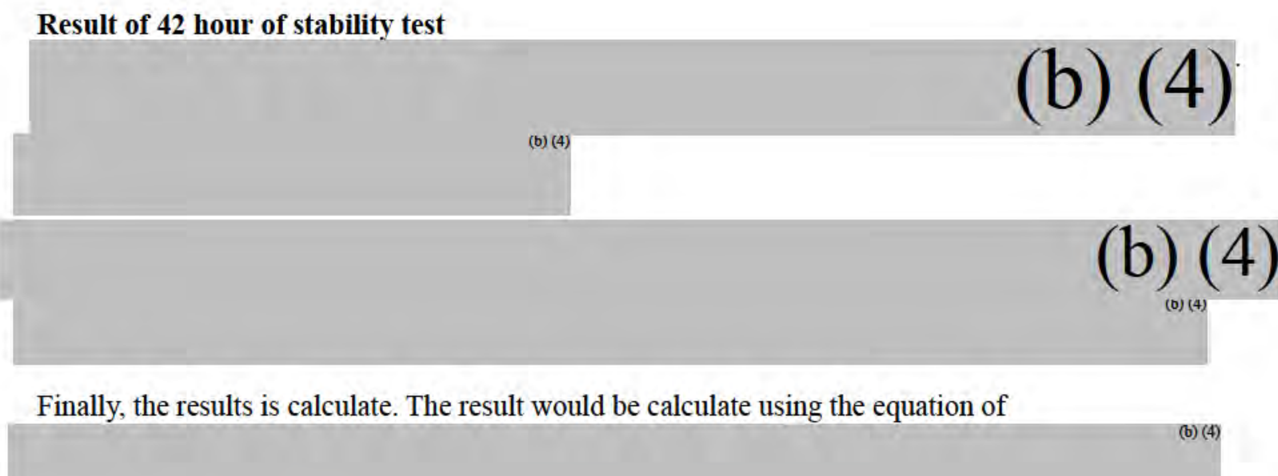
Table 26. Injection of 42 hour

42 hour		
injection No.	file name	information
50	MSSTD42_1	standard solution 1
51	MSSAM42_1	sample solution 1
52	MSSTD42_2	standard solution 2
53	MSSAM42_2	sample solution 2
54	MSSTD42_3	standard solution 3
55	MSSAM42_3	sample solution 3
56	MSSTD42_4	standard solution 4

Table 27. Calculation the result 42 hour

	(b) (4)
Weight	
Prepared concentration	
Area 1	
Area 2	
Area 3	
Area 4	
Average	
STDEV	
%RSD*	
R.F (Response factor)	
Measurement concentration	
Result	

Result of 42 hour of stability test



Finally, the results is calculate. The result would be calculate using the equation of

Table 28. Injection of 48 hour

48 hour		
injection No.	file name	information
57	MSSTD48_1	standard solution 1
58	MSSAM48_1	sample solution 1
59	MSSTD48_2	standard solution 2
60	MSSAM48_2	sample solution 2
61	MSSTD48_3	standard solution 3
62	MSSAM48_3	sample solution 3
63	MSSTD48_4	standard solution 4

Table 29. Calculation the result of 48 hour

	Standard solution	Sample solution
Weight		
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of 48 hour of stability test

(b) (4)

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

4.3 Precision

(b) (4)

Table 30. sample solution (2.7 g/L)

injection No.	file name
1	SP_1
2	SP_2
3	SP_3
4	SP_4
5	SP_5
6	SP_6
7	SP_7
8	SP_8
9	SP_9
10	SP_10
%RSD	<1%

Table 31. certified reference material solution (2g/L)

injection No.	file name
1	QP_1
2	QP_2
3	QP_3
4	QP_4
5	QP_5
6	QP_6
7	QP_7
8	QP_8
9	QP_9
10	QP_10
%RSD	<1%

%RSD = relative standard deviation = standard deviation / mean value x 100

5. Linearity

(b) (4)

Table 32. Injection of calibration curve

	Conc. (g/L)	file name	Mean area
STD 1(25%)	0.50	L1_1	Mean area of L1_1 to L1_3
		L1_2	
		L1_3	
STD 2(50%)	1.00	L2_1	Mean area of L2_1 to L2_3
		L2_2	
		L2_3	
STD 3(80%)	1.60	L3_1	Mean area of L3_1 to L3_3
		L3_2	
		L3_3	
STD 4(100%)	2.00	L4_1	Mean area of L4_1 to L4_3
		L4_2	
		L4_3	
STD 5(120%)	2.40	L5_1	Mean area of L5_1 to L5_3
		L5_2	
		L5_3	

Evaluate calibration curve was using the data as table below (using mean area of triplet injection)

Table 33. Calibration curve

	Conc. (g/L)	Mean area
STD 1(25%)	0.5	Mean area of L1_1 to L1_3
STD 2(50%)	1.0	Mean area of L2_1 to L2_3
STD 3(80%)	1.6	Mean area of L3_1 to L3_3
STD 4(100%)	2.0	Mean area of L4_1 to L4_3
STD 5(120%)	2.4	Mean area of L5_1 to L5_3

6. Limit of detection and limit of quantification

To determine LOD (Limit of detection) and LOQ (Limit of quantification) is calculated from the slope and standard deviation (SD) of the linearity regression curve.

- Limit of detection, LOD = SD × (b) (4)/slope
- Limit of quantification, LOQ = SD × (b) (4)/slope

Where:

- (b) (4)
- Slope = slope of the linearity curve obtained by regression analysis
- n = number of points
- y = experimental response
- y'= calculated response found using the x-value (concentration) and the regression equation
- (y-y') = difference or residual between experimental and calculated response for each x-value

After calculating the LOD and LOQ, the concentration is actually prepared and analyzed.

7. Accuracy

According to '4. ACCURACY' part in 'ICH guideline, Validation of Analytical Procedures: Text and Methodology Q2 (R1)', accuracy test was conducted. This method is assay for chemical substance. The analytical procedure to analyze of known purity of sample, which is CRM in this test, was applied and compared the results with uncertainty.

Accuracy of Dried L-Valine Fermentation Product was evaluated using CRM (certified material reference, PHR1172).

When the analysis is performed using CRM as a sample and statistical processing is performed, the En value of the result should be less than or equal to an absolute value of 1 (with reference to ISO 17043, General requirements for proficiency testing). The meaning of the En value indicates how many times the difference between the CRM value and the tester's result value is in the CRM and the tester's combined uncertainty values. At this time, when | En | value is within 1, it is judged as satisfactory, and the difference value should not be larger than the uncertainty value.

We considered uncertainty arising from the reference material, balances, volumetric measuring devices, sample preparation, and instrumental factors. The measurement uncertainty (U), which is the expanded uncertainty, was obtained by multiplying the combined standard uncertainty by a coverage factor, k = 2, which yields a confidence level of approximately 95 %. The equations for the measurement uncertainty (U) are as follows:

The purity and uncertainty of the test report of PHR1172 were 98.90 % ± 0.07 % (approximate level of confidence, k = 2).

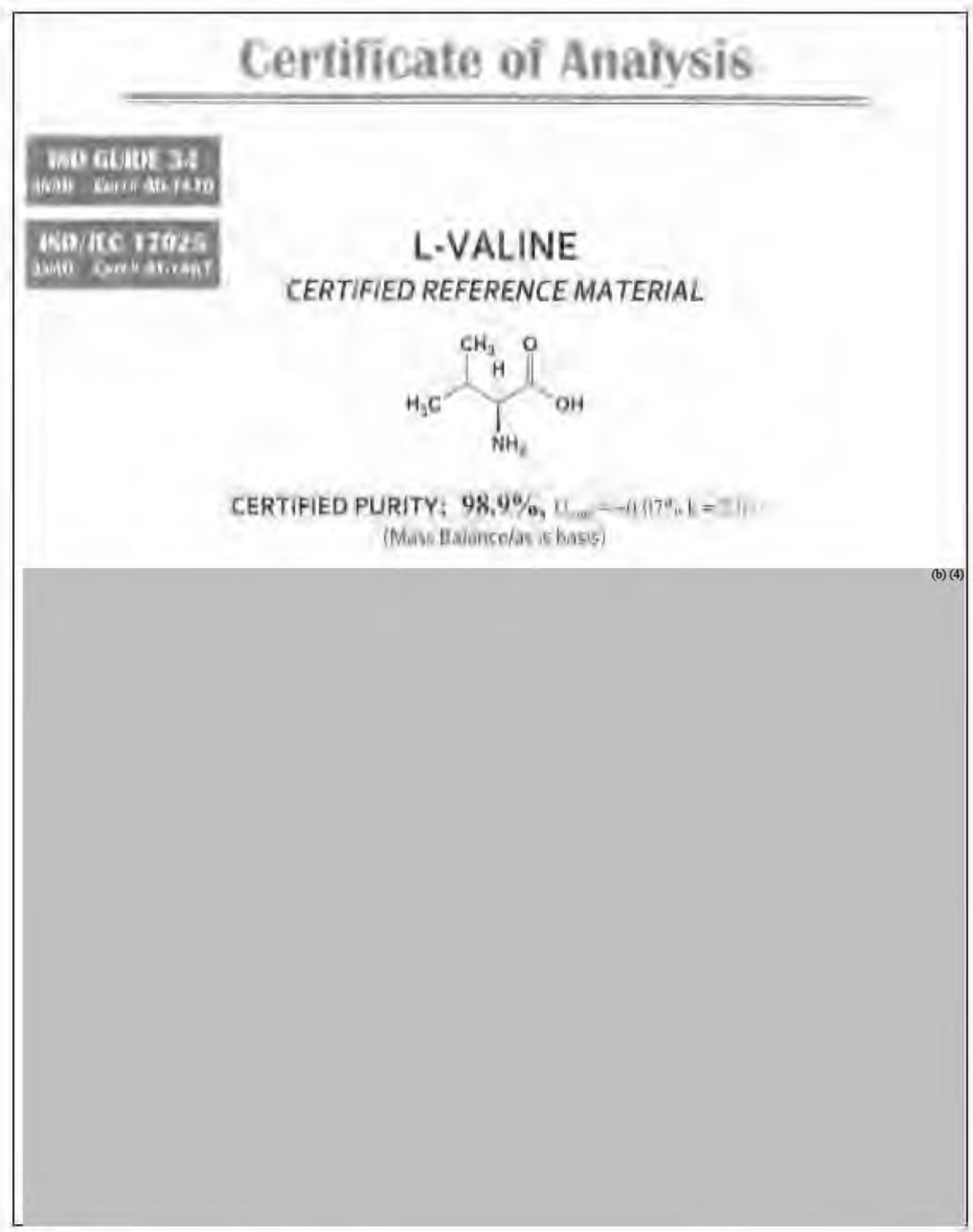

Certificate of Analysis
ISO GUIDE 34
ISO/IEC 17025
L-VALINE
CERTIFIED REFERENCE MATERIAL
CERTIFIED PURITY: 98.9%,
(b) (4)

Table 34. Injection of CRM test

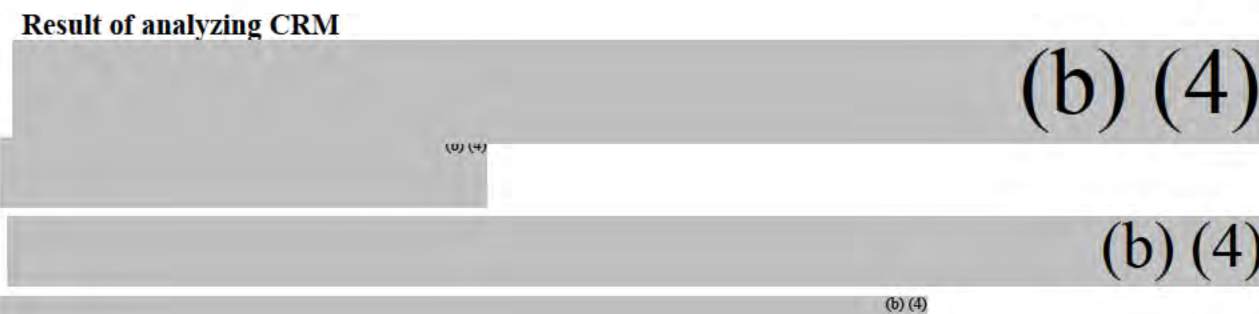
injection No.	file name	information
1	ASTD_1	standard solution 1
2	CM_1	CRM solution 1
3	ASTD_2	standard solution 2
4	CM_2	CRM solution 2
5	ASTD_3	standard solution 3
6	CM_3	CRM solution 3
7	ASTD_4	standard solution 4

Inject the order of standard solution1-CRM solution1-standard solution2-CRM solution2-standard solution3-CRM solution3- standard solution4

Table 35. Calculation the result of CRM

	Standard solution	CRM solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result of analyzing CRM



Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

Calculation of |En| value

(b) (4)

- X_{lab}: Result of lab
- X_{ref}: Content of CRM 1172
- U_{lab}: Measurement uncertainty of lab
- U_{ref}: Measurement uncertainty of CRM 1172

6. Robustness

ICH defined the robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness partly assured by good system suitability specifications. Thus, it is important to set tight, but realistic and systematically suitable specifications. Test conditions such as column temperature, pH, and composition of mobile phase were varied in this test. Robustness of the method was evaluated by changing the column temperature (b) (4)

Recovery was calculated compare to the mean results of homogeneity. The recovery of sample was satisfied with the acceptance criteria of 99%-101%.
All sample and standard solution was prepared before robustness test and it was stored in refrigerator.
During the robustness test, if some impurity peak is overlapped or the results of changed condition is not in acceptance criteria, it should reported and change the condition of robustness.

6.1. Change column temperature to 35°C

Table 36. HPLC condition (change column temperature to 35°C)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV (b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size (b) (4)
Column Temp.	**35 °C**
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, pH 2.5 (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Injection volume	5 µl

Changed column temperature to 35 °C (from 40°C) and other condition was same to reference condition.

6.1.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)		(b) (4)	1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			1137 mL

(b) (4)

Table 37. Injection (change column temperature to 35℃)

injection No.	file name	information
1	35STD_1	standard solution 1
2	35SAM_1	sample solution 1
3	35STD_2	standard solution 2
4	35SAM_2	sample solution 2
5	35STD_3	standard solution 3
6	35SAM_3	sample solution 3
7	35STD_4	standard solution 4

Table 38. Calculation the results (change column temperature to 35℃)

	Standard solution	Sample solution
Weight		(b) (4)
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		

Result	(b) (4)

Result when change column temperature to 35°C

(b) (4)

(b) (4)

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

6.2. Change column temperature to 45°C

Table 39. HPLC condition (change column temperature to 45°C)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV (b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size (b) (4)
Column Temp.	**45 °C**
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, pH (b) (4) (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Injection volume	5 µl

Changed column temperature to 45 °C (from 40°C) and other condition was same to reference condition.

6.2.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			1137 mL

(b) (4)

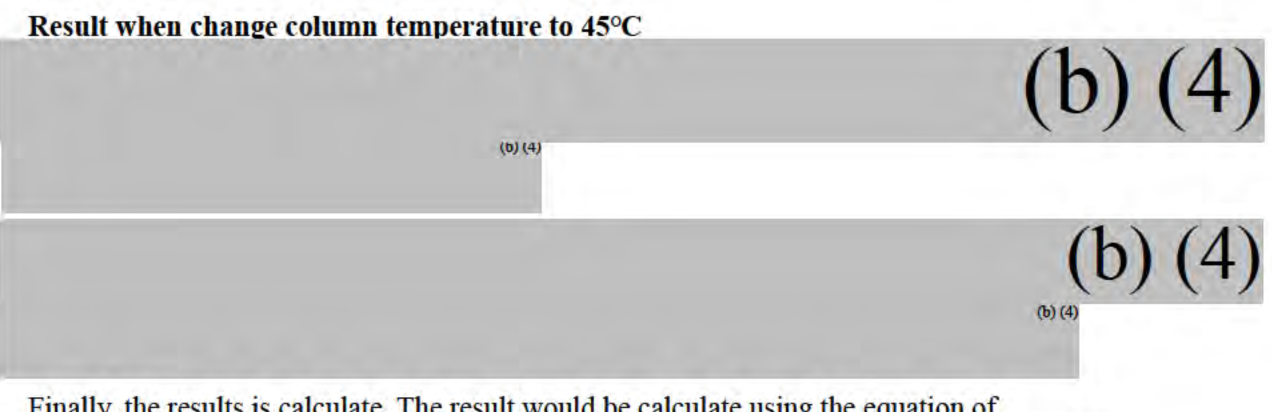
Table 40. Injection (change column temperature to 45°C)

injection No.	file name	information
1	45STD_1	standard solution 1
2	45SAM_1	sample solution 1
3	45STD_2	standard solution 2
4	45SAM_2	sample solution 2
5	45STD_3	standard solution 3
6	45SAM_3	sample solution 3
7	45STD_4	standard solution 4

Table 41. Calculation the results (change column temperature to 45°C)

	Standard solution	Sample solution
Weight		(b) (4)
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result when change column temperature to 45°C



Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

concentration of sample soltuion(45SAM)×100".

6.3. Change flow rate of mobile phase to 0.8 mL/min

Table 42. HPLC condition (change flow rate to 0.8 mL/min)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV(b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size(b) (4) μm
Column Temp.	40 ℃
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, pH 2.5 (by H_3PO_4)
Flow rate of mobile phase	**0.8 ml/min**
Injection volume	5 μl

Changed flow rate to 0.8 mL/min (from 1.0mL/min) and other condition was same to reference condition.

6.3.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			1137 mL

(b) (4)

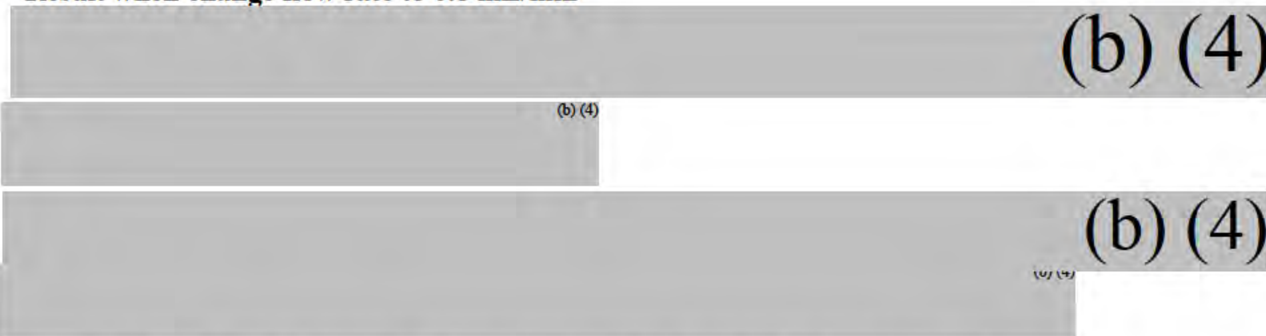
Table 43. Injection (change flow rate to 0.8 mL/min)

flow rate 0.8 mL/min		
injection No.	file name	information
1	F1STD_1	standard solution 1
2	F1SAM_1	sample solution 1
3	F1STD_2	standard solution 2
4	F1SAM_2	sample solution 2
5	F1STD_3	standard solution 3
6	F1SAM_3	sample solution 3
7	F1STD_4	standard solution 4

Table 44. Calculation the results (change flow rate to 0.8 mL/min)

	Standard solution	Sample solution
Weight		(b) (4)
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result when change flow rate to 0.8 mL/min



Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

6.4. Change flow rate of mobile phase to 1.2 mL/min

Table 45. HPLC condition (change flow rate to 1.2 mL/min)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV(b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size(b) (4)
Column Temp.	40 °C

Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, pH 2.5 (by H_3PO_4)
Flow rate of mobile phase	**1.2 ml/min**
Injection volume	5 µl

Changed flow rate to 1.2 mL/min (from 1.0mL/min) and other condition was same to reference condition.

6.4.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			1137 mL

(b) (4)

Table 46. Injection (change flow rate to 1.2 mL/min)

injection No.	file name	information
1	F2STD_1	standard solution 1
2	F2SAM_1	sample solution 1
3	F2STD_2	standard solution 2
4	F2SAM_2	sample solution 2
5	F2STD_3	standard solution 3
6	F2SAM_3	sample solution 3
7	F2STD_4	standard solution 4

Table 47. Calculation the results (change flow rate to 1.2 mL/min)

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		

R.F (Response factor)	(b) (4)
Measurement concentration	
Result	

Result when change flow rate to 1.2 mL/min

(b) (4)

(b) (4)

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

6.5. Change the pH of mobile phase to pH 2.3

Table 48. HPLC condition (change pH to 2.3)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV(b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size(b) (4)
Column Temp.	40 °C
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, **pH 2.3** (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Injection volume	5 µl

Changed pH of mobile phase to pH 2.3 (from pH 2.5) and other condition was same to reference condition.

6.5.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		1 000 mL
1-Octanfonic acid sodium salt (OSA)			

Phosphoric Acid(H_3PO_4)	(b) (4)
Acetonitrile (CH_3CN)	(b) (4)

(b) (4)

Table 49. Injection (change pH to 2.3)

injection No.	file name	information
1	P1STD_1	standard solution 1
2	P1SAM_1	sample solution 1
3	P1STD_2	standard solution 2
4	P1SAM_2	sample solution 2
5	P1STD_3	standard solution 3
6	P1SAM_3	sample solution 3
7	P1STD_4	standard solution 4

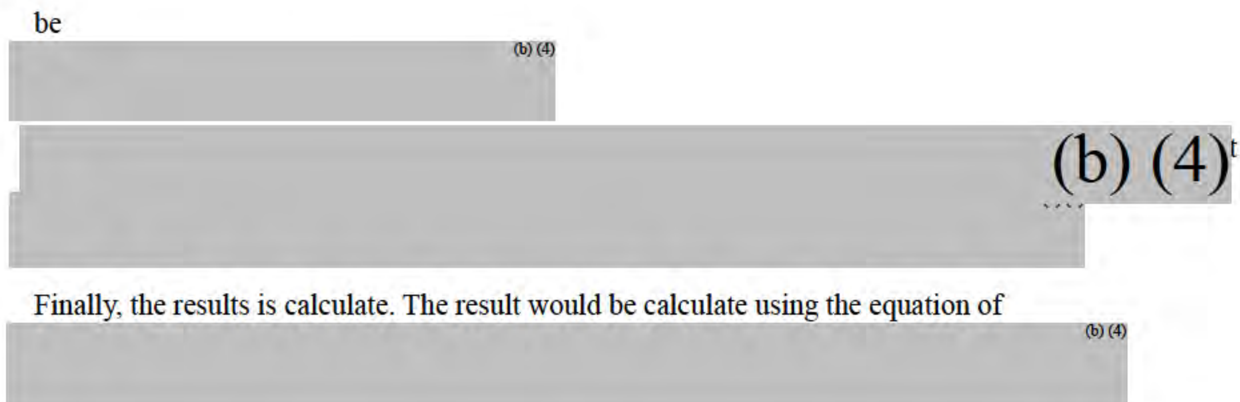
Table 50. Calculation the results (change pH to 2.3)

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result when change pH of mobile phase to pH 2.3

(b) (4)

be



Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

6.6. Change the pH of mobile phase to pH 2.7

Table 51. HPLC condition (change pH to 2.7)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV(b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size (b) (4)
Column Temp.	40 °C
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, **pH 2.7** (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Injection volume	5 µl

Changed pH of mobile phase to pH 2.7 (from pH 2.5) and other condition was same to reference condition.

6.6.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			(b) (4) mL

(b) (4)

Table 52. Injection (change pH to 2.7)

injection No.	file name	information
1	P2STD_1	standard solution 1
2	P2SAM_1	sample solution 1

3	P2STD_2	standard solution 2
4	P2SAM_2	sample solution 2
5	P2STD_3	standard solution 3
6	P2SAM_3	sample solution 3
7	P2STD_4	standard solution 4

Table 53. Calculation the results (change pH to 2.7)

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result when change pH of mobile phase to pH 2.7

(b) (4)

The next step is calculate measurement concentration using R.F. value. The equation of measurement concentration of sample solution would be

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

6.7. Change the CH_3CN ratio of mobile phase to 10%

Table 54. HPLC condition (change CH_3CN ratio to 10%)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV (b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size (b)(4) µm
Column Temp.	40 °C
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in **10% CH_3CN**, pH 2.5 (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Injection volume	5 µl

Changed CH_3CN ratio of mobile phase to 10% (from 12%) and other condition was same to reference condition.

6.7.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			**1111 mL**

(b) (4)

Table 55. Injection (change CH_3CN ratio to 10%)

injection No.	file name	information
1	8STD1_1	standard solution 1
2	8SAM1_1	sample solution 1
3	8STD1_2	standard solution 2
4	8SAM1_2	sample solution 2
5	8STD1_3	standard solution 3
6	8SAM1_3	sample solution 3
7	8STD1_4	standard solution 4

Table 56. Calculation the results (change CH_3CN ratio to 10%)

	Standard solution	Sample solution
Weight		(b) (4)
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		

Result when change CH_3CN ratio of mobile phase to 10%

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

6.8. Change the CH3CN ratio of mobile phase to 14%

Table 57. HPLC condition (change CH_3CN ratio to 14%)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV (b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size (b)(µm
Column Temp.	40 °C
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in **14% CH_3CN**, pH 2.5 (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Injection volume	5 µl

Changed CH_3CN ratio of mobile phase to 14% (from 12%) and other condition was same to reference condition.

6.8.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			**1163 mL**

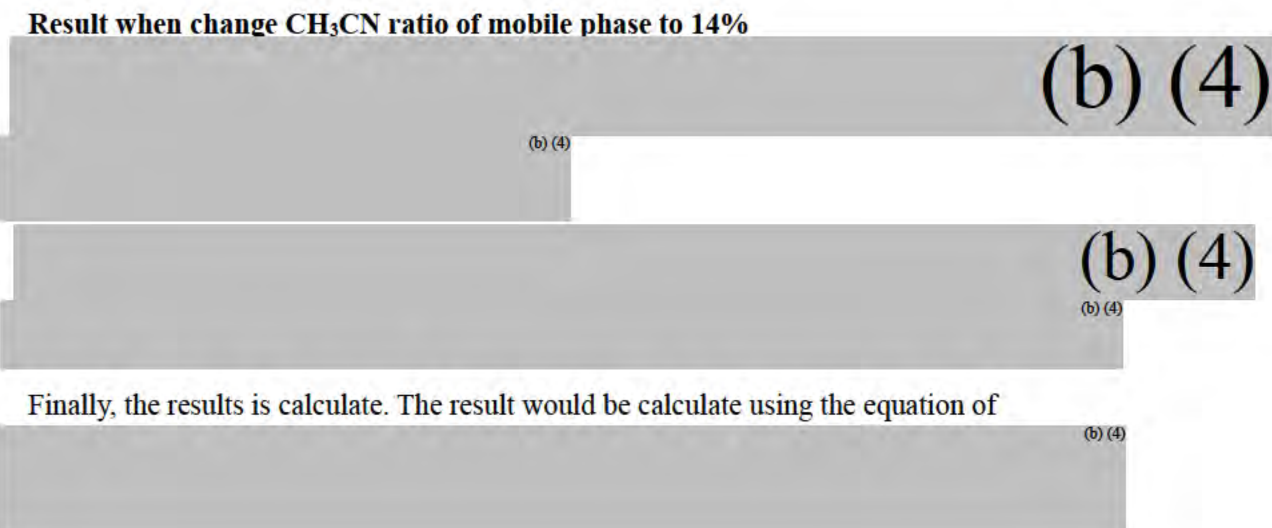
(b) (4)

Table 58. Injection (change CH_3CN ratio to 14%)

injection No.	file name	information
1	12STD1_1	standard solution 1
2	12SAM1_1	sample solution 1
3	12STD1_2	standard solution 2
4	12SAM1_2	sample solution 2
5	12STD1_3	standard solution 3
6	12SAM1_3	sample solution 3
7	12STD1_4	standard solution 4

Table 59. Calculation the results (change CH_3CN ratio to 14%)

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		
Result		



Result when change CH_3CN ratio of mobile phase to 14%

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

6.9. Change the column particle size to 3.5 μm (run 1)

Table 60. HPLC condition (column particle size to 3.5 μm, run 1)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV (b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size (b) (4) μm
Column Temp.	40 °C
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, pH 2.5 (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Injection volume	5 μl

Changed particle size of column to 3.5 μm (from 3.0 μm) and other condition was same to reference condition.

6.9.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)	(b) (4)		1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			1137 mL

(b) (4)

Table 61. Injection (column particle size to 3.5 μm, run 1)

injection No.	file name	information
1	XSTD1_1	standard solution 1
2	XSAM1_1	sample solution 1
3	XSTD1_2	standard solution 2
4	XSAM1_2	sample solution 2
5	XSTD1_3	standard solution 3
6	XSAM1_3	sample solution 3
7	XSTD1_4	standard solution 4

Table 62. Calculation the results (column particle size to 3.5 μm, run 1)

	Standard solution	Sample solution
Weight	(b) (4)	
Prepared concentration		

Area 1	(b) (4)
Area 2	
Area 3	
Area 4	
Average	
STDEV	
%RSD*	
R.F (Response factor)	
Measurement concentration	
Result	

Result when change particle size of column to 3.5 µm

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

6.10. Change the column particle size to 3.5 µm (run 2)

Table 63. HPLC condition (column particle size to 3.5 µm, run 2)

	Condition
System	HPLC
Detector	Photodiode array detector (Shimadzu) UV (b) (4) nm
Column	ODS C18, 150 × 4.6 mm, particle size (b) (4) µm
Column Temp.	40 ℃
Mobile phase	16.7 mM-KH_2PO_4 + 5 mM OSA in 12% CH_3CN, pH 2.5 (by H_3PO_4)
Flow rate of mobile phase	1.0 ml/min
Injection volume	5 µl

Changed particle size of column to 3.5 μm (from 3.0 μm) and other condition was same to reference condition.

6.10.1. Mobile phase solution preparation method

Reagent Name	Concentration (mM)	Sampling amount (g)	Total volume (mL)
Potassium dihydrogenphosphate (KH_2PO_4)		(b) (4)	1 000 mL
1-Octanfonic acid sodium salt (OSA)			
Phosphoric Acid(H_3PO_4)			
Acetonitrile (CH_3CN)			1137 mL

(b) (4)

Table 64. Injection (column particle size to 3.5 μm, run 2)

injection No.	file name	information
1	XSTD2_1	standard solution 1
2	XSAM2_1	sample solution 1
3	XSTD2_2	standard solution 2
4	XSAM2_2	sample solution 2
5	XSTD2_3	standard solution 3
6	XSAM2_3	sample solution 3
7	XSTD2_4	standard solution 4

Table 65. Calculation the results (column particle size to 3.5 μm, run 2)

	Standard solution	Sample solution
Weight		(b) (4)
Prepared concentration		
Area 1		
Area 2		
Area 3		
Area 4		
Average		
STDEV		
%RSD*		
R.F (Response factor)		
Measurement concentration		

	(b) (4)
Result	

Result when change particle size of column to 3.5 µm (run 2)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Finally, the results is calculate. The result would be calculate using the equation of

(b) (4)

Cerrito, Chelsea

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Monday, July 27, 2020 3:05 PM
To: Tang, Lei; Wong, Geoffrey K; Animalfood-premarket
Cc: '강민경님 [Min Kang]'; Keith D. Haydon; Biesiada,Thomas님
Subject: RE: GRAS AGRN 35 --AMENDMENT --Email 3---supplements 7-13
Attachments: Supplement 7. REVISED Appendix 1_Method Validation .pdf; Supplement 8. REVISED Appendix 1 _Method Validation-raw data.pdf; Supplement 9. REVISED Appendix 1_Method Validation_Amendment Report.pdf; Supplement 10. The Detailed Description of Extra Peaks.pdf; Supplement 11. REVISED Appendix 1_Method Validation-CRM1172 VAL COA.pdf; Supplement 12. Test report_Biogenic amine analysis_Parental strain vs Production strain.pdf; Supplement 12-1 _RawData_Biogenic amine analysis_ATCC14067.pdf; Supplement 12-2_RawData_Biogenic amine analysis_KCCM11201P.pdf; Supplement 12-3_RawData_Biogenic amine analysis_KCCM80058.pdf; Supplement 13. REVISED Appendix 3_Pre-fermentation_LVFP.pdf

Dr. Tang:

This email contains supplements (attachments) 7-13 for AGRN 35.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

From: Kristi Smedley [mailto:smedley@cfr-services.com]
Sent: Monday, July 27, 2020 3:02 PM
To: 'Tang, Lei'; 'Wong, Geoffrey K'; Animalfood-premarket (Animalfood-premarket@fda.hhs.gov)
Cc: '강민경님 [Min Kang]' (mg.kang@cj.net); Keith D. Haydon (keith.haydon@cj.net); Biesiada,Thomas님 (thomas.biesiada@cj.net)
Subject: RE: GRAS AGRN 35 --AMENDMENT --Email 2---supplements 1-6

Dr. Tang:

This email contains Supplements (attachments 1-6) to support AGRN 35 amendment.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)

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From: Kristi Smedley [mailto:smedley@cfr-services.com]
Sent: Monday, July 27, 2020 3:00 PM
To: 'Tang, Lei'; 'Wong, Geoffrey K'; Animalfood-premarket (Animalfood-premarket@fda.hhs.gov)
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Subject: GRAS AGRN 35 --AMENDMENT --Email 1

Dr. Tang:

On behalf of CJ, I am providing the amendment of AGRN 35, L-Valine fermentation product , as requested. This email provides a part of the supporting material for this amendment.

You will be receiving a series of emails, to assure that all supplements (attachments) are received. I will number them in the subject line of the email, as well as provide a brief description in the body of each email. You should receive 4 reference files and 15 supplements (attachments).

This email will include the signed amendment letter as well as all reference publications.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

From: Tang, Lei [mailto:Lei.Tang@fda.hhs.gov]
Sent: Wednesday, July 08, 2020 12:02 PM
To: Kristi Smedley
Cc: Wong, Geoffrey K
Subject: RE: GRAS AGRN 35

Dear Dr. Smedley,

This letter is in response to your email dated July 3, 2020 requesting for meeting minutes from the July 1, 2020 meeting between the Center for Veterinary Medicine and CJ CheilJedang Corporation.

Please find enclosed a copy of our meeting minutes for the referenced meeting.

If you have any questions concerning this letter, please contact me via email at Lei.Tang@fda.hhs.gov or by phone at 240-402-5922. Please refer to AGRN #35 in any future correspondences.

Sincerely,

Lei Tang, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal feeds
U.S. Food and Drug Administration

Tel: 240-402-5922
lei.tang@fda.hhs.gov

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From: Kristi Smedley <smedley@cfr-services.com>
Sent: Friday, July 3, 2020 9:32 AM
To: Tang, Lei <Lei.Tang@fda.hhs.gov>
Cc: Wong, Geoffrey K <Geoffrey.Wong@fda.hhs.gov>; Keith D. Haydon <keith.haydon@cj.net>; '강민경님 [Min Kang]' <mg.kang@cj.net>
Subject: RE: GRAS AGRN 35

Dr. Tang

This is a request for the notes of the FDA teleconference (July 1, 2020) specific to issues raised during the AGRN 35 review.

We will accept these notes by email.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

REPORT

Analytical Method Validation of Dried L-Valine Fermentation Product using HPLC
(Confidential)

Original final report date: Aug 28, 2018
Revised version report date: July 22, 2020

Study Director	Quality Assurance Manager
정 다미	(b) (4)
Dami Jeong	

CJ Research Institute of Biotechnology

1. Introduction

There are several official methods to analyze L-valine. The commonly used method of L-valine analysis is potentiometric titration with perchloric acid, however, most other amino acids could also be detected by this method. Therefore, titration method is not applicable in case of sample containing the other amino acids as an impurity. Another analytical method for L-valine is HPLC analysis using the sample reacted with ninhydrin or orthophtalaldehyde (OPA). The analytical intensity of this method is very high that meant there is a possibility of error-prone from the high dilution factor.

For this reason, CJ developed the analytical method for 'L-valine' and this analytical method was verified by method validation.

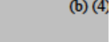
2. Test Article

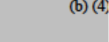
(1) Test Article

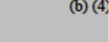
1) Identity: Dried L-Valine Fermentation Product (VAL Pro)
2) Lot number: GVAL180116
3) Purity: > 72.0% (L-Valine, Refer to Attachment 2-Raw data 6-COA)
4) Date of Receipt: Jan 22, 2018
5) Amount of Receipt: Approximately 100 g
6) Storage Conditions: Room temperature
7) Supplier: CJ Research Institute of Biotechnology

(2) Reference Standard

1) Identity: L-Valine
2) Product No.: V0500
3) Purity: > 98.0%
4) Date of Receipt: May 15, 2017
5) Amount of Receipt: 25 g
6) Storage Conditions: Room temperature
7) Supplier: (b) (4)
8) Expiry Date (Retest date): Mar, 2020







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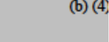

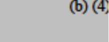

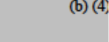

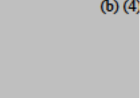

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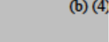

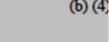

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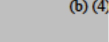

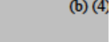

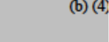

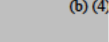

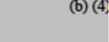

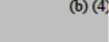

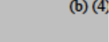

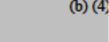

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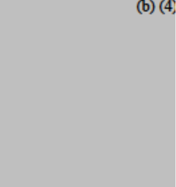
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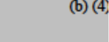

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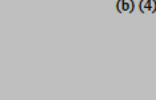
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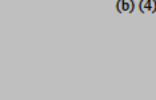
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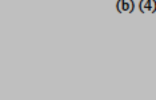
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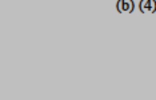
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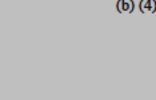
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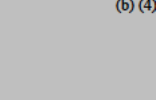
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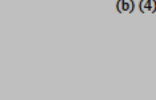
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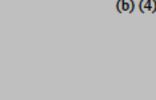
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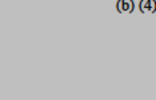
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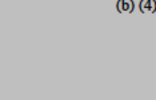
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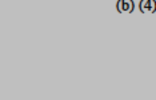

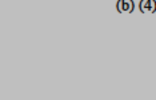
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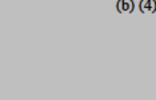

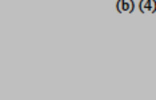
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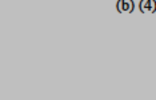
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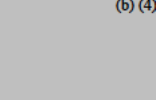
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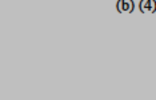
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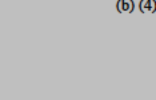
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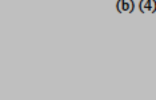
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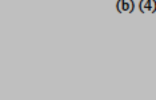
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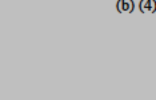
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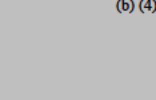
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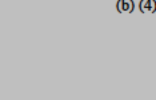

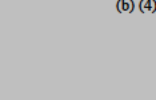
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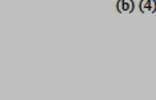
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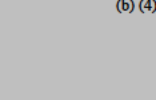
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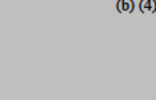
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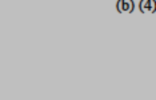
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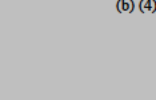
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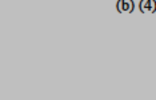
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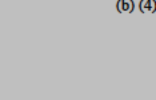

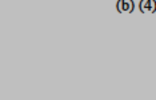
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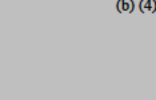
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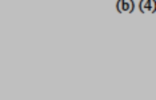

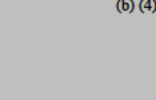
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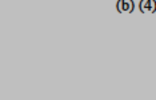

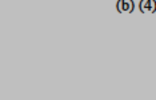

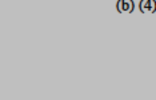
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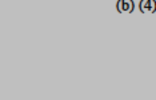
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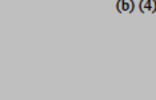
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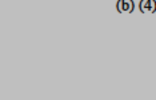
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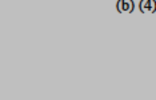
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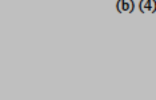
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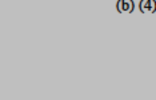
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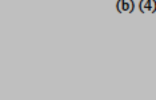
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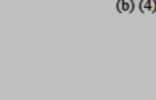
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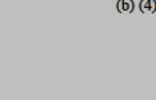

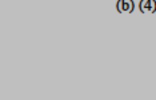

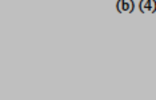
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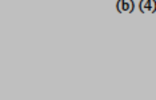
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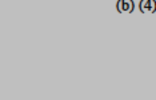
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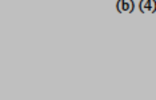
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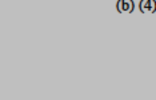
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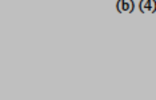
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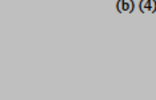

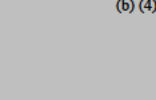
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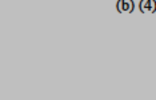
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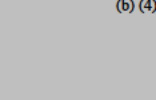
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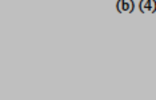
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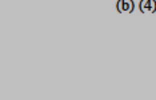
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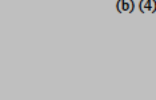
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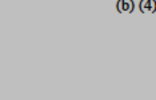
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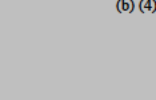
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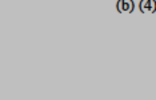
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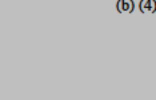
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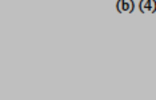
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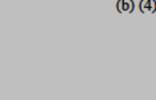

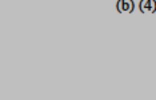
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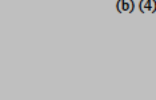

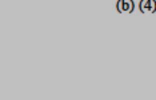
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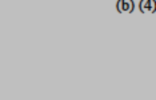

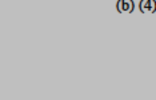

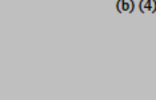
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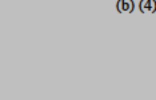

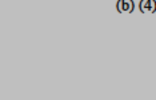
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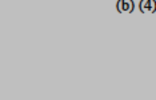

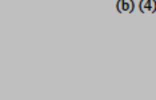
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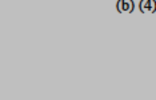
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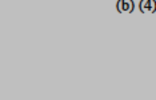
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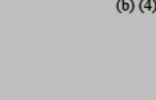
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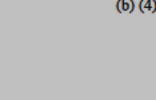
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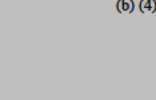

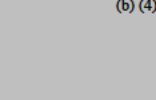
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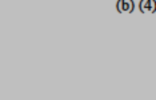

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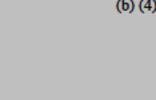

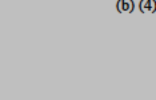
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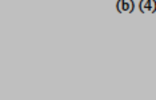

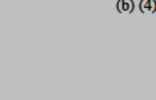
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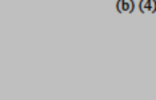
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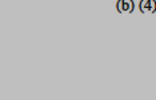

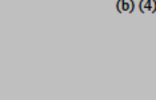
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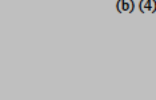

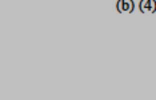
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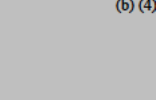

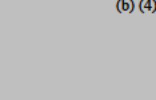
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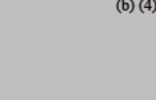
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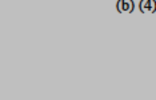
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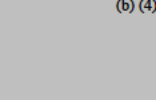
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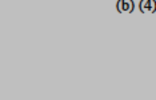
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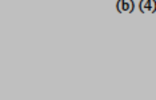

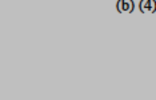
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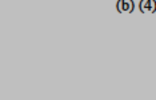

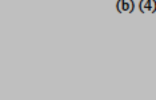
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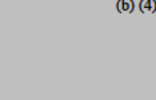
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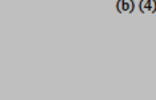
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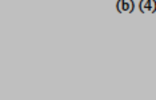
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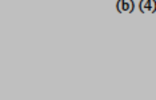
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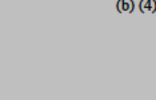
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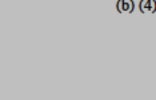
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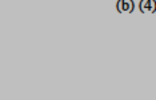
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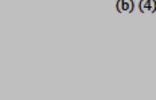
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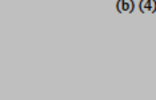

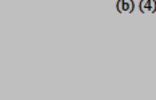
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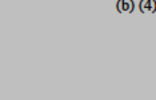
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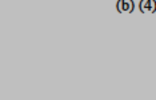
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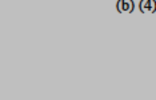

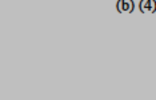

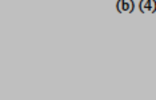
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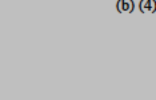
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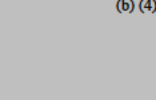
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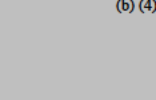
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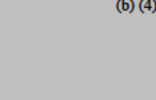
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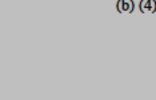
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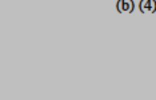
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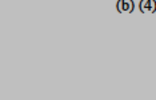
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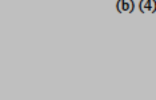
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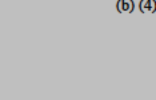

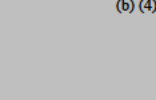
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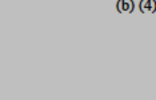
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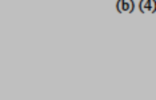
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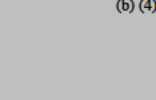
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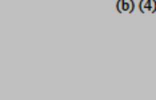
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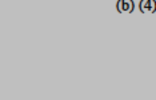
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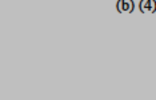
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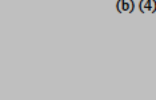
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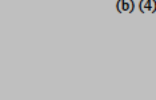
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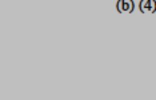
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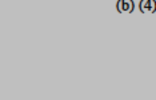
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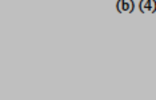
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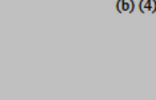
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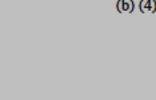
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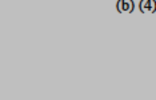
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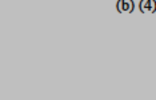
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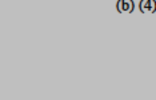
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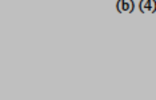
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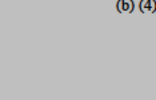
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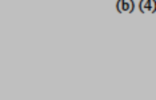
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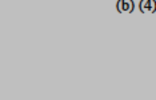
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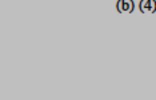
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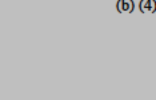

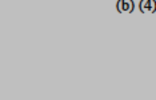
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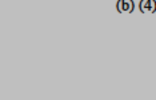
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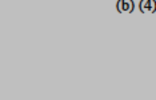
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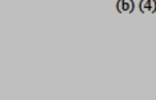
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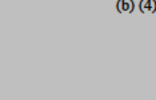
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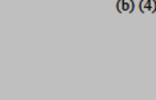
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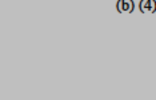
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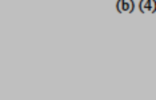

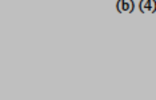
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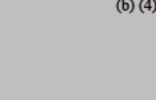
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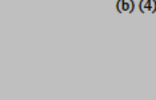

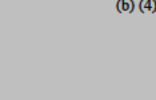
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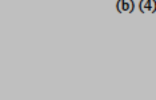
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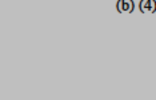
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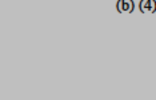

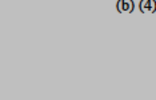
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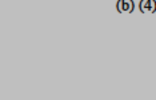
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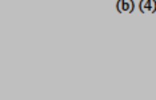

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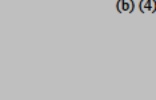
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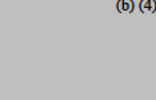
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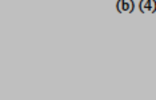
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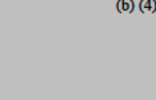

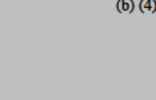
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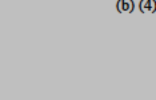

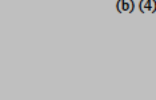
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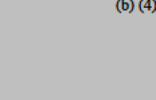
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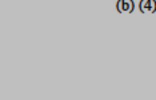

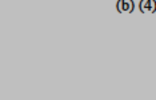
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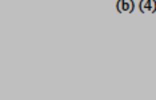
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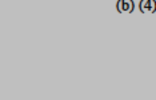
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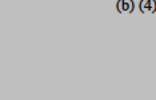
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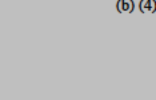
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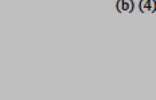
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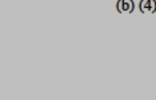
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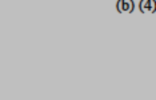
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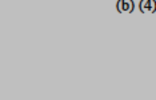
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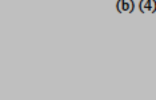

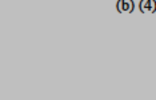

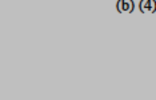
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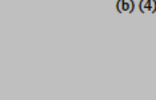
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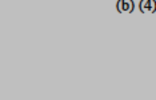

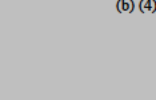
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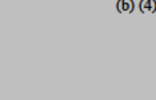

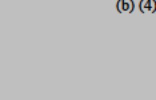
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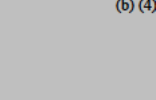

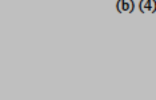

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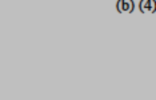
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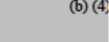

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Certificate of Analysis

ISO GUIDE 34
ANAB Cert# AR-1470

ISO/IEC 17025
ANAB Cert# AT-1467

L-VALINE

CERTIFIED REFERENCE MATERIAL

CH_3 O H H_3C OH NH_2

CERTIFIED PURITY: 98.9%, $U_{crm} = \pm 0.07\%$ k = 2.07
(Mass Balance/as is basis)

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QC Supervisor

QA Supervisor

(b) (4)

Test Report

(b) (4)	**Report number** T200311 **Page (1) / Total (4)**	(b) (4)

1. Client

* Company Name : CJ CheilJedang Corp.
* Name : Tae yeon, Kim
* Adress : 55, Gwanggyo-ro 42beon-gil, Yeongtong-gu, Suwon-si, Gyeonggi-do, Republic of Korea

TEL : 031-8099-2385 FAX : -

* Date : 2020-07-01

2. A use of Report : QC

3. Sample Type : Sample-3-

4. A term of Test : 20.07.01 ~ 20.07.20

5. Method of Analysis : HPLC-UVD

6. Result of Anaylysis

* unit: mg/L

Confirmation	Writer		Supervisor	
	Position :	Researcher	Technical manager :	Team Leader
	Name :	(b) (4)	Name :	(b) (4) (b) (4)

July 20, 2020

(b) (4)

* The Result data cannot be used for advertisement, PR and prosecution.
* The Result data is only from what you offered us and cannot be used other similar samples.
* The customers may use these data, after getting the agreement with (b) (4) .

(b) (4)

TEST RESULT

Test No.	T200311	Page (2) / Total (4)

Sample	Element	Result	LOD	LOQ	Unit	Analysis
ATCC14067	Tryptamine	ND	0.3	1.0	mg/L	HPLC-UVD
	2-phenylethylamine	ND	0.3	1.0	mg/L	HPLC-UVD
	Putrescine	ND	0.3	1.0	mg/L	HPLC-UVD
	Cadaverine	ND	0.3	1.0	mg/L	HPLC-UVD
	Histamine	ND	0.3	1.0	mg/L	HPLC-UVD
	Tyramine	8.88	0.3	1.0	mg/L	HPLC-UVD
	Spermidine	ND	0.3	1.0	mg/L	HPLC-UVD
	Spermine	ND	0.3	1.0	mg/L	HPLC-UVD

Remark	LOD : Limit of detection LOQ : Limit of quantification ND : Not detected

(b) (4)

TEST RESULT

Test No.	T200311	Page (3) / Total (4)

Sample	Element	Result	LOD	LOQ	Unit	Analysis
KCCM11201P	Tryptamine	(b) (4)			mg/L	HPLC-UVD
	2-phenylethylamine				mg/L	HPLC-UVD
	Putrescine				mg/L	HPLC-UVD
	Cadaverine				mg/L	HPLC-UVD
	Histamine				mg/L	HPLC-UVD
	Tyramine				mg/L	HPLC-UVD
	Spermidine				mg/L	HPLC-UVD
	Spermine				mg/L	HPLC-UVD

Remark	LOD : Limit of detection LOQ : Limit of quantification ND : Not detected

(b) (4)

TEST RESULT

Test No.	T200311	Page (4) / Total (4)

Sample	Element	Result	LOD	LOQ	Unit	Analysis
KCCM80058	Tryptamine	(b) (4)			mg/L	HPLC-UVD
	2-phenylethylamine				mg/L	HPLC-UVD
	Putrescine				mg/L	HPLC-UVD
	Cadaverine				mg/L	HPLC-UVD
	Histamine				mg/L	HPLC-UVD
	Tyramine				mg/L	HPLC-UVD
	Spermidine				mg/L	HPLC-UVD
	Spermine				mg/L	HPLC-UVD

Remark	LOD : Limit of detection LOQ : Limit of quantification ND : Not detected

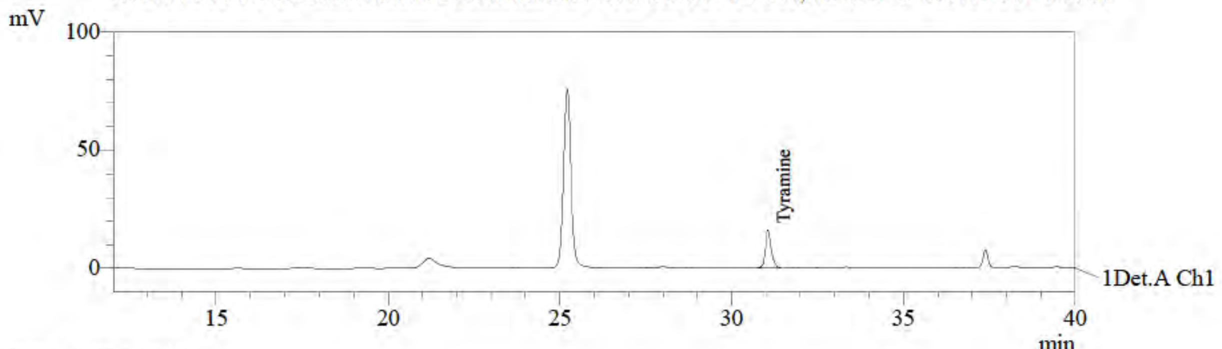
(b) (4)

REPORT

Sample Information

Acquired by : Admin
Sample Name : 200714-311-1-2
Sample ID : 200714-311-1-2
Tray# : 1
Vail# : 9
Injection Volume : 10 uL
Data Filename : 200714-311-1-2.lcd

Chromatogram
200714-311-1-2 C:\LabSolutions\LCsolution\Data\BAs\2020\07\14-311,ck-0054-59\200714-311-1-2.lcd



1 Det.A Ch1 / 254nm

Quantitative Results

Detector A

ID#	Name	Ret. Time	Area	Height	Conc.
1	Tryptamine	0.000	0	0	0.000
2	2-Phenylethylamine	0.000	0	0	0.000
3	Putrescine	0.000	0	0	0.000
4	Cadaverine	0.000	0	0	0.000
5	Histamine	0.000	0	0	0.000
6	Tyramine	31.057	194738	16015	1.775
7	Spermidine	0.000	0	0	0.000
8	Spermine	0.000	0	0	0.000

Calibration Curve

(b) (4)

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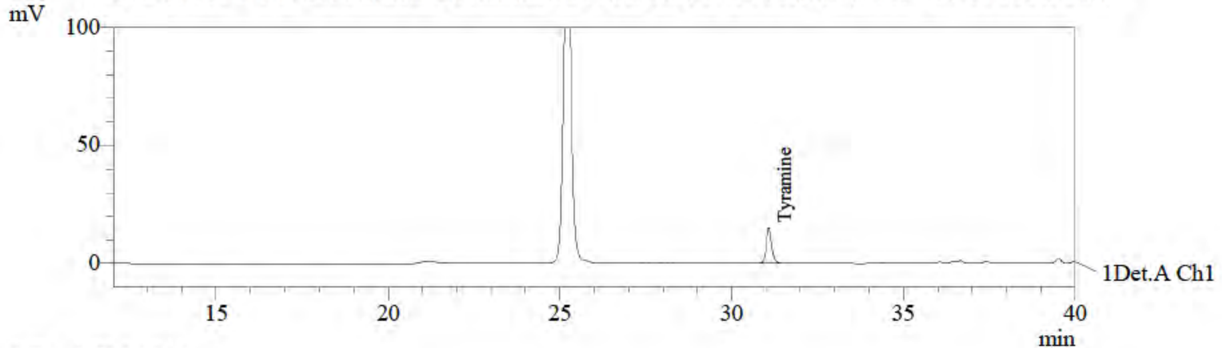
REPORT

Sample Information

Acquired by : Admin
Sample Name : 200714-311-2-1
Sample ID : 200714-311-2-1
Tray# : 1
Vail# : 10
Injection Volume : 10 uL
Data Filename : 200714-311-2-1.lcd

Chromatogram
200714-311-2-1 C:\LabSolutions\LCsolution\Data\BAs\2020\07\14-311,ck-0054-59\200714-311-2-1.lcd



1 Det.A Ch1 / 254nm

Quantitative Results

Detector A

ID#	Name	Ret. Time	Area	Height	Conc.
1	Tryptamine	0.000	0	0	0.000
2	2-Phenylethylamine	0.000	0	0	0.000
3	Putrescine	0.000	0	0	0.000
4	Cadaverine	0.000	0	0	0.000
5	Histamine	0.000	0	0	0.000
6	Tyramine	31.080	169047	14390	1.553
7	Spermidine	0.000	0	0	0.000
8	Spermine	0.000	0	0	0.000

Calibration Curve

(b) (4)

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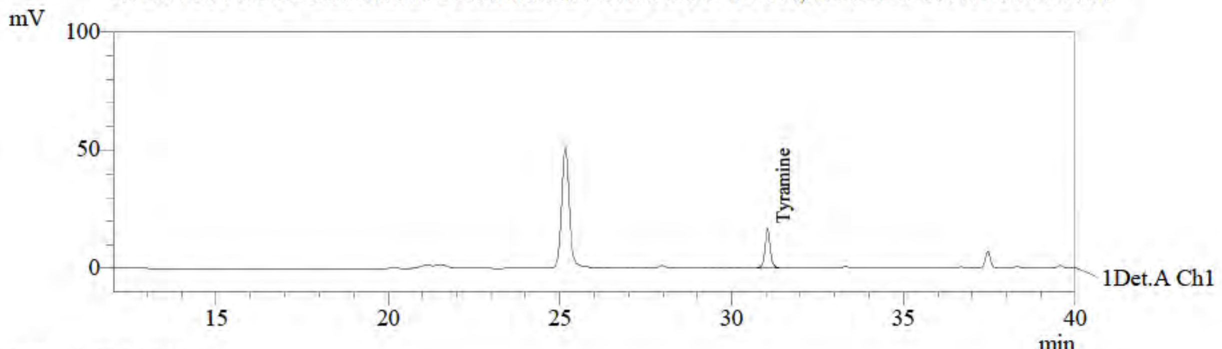
(b) (4)

REPORT

Sample Information

Acquired by : Admin
Sample Name : 200714-311-3-2
Sample ID : 200714-311-3-2
Tray# : 1
Vail# : 13
Injection Volume : 10 uL
Data Filename : 200714-311-3-2.lcd

Chromatogram
200714-311-3-2 C:\LabSolutions\LCsolution\Data\BAs\2020\07\14-311,ck-0054-59\200714-311-3-2.lcd



1 Det.A Ch1 / 254nm

Quantitative Results

Detector A

ID#	Name	Ret. Time	Area	Height	Conc.
1	Tryptamine	0.000	0	0	0.000
2	2-Phenylethylamine	0.000	0	0	0.000
3	Putrescine	0.000	0	0	0.000
4	Cadaverine	0.000	0	0	0.000
5	Histamine	0.000	0	0	0.000
6	Tyramine	31.042	182081	15284	1.666
7	Spermidine	0.000	0	0	0.000
8	Spermine	0.000	0	0	0.000

Calibration Curve

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APPENDIX 3. PRE-FERMENTATION INFORMATION (CONFIDENTIAL)

Contents

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List of Tables

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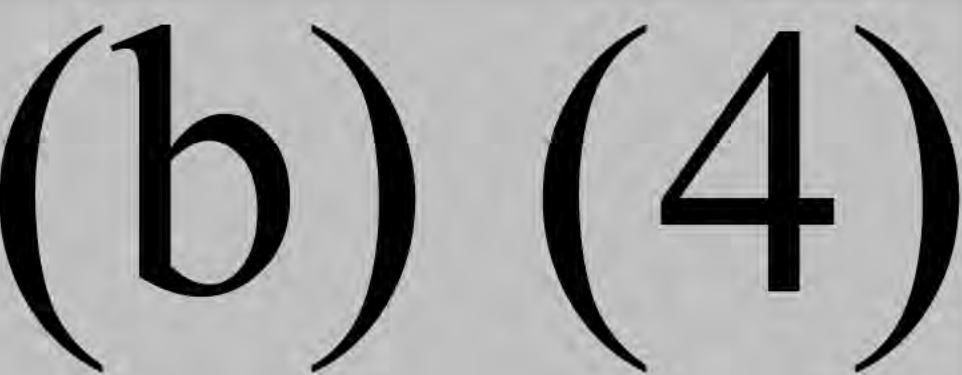
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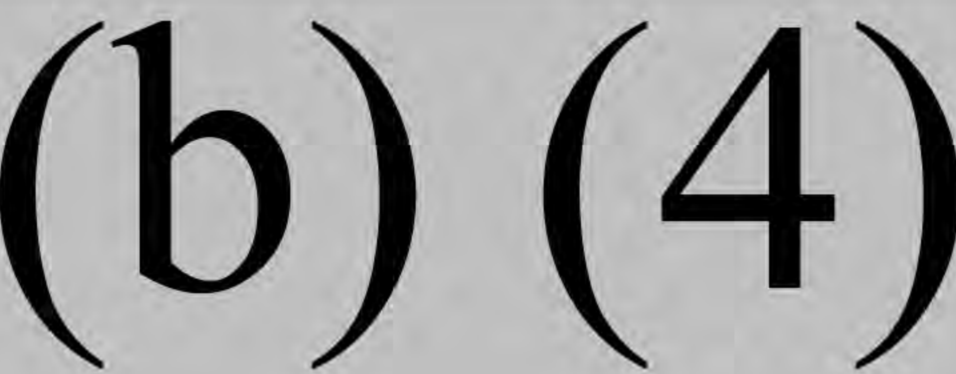

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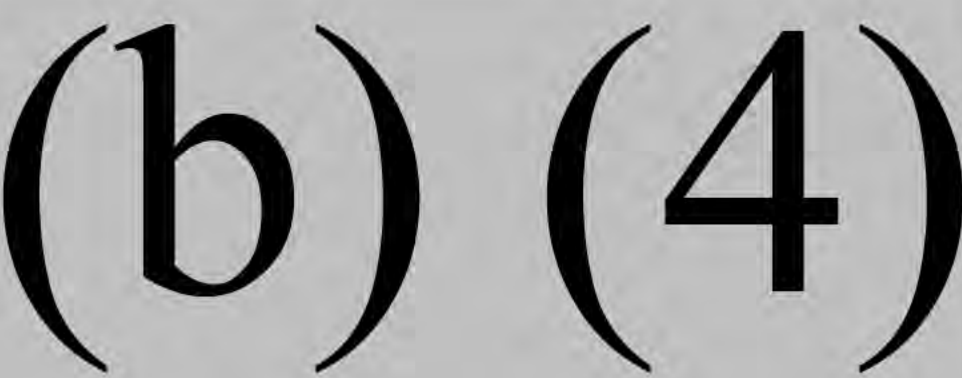
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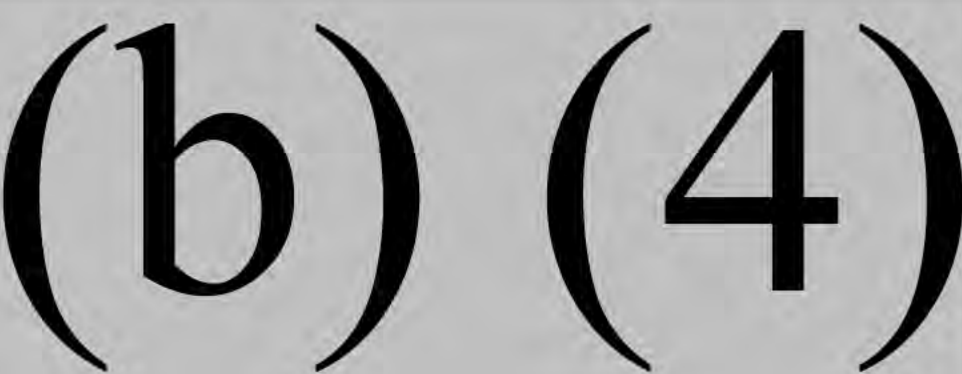
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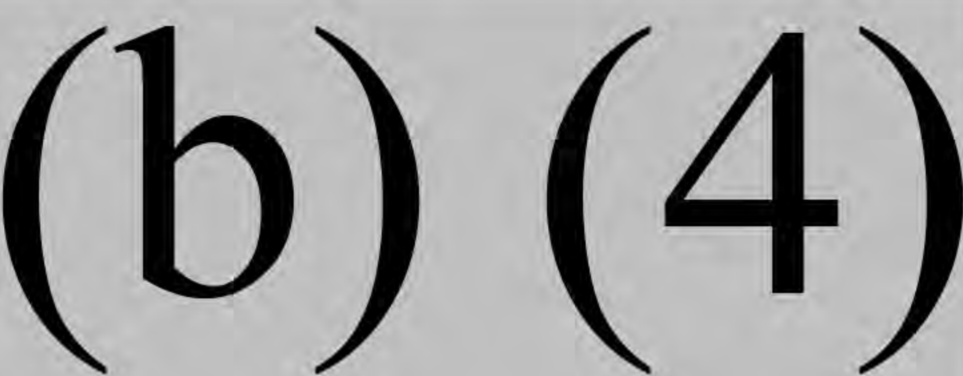
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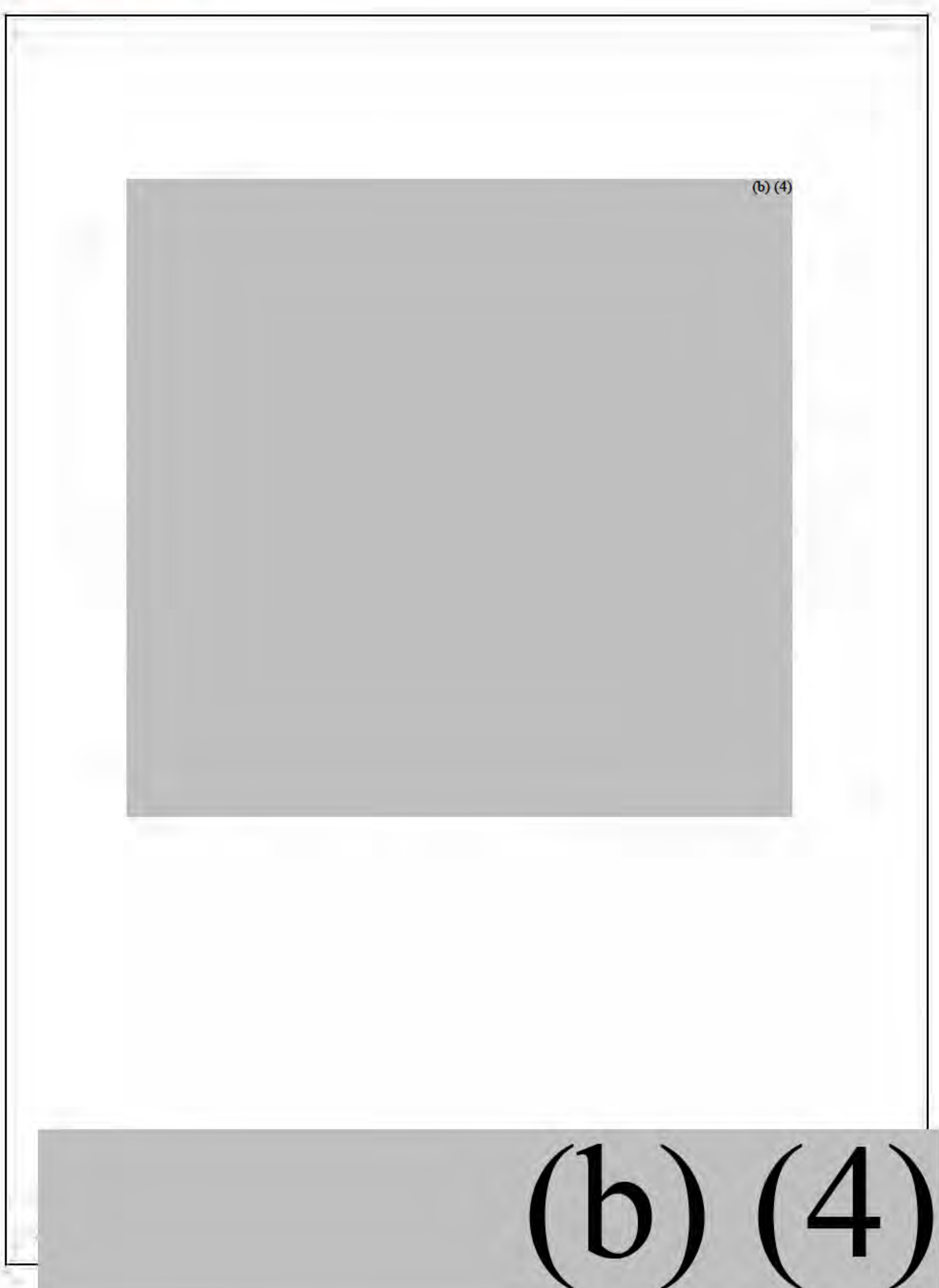
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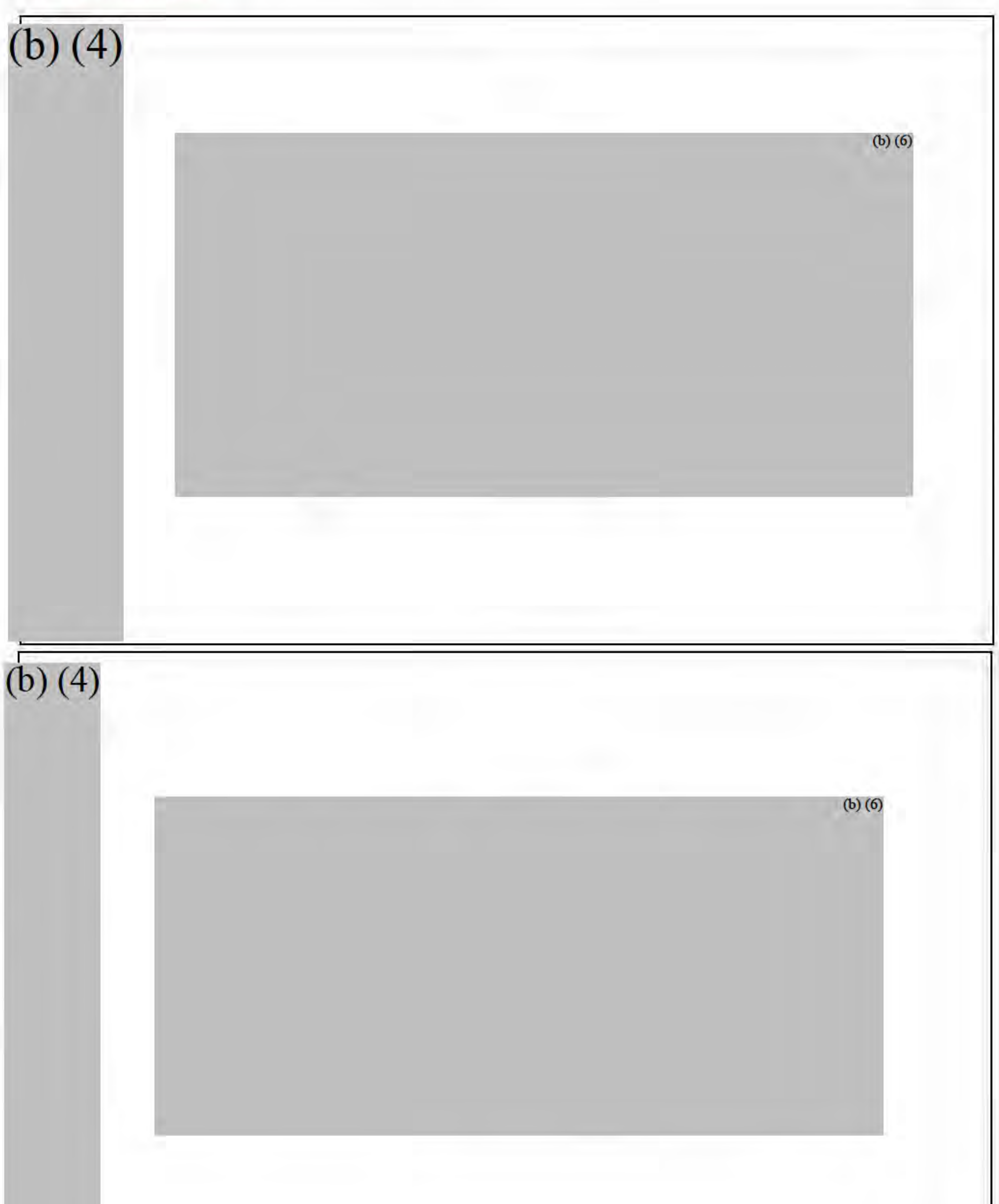
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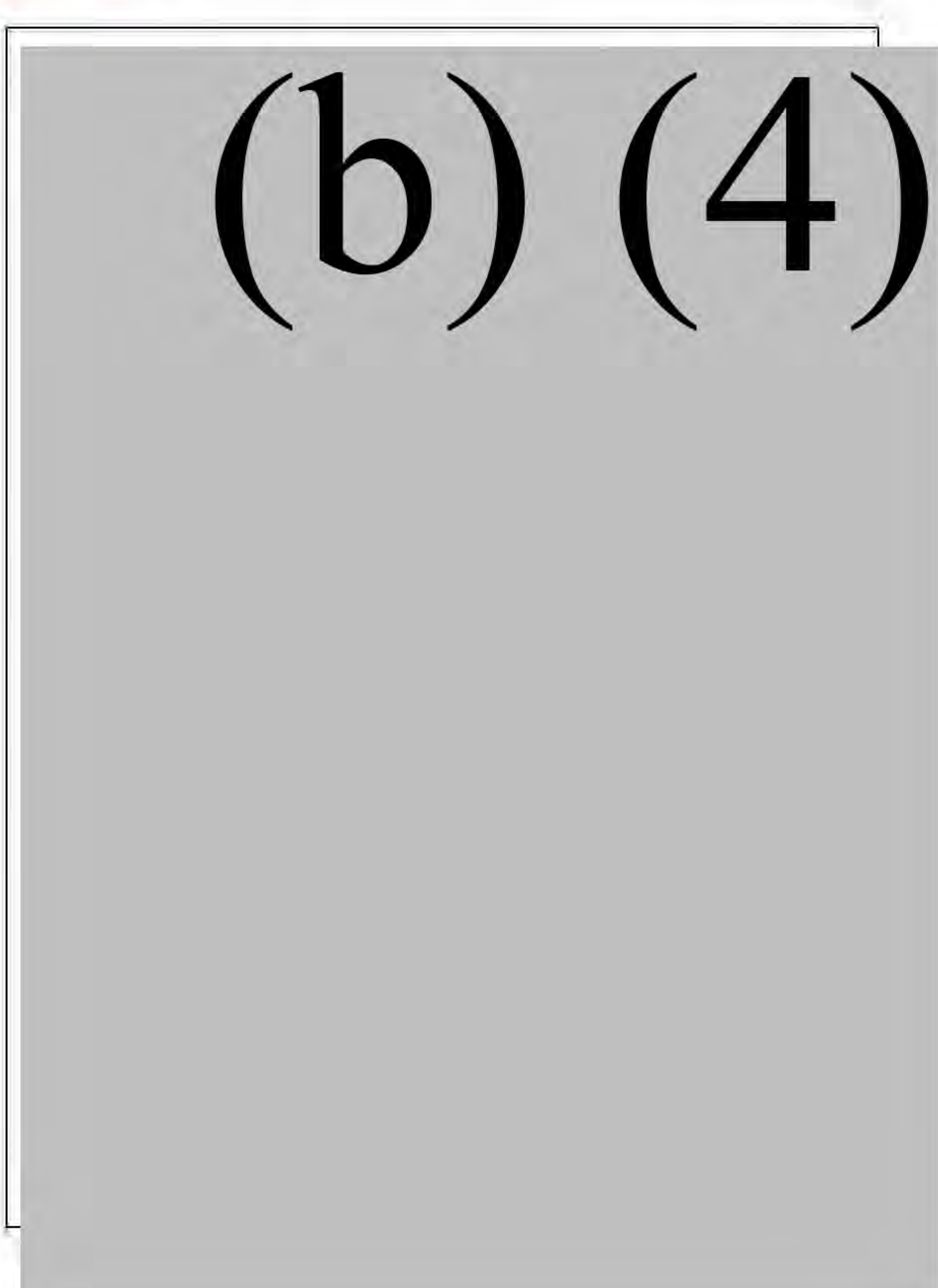
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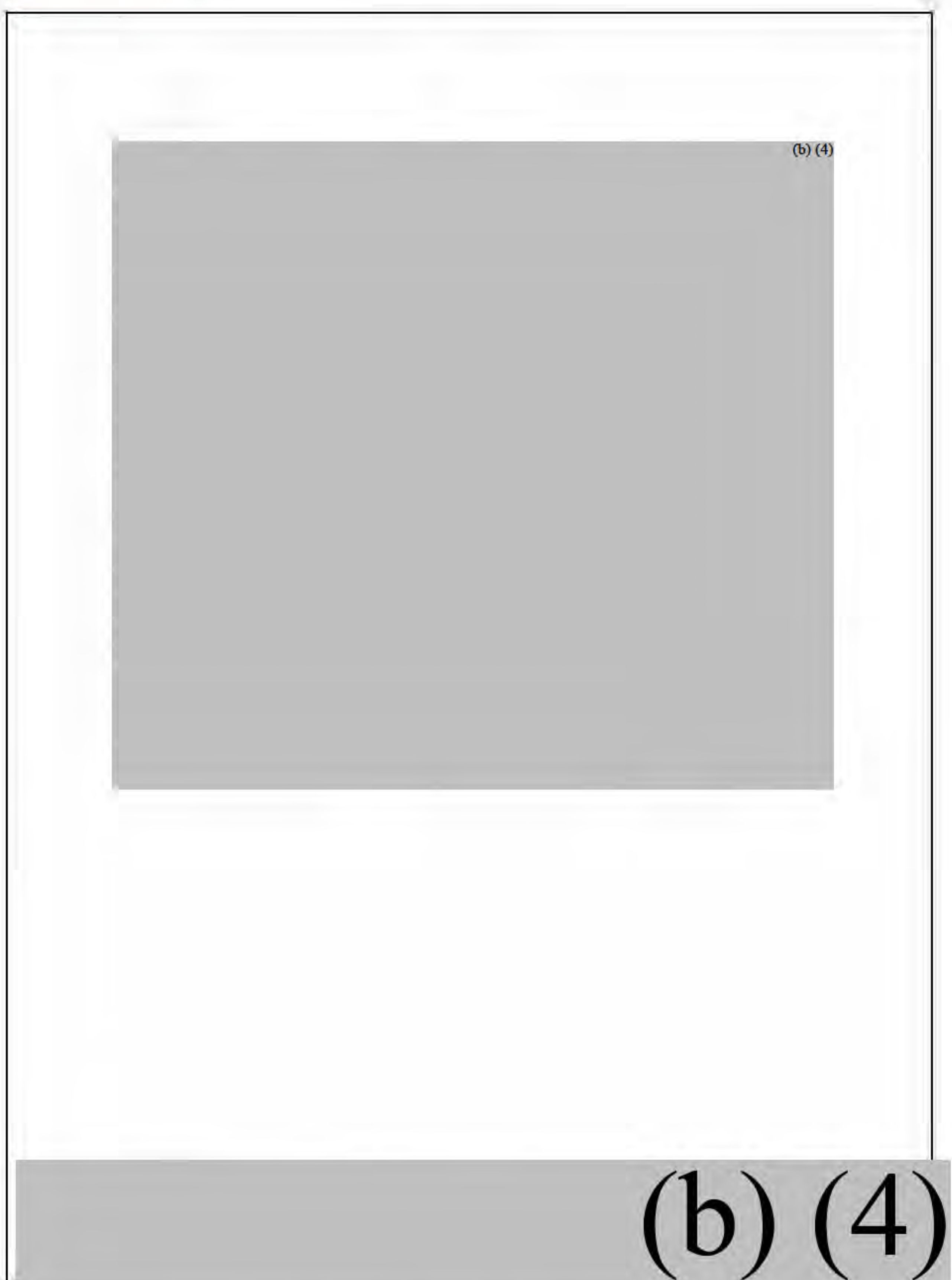
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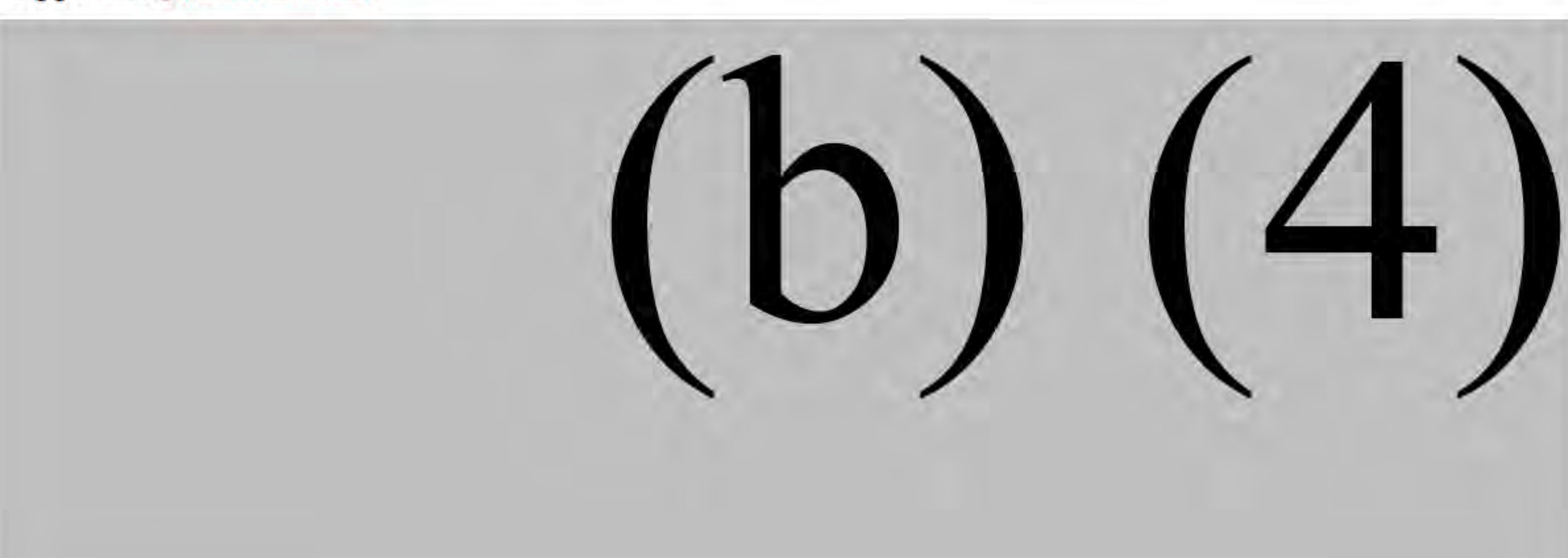
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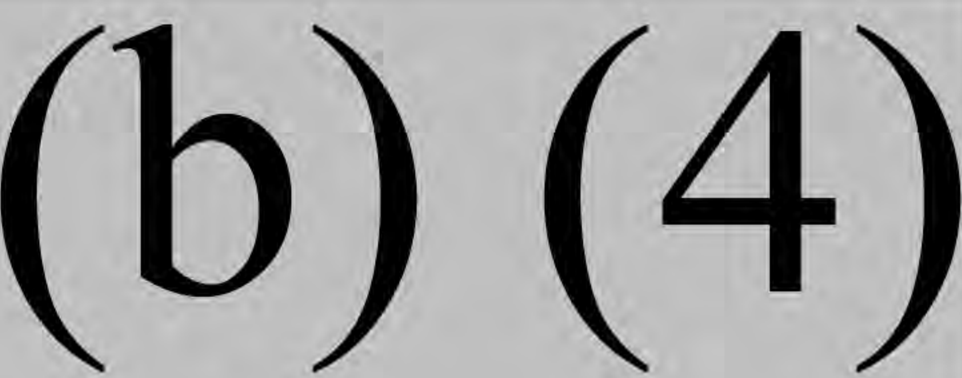
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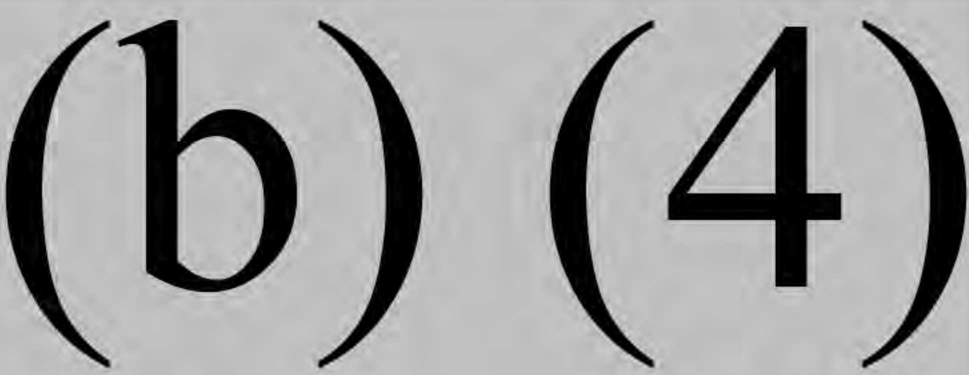
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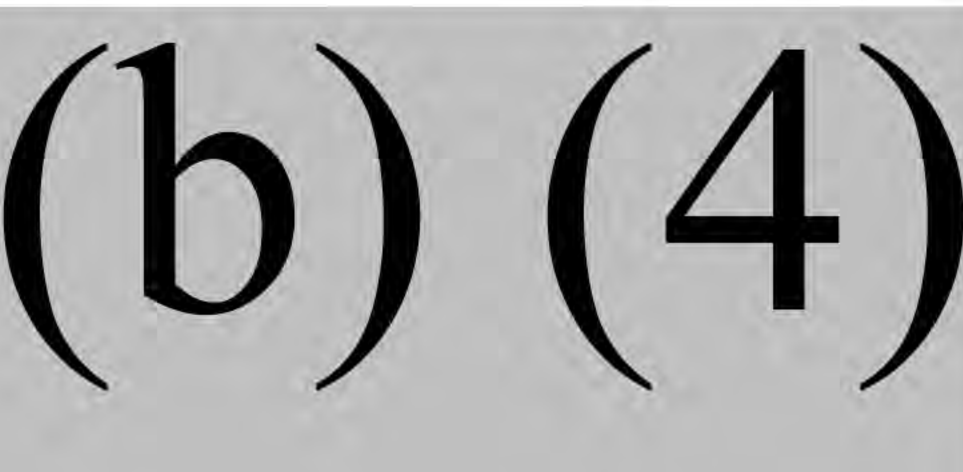
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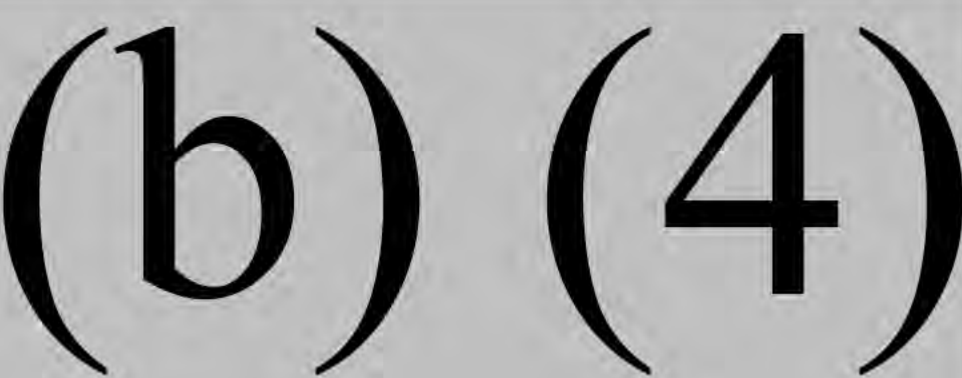
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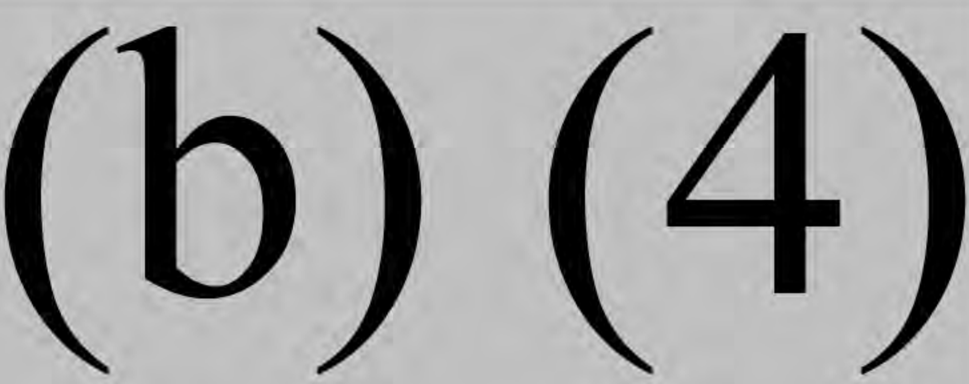
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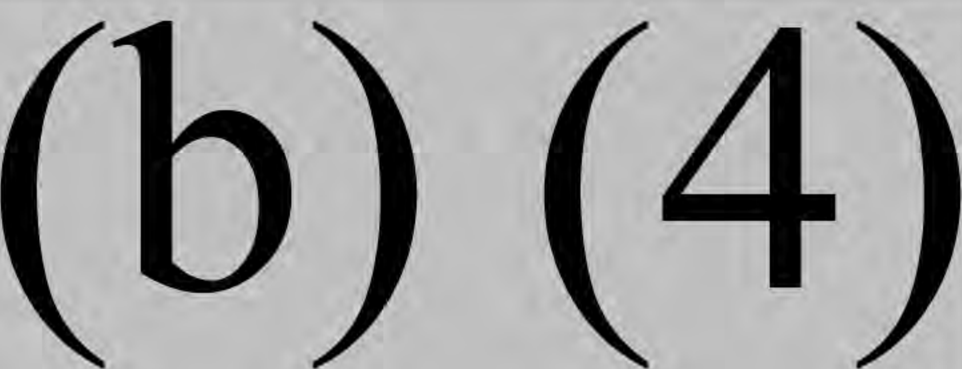
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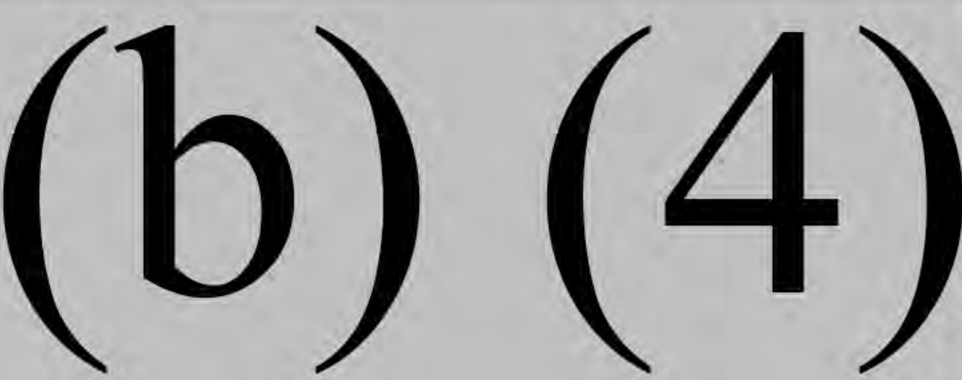
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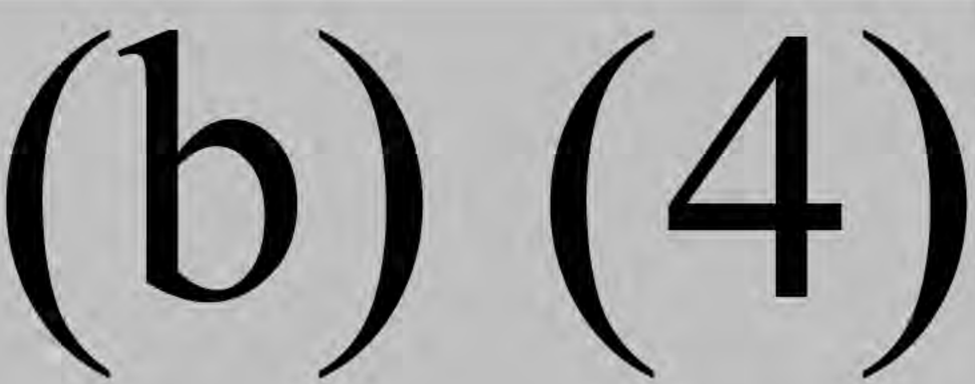
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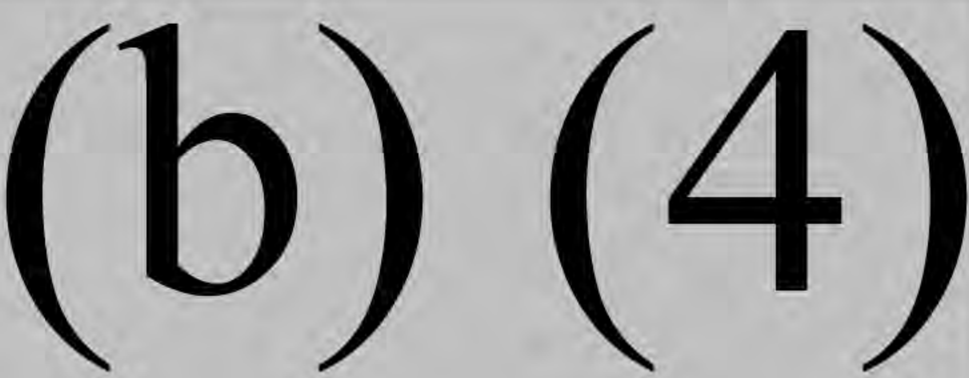
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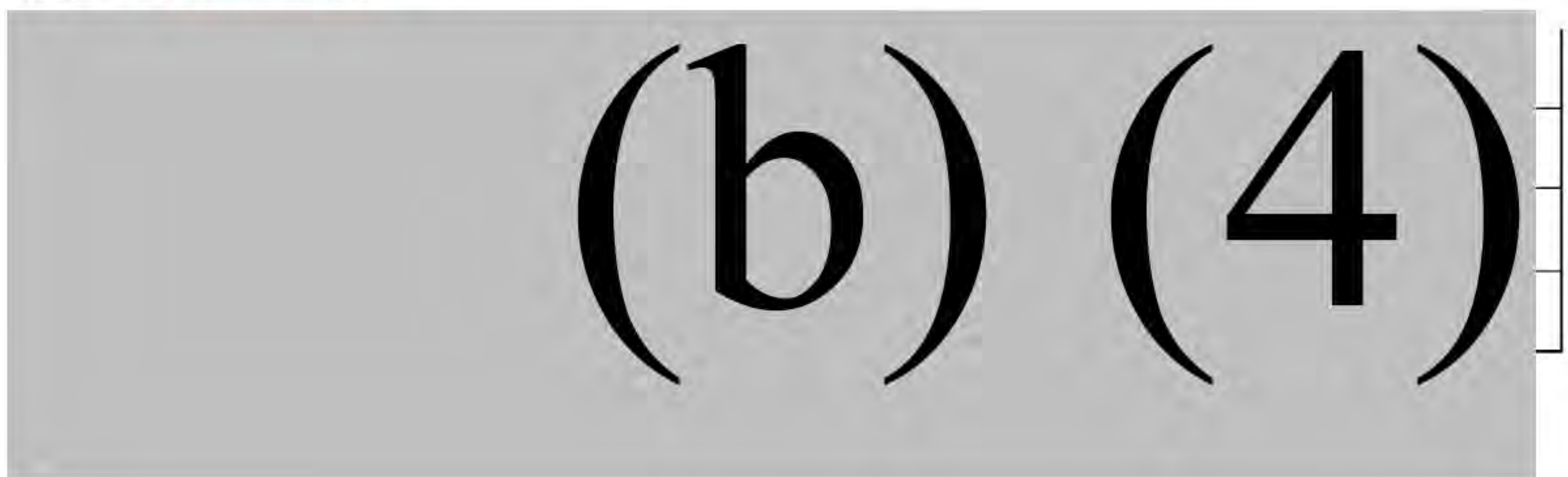
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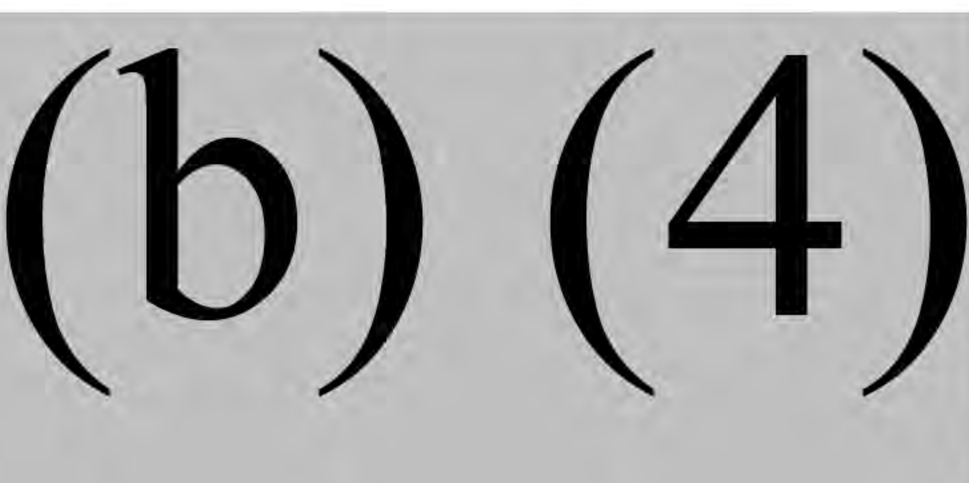
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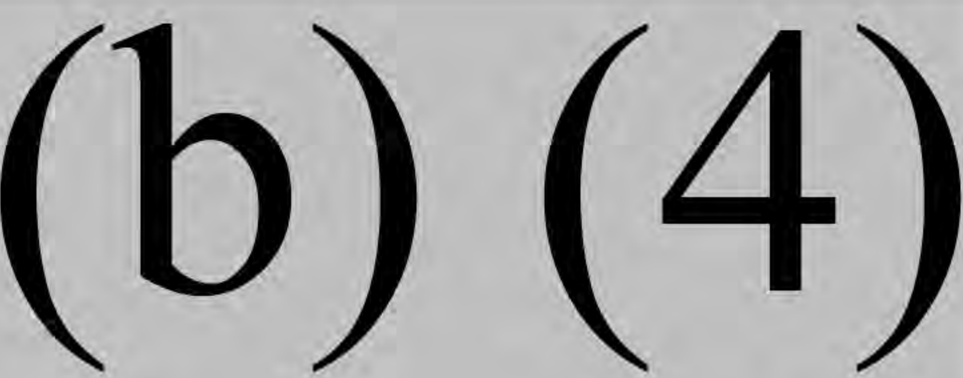
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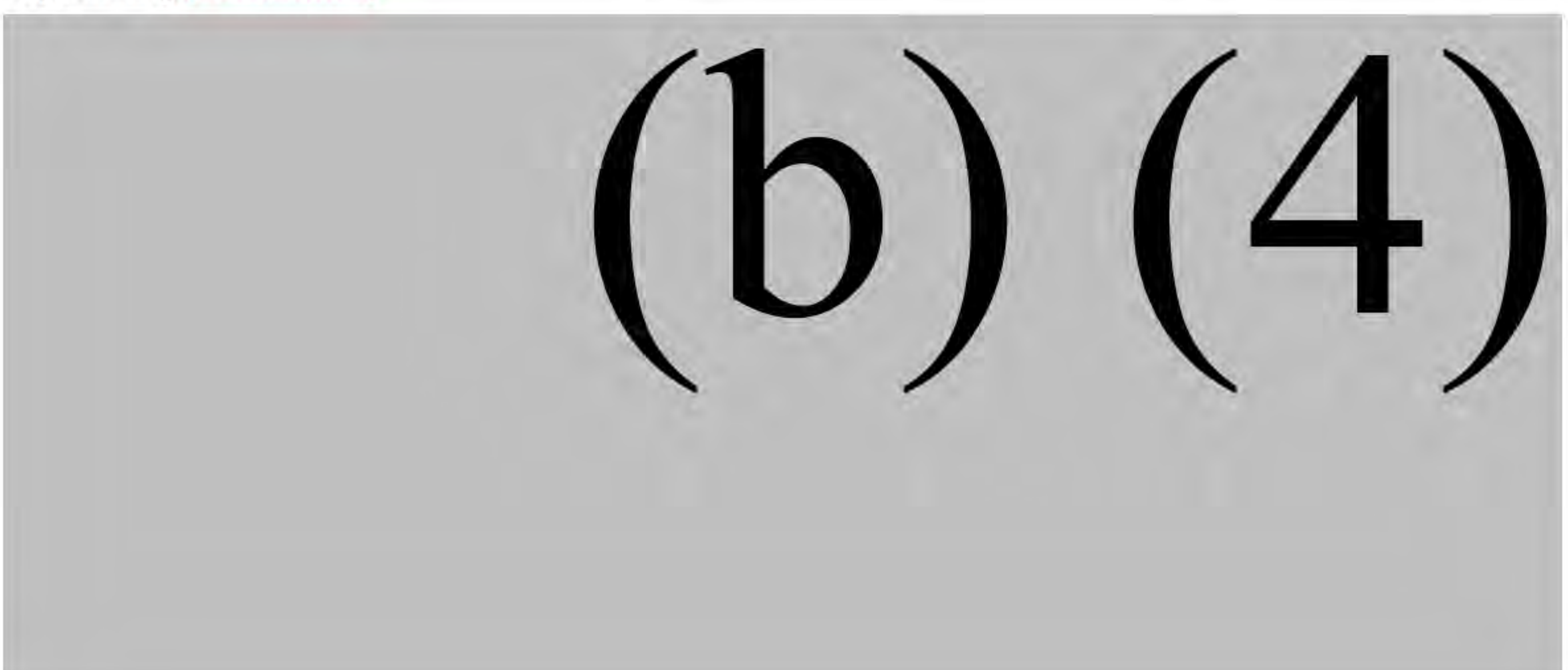
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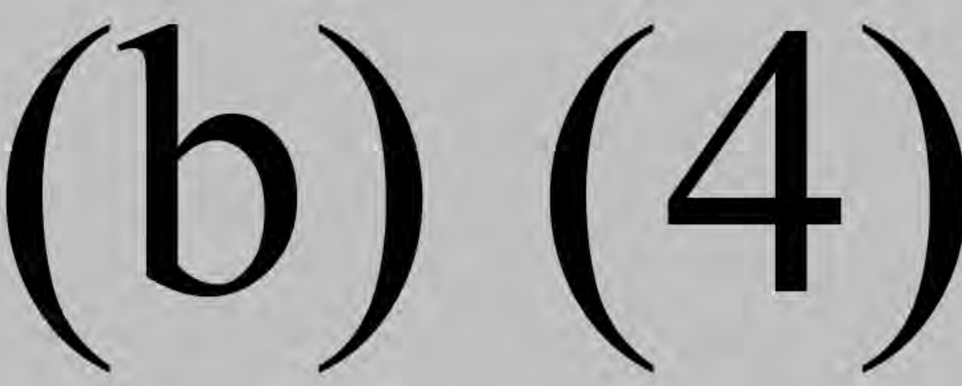
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Cerrito, Chelsea

From:	Kristi Smedley <smedley@cfr-services.com>
Sent:	Monday, July 27, 2020 3:08 PM
To:	Tang, Lei; Wong, Geoffrey K; Animalfood-premarket
Cc:	'강민경님 [Min Kang]'; Keith D. Haydon; Biesiada,Thomas님
Subject:	RE: GRAS AGRN 35 --AMENDMENT --Email 4 (of 4)---supplements 14-15
Attachments:	Supplement 14. ORF Analysis for the Full Genome Sequence.pdf; Supplement 15. Full genome Sequence Analysis.pdf

Dr. Tang:

This email contains supplements (attachments) 14-15 for AGRN 35. This is the 4 of 4 emails specific to this AGRN amendment.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (4)
Fax 703-580-8637

From: Kristi Smedley [mailto:smedley@cfr-services.com]
Sent: Monday, July 27, 2020 3:05 PM
To: 'Tang, Lei'; 'Wong, Geoffrey K'; Animalfood-premarket (Animalfood-premarket@fda.hhs.gov)
Cc: '강민경님 [Min Kang]' (mg.kang@cj.net); Keith D. Haydon (keith.haydon@cj.net); Biesiada,Thomas님 (thomas.biesiada@cj.net)
Subject: RE: GRAS AGRN 35 --AMENDMENT --Email 3---supplements 7-13

Dr. Tang:

This email contains supplements (attachments) 7-13 for AGRN 35.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

From: Kristi Smedley [mailto:smedley@cfr-services.com]
Sent: Monday, July 27, 2020 3:02 PM

To: 'Tang, Lei'; 'Wong, Geoffrey K'; Animalfood-premarket (Animalfood-premarket@fda.hhs.gov)
Cc: '강민경님 [Min Kang]' (mg.kang@cj.net); Keith D. Haydon (keith.haydon@cj.net); Biesiada,Thomas님 (thomas.biesiada@cj.net)
Subject: RE: GRAS AGRN 35 --AMENDMENT --Email 2---supplements 1-6

Dr. Tang:

This email contains Supplements (attachments 1-6) to support AGRN 35 amendment.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

From: Kristi Smedley [mailto:smedley@cfr-services.com]
Sent: Monday, July 27, 2020 3:00 PM
To: 'Tang, Lei'; 'Wong, Geoffrey K'; Animalfood-premarket (Animalfood-premarket@fda.hhs.gov)
Cc: '강민경님 [Min Kang]' (mg.kang@cj.net); Keith D. Haydon (keith.haydon@cj.net); Biesiada,Thomas님 (thomas.biesiada@cj.net)
Subject: GRAS AGRN 35 --AMENDMENT --Email 1

Dr. Tang:

On behalf of CJ, I am providing the amendment of AGRN 35, L-Valine fermentation product , as requested. This email provides a part of the supporting material for this amendment.

You will be receiving a series of emails, to assure that all supplements (attachments) are received. I will number them in the subject line of the email, as well as provide a brief description in the body of each email. You should receive 4 reference files and 15 supplements (attachments).

This email will include the signed amendment letter as well as all reference publications.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

From: Tang, Lei [mailto:Lei.Tang@fda.hhs.gov]
Sent: Wednesday, July 08, 2020 12:02 PM
To: Kristi Smedley

Cc: Wong, Geoffrey K
Subject: RE: GRAS AGRN 35

Dear Dr. Smedley,

This letter is in response to your email dated July 3, 2020 requesting for meeting minutes from the July 1, 2020 meeting between the Center for Veterinary Medicine and CJ CheilJedang Corporation.

Please find enclosed a copy of our meeting minutes for the referenced meeting.

If you have any questions concerning this letter, please contact me via email at Lei.Tang@fda.hhs.gov or by phone at 240-402-5922. Please refer to AGRN #35 in any future correspondences.

Sincerely,

Lei Tang, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal feeds
U.S. Food and Drug Administration

Tel: 240-402-5922
lei.tang@fda.hhs.gov

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From: Kristi Smedley <smedley@cfr-services.com>
Sent: Friday, July 3, 2020 9:32 AM
To: Tang, Lei <Lei.Tang@fda.hhs.gov>
Cc: Wong, Geoffrey K <Geoffrey.Wong@fda.hhs.gov>; Keith D. Haydon <keith.haydon@cj.net>; '강민경님 [Min Kang]' <mg.kang@cj.net>
Subject: RE: GRAS AGRN 35

Dr. Tang

This is a request for the notes of the FDA teleconference (July 1, 2020) specific to issues raised during the AGRN 35 review.

We will accept these notes by email.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b) (6)
Fax 703-580-8637

Open Reading Frame Analysis for the Full Genome Sequence of *Corynebacterium glutamicum* KCCM80058 (CONFIDENTIAL)

REPORT DATE: November 20, 2018

CJ BLOSSOM PARK

TITLE: The analysis of open reading frames (ORFs) for the full genome sequence of the *Corynebacterium glutamicum* KCCM80058

OBJECTIVE OF THE STUDY

This study was done to analyze the ORFs for the full genome sequence of the *Corynebacterium glutamicum* KCCM80058

SCHEDULE OF THE STUDY

Data of Receipt: Oct 01, 2018

Data of Test: Oct 05, 2018

Data of Final report: Nov 20, 2018

TESTING FACILITY

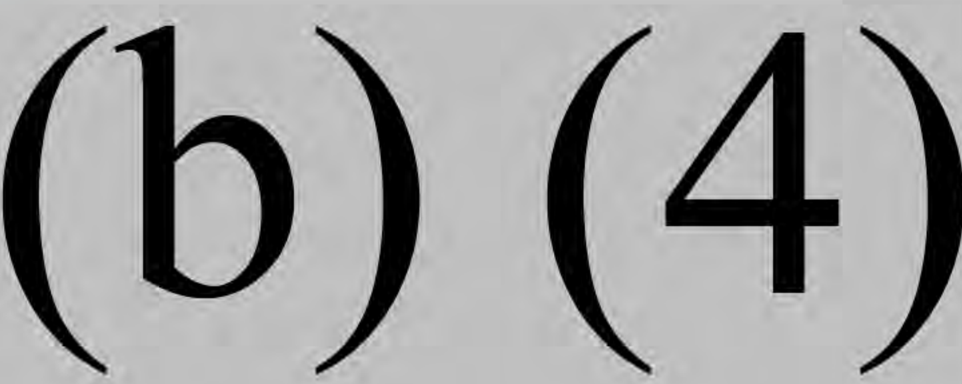
Name: (b) (4)

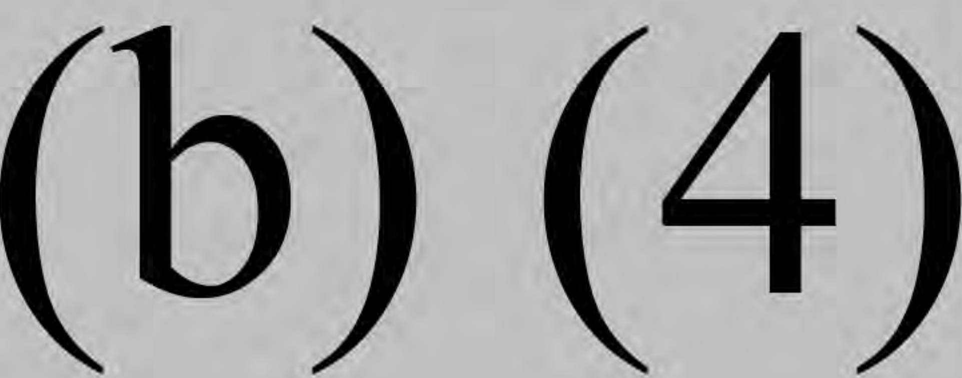
(b) (4)

RESPONSIBLE STAFFS

Study Director	Sang Jun Kim	03/04/2019
Quality Assurance Manager	Sung Gun Lee	03/04/2019

Summary

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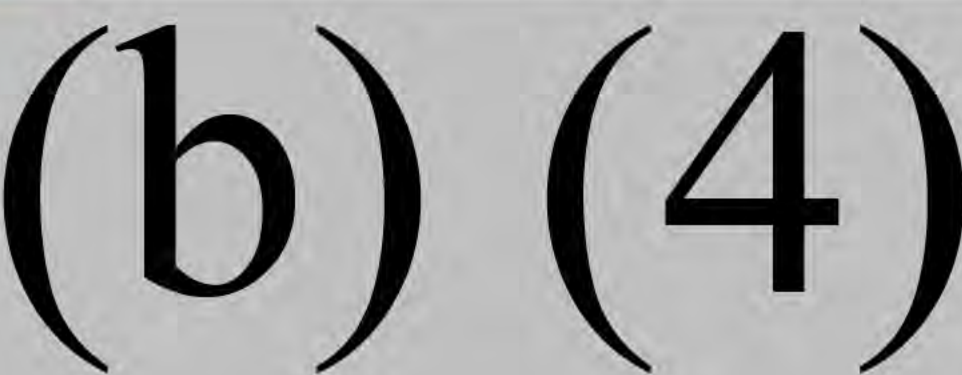
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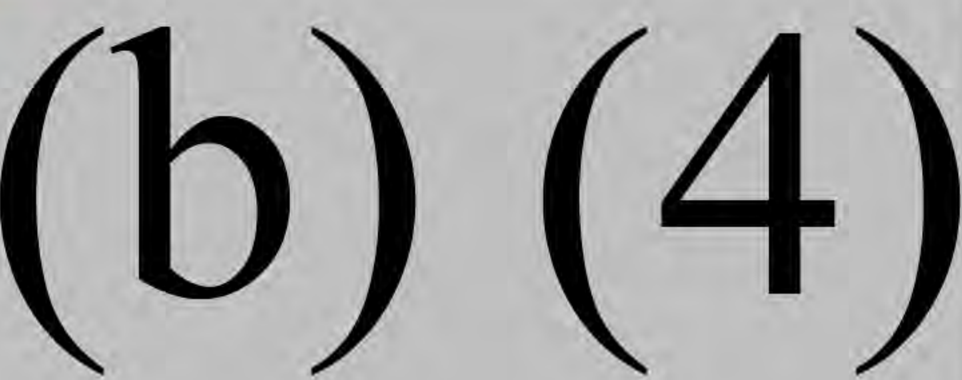
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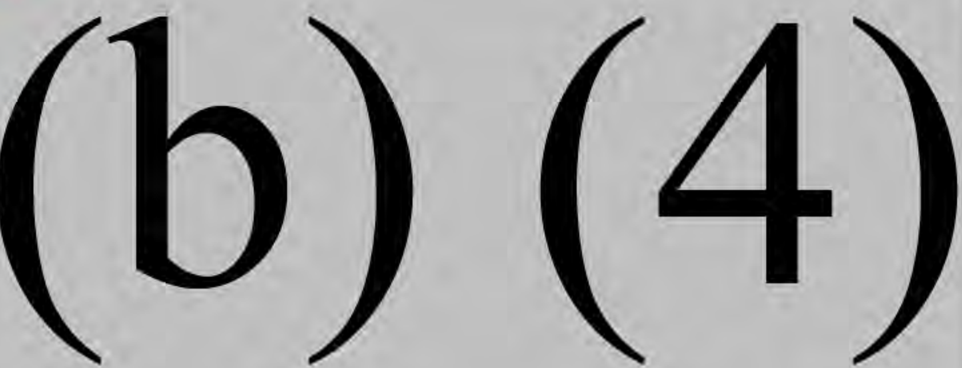
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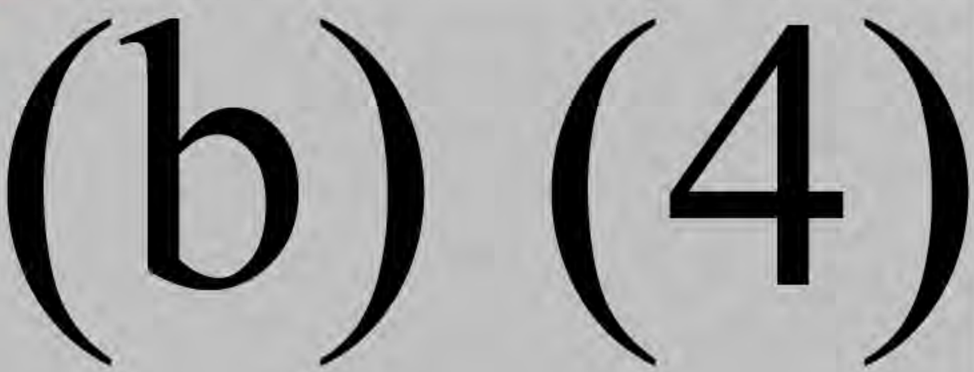
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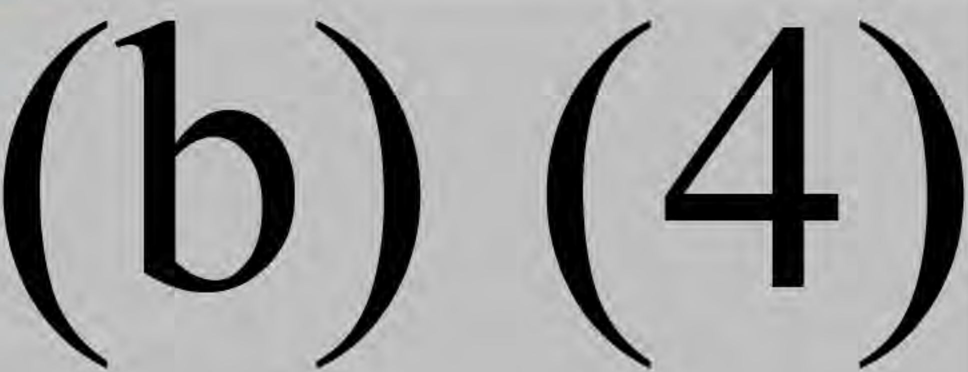
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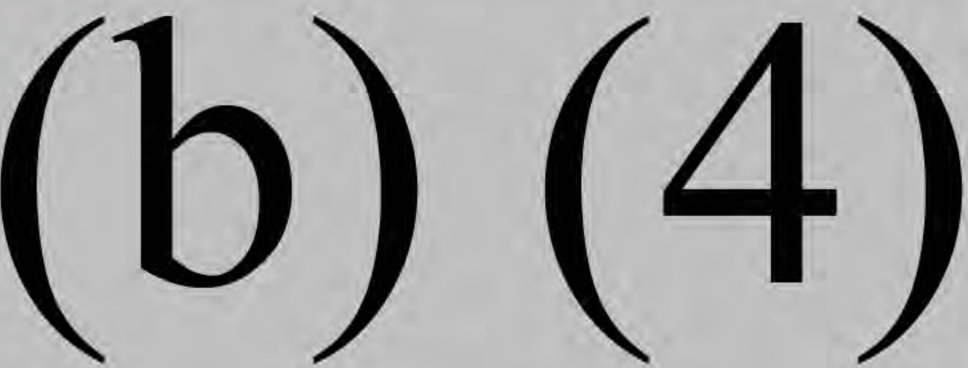
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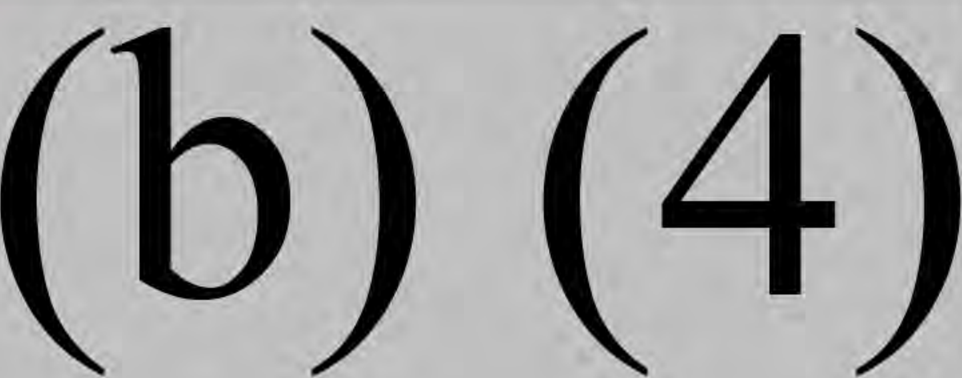
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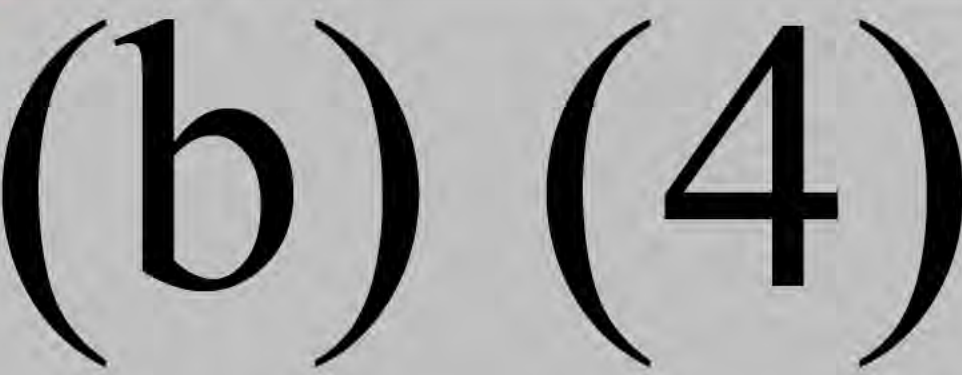
Attachment 3 (Confidential)



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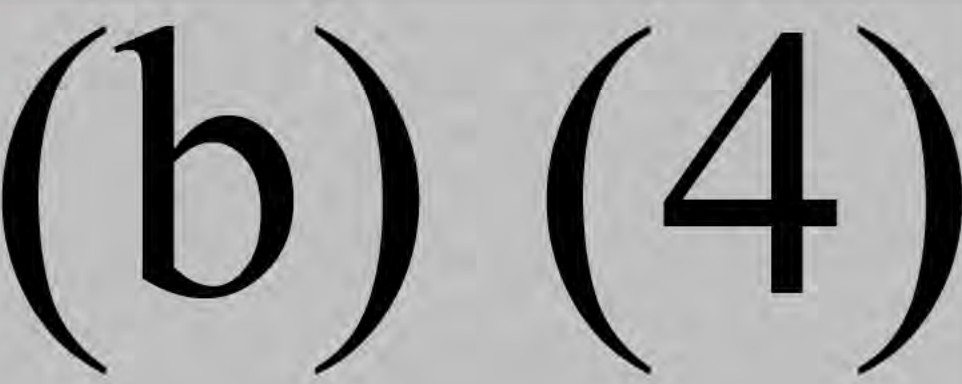
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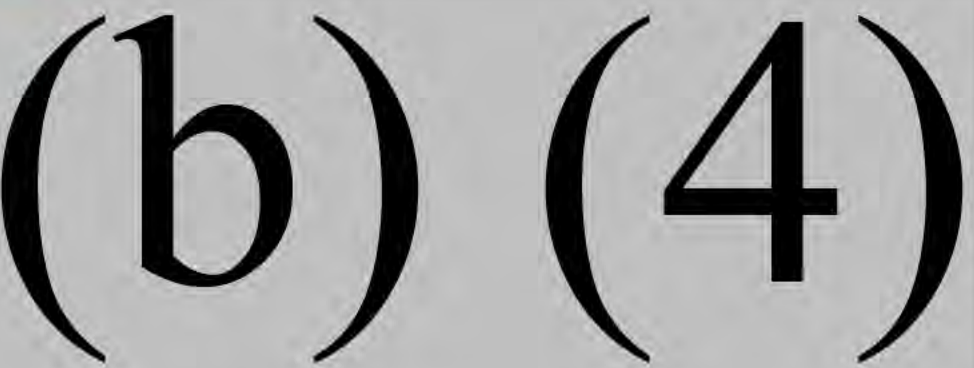
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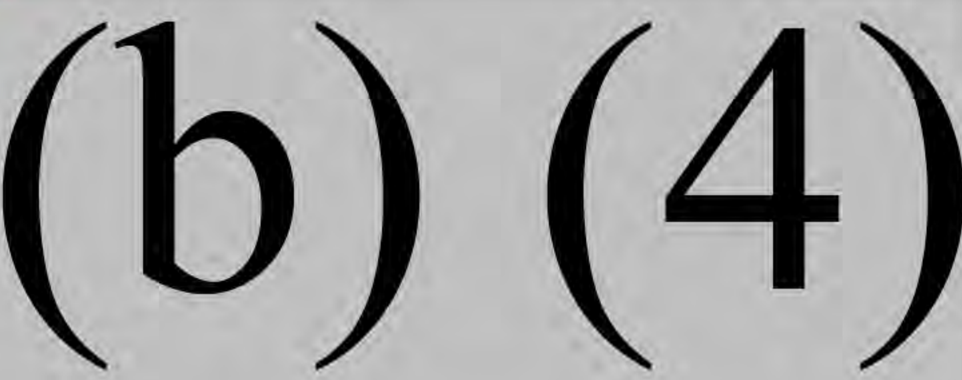
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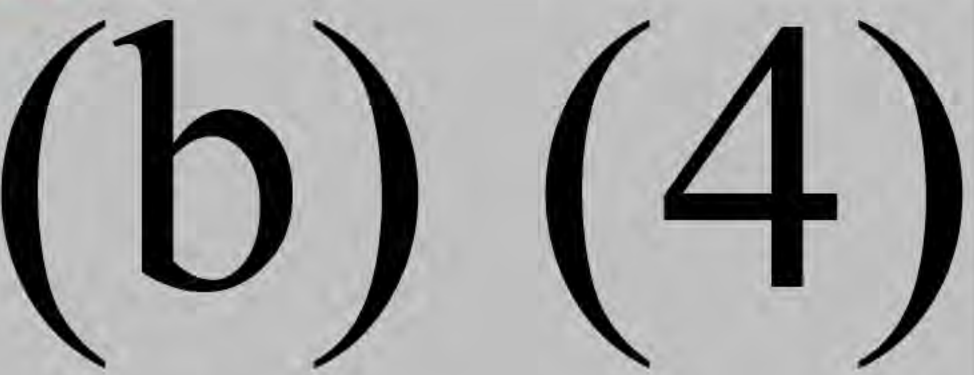
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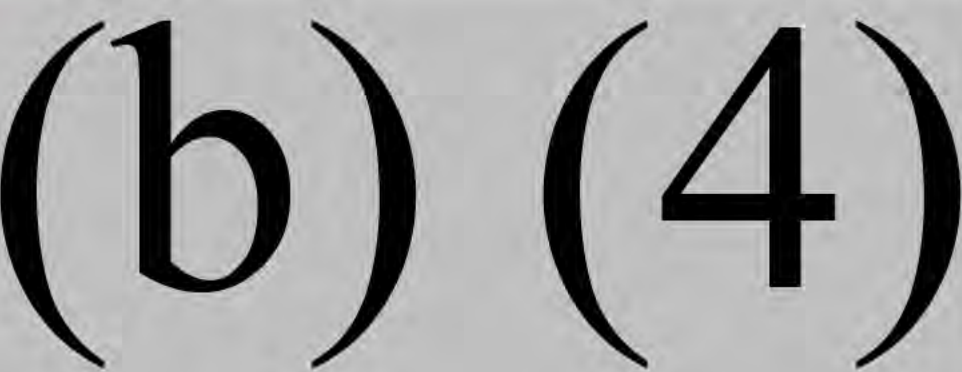
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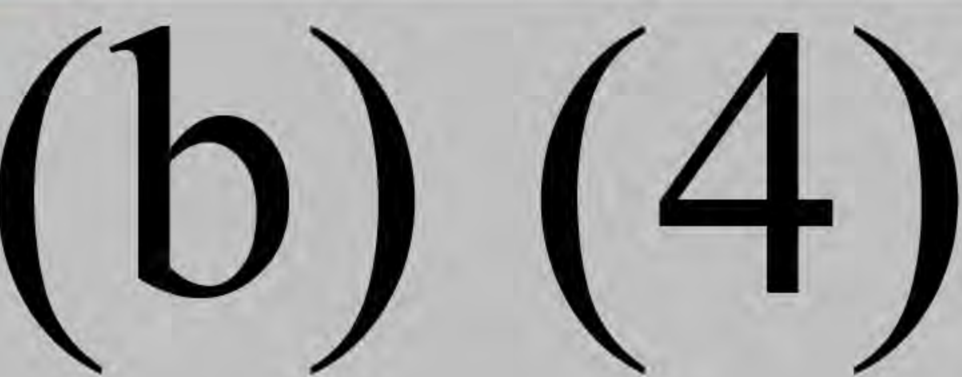
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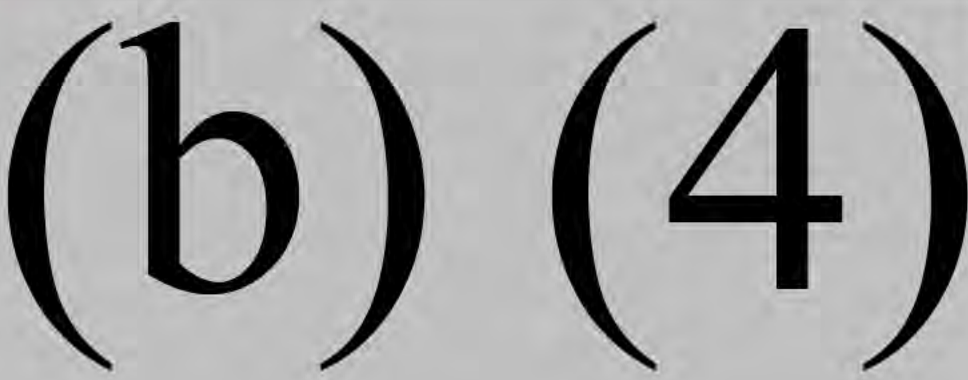
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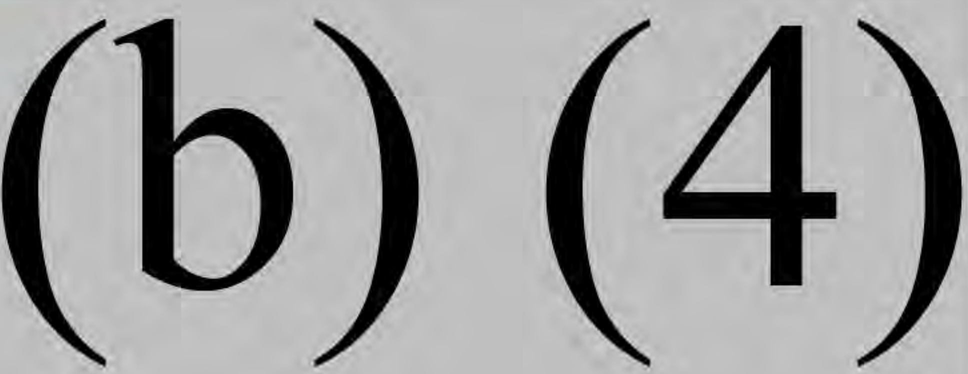
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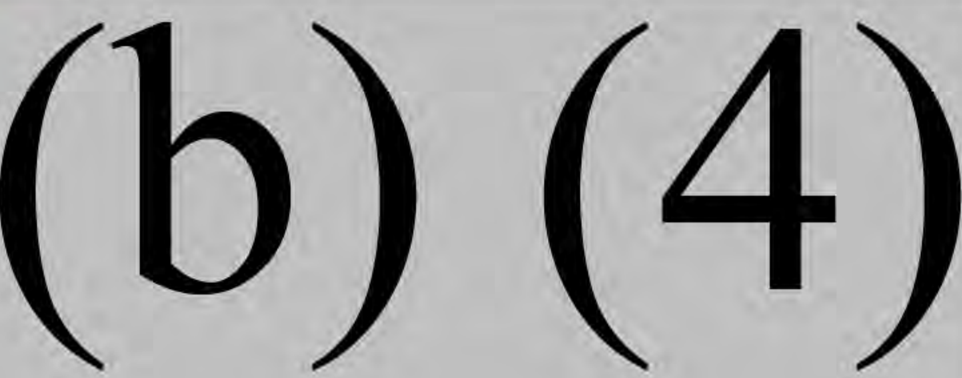
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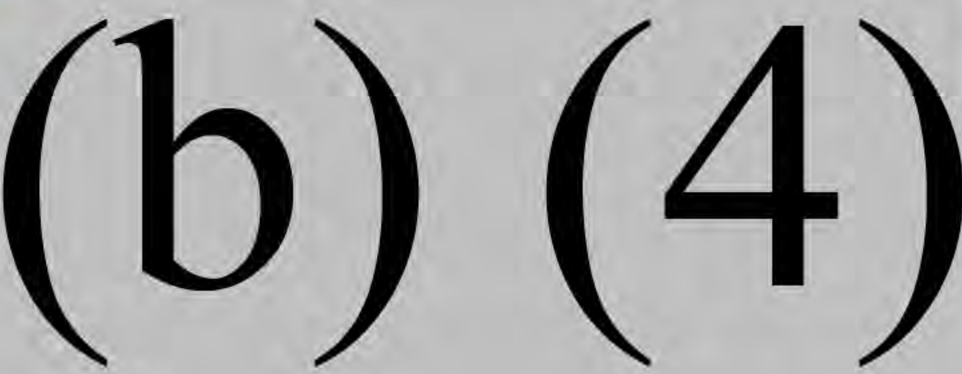
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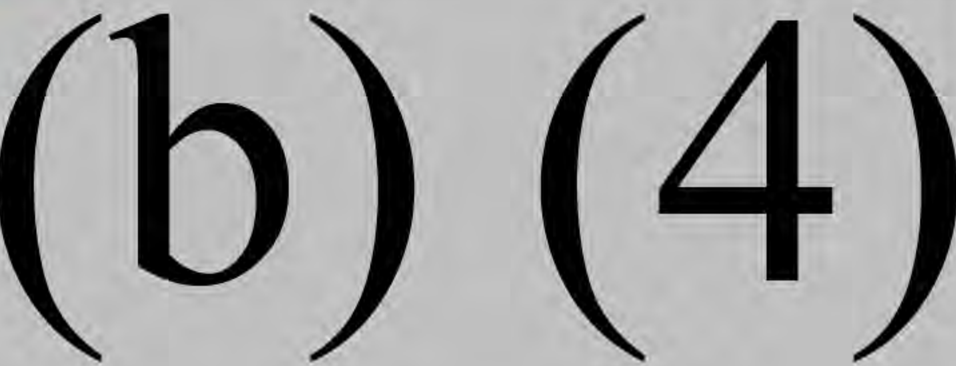
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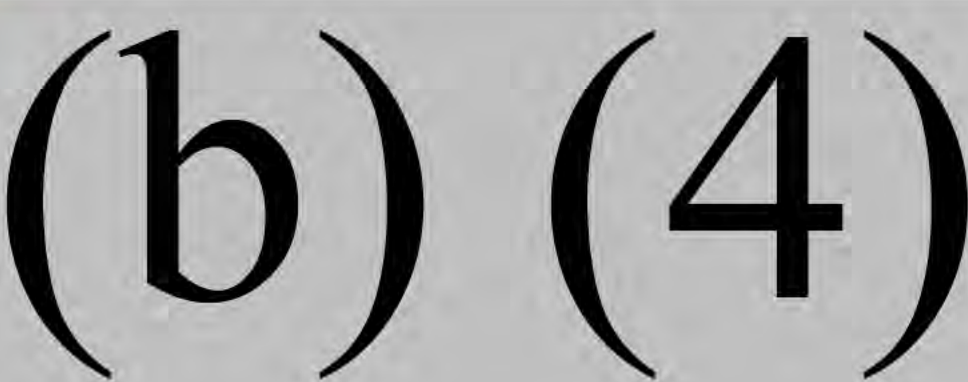
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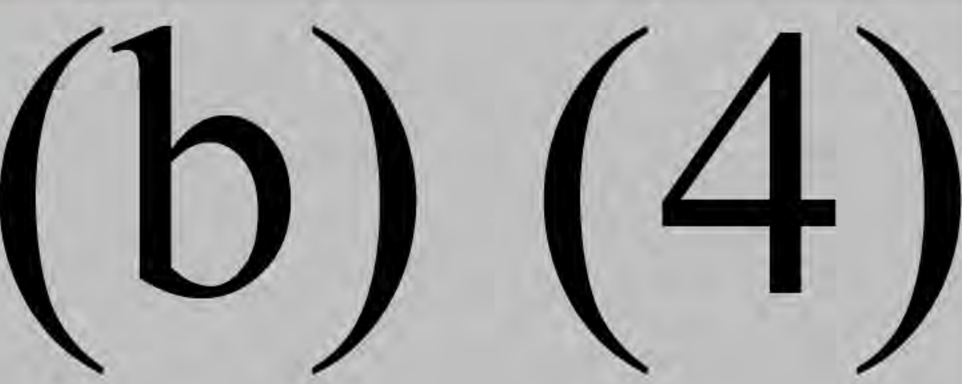
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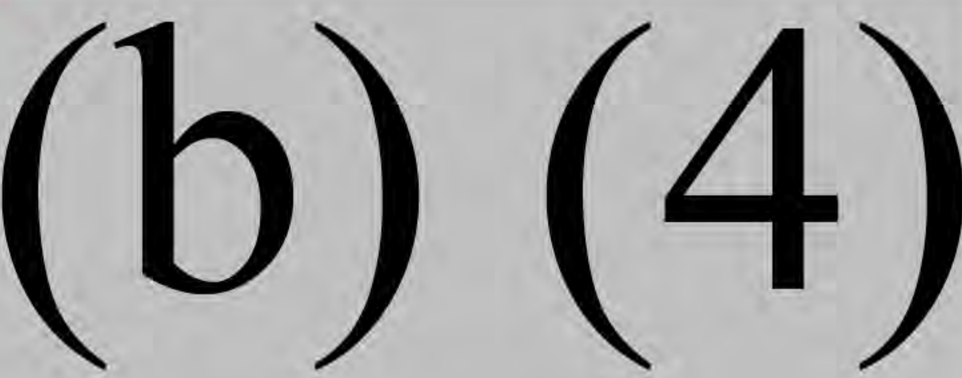
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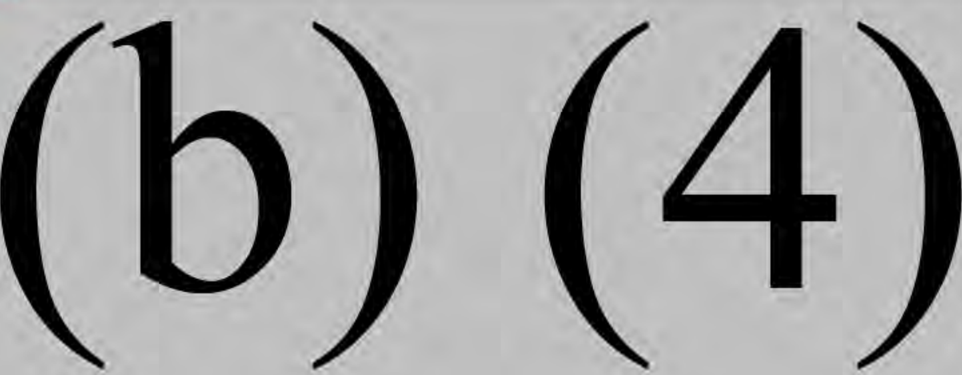
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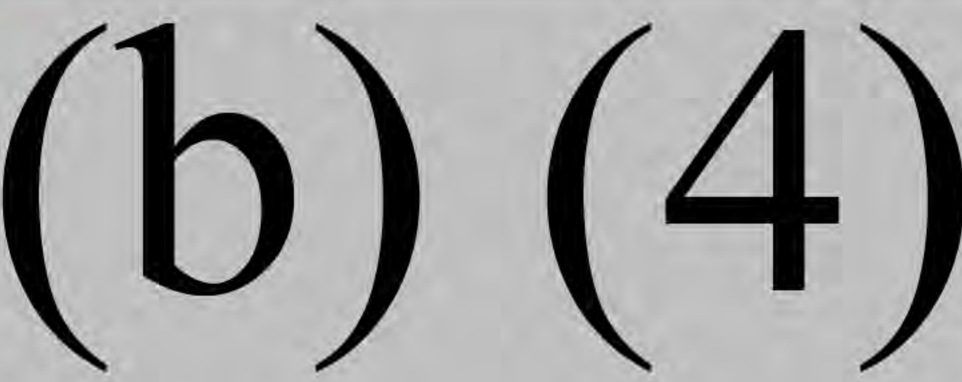
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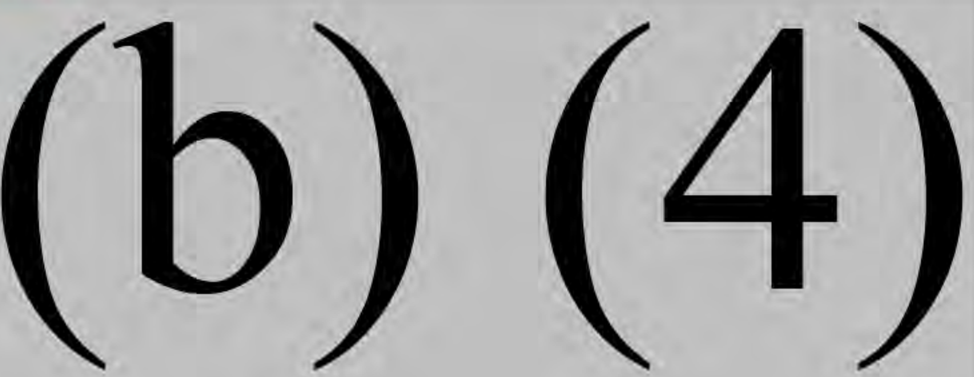
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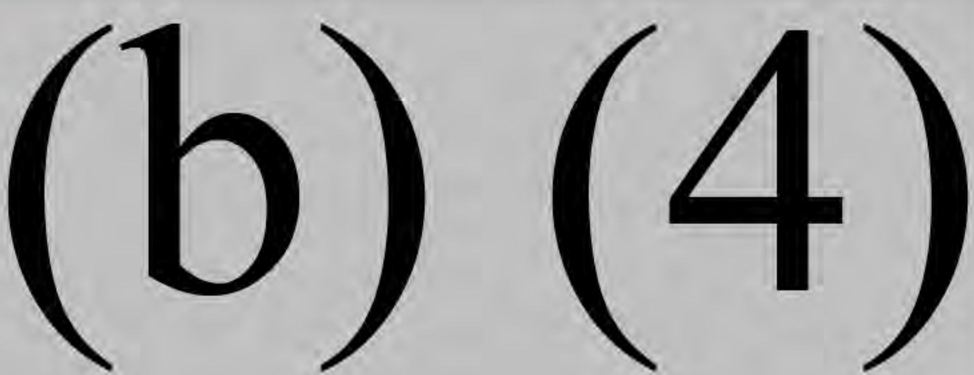
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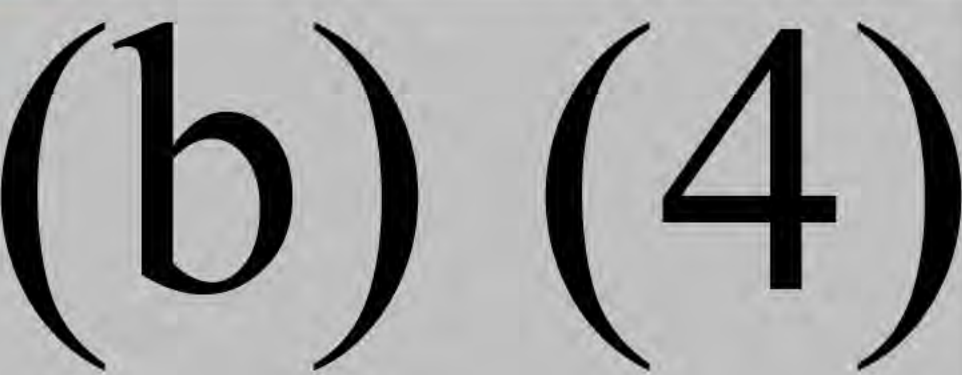
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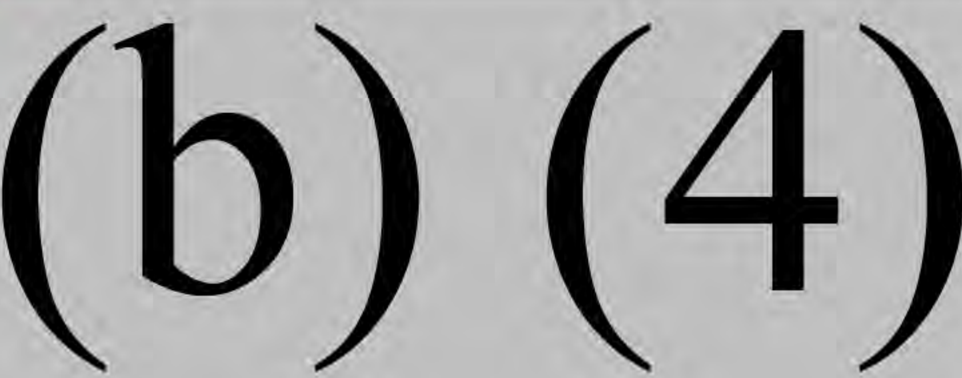
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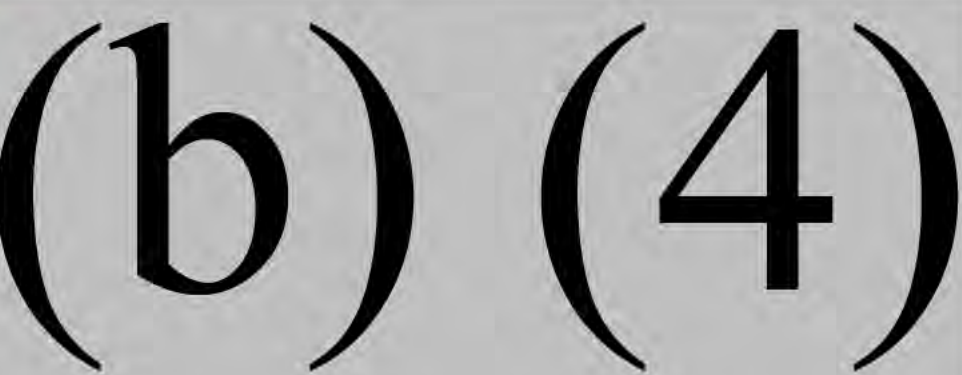
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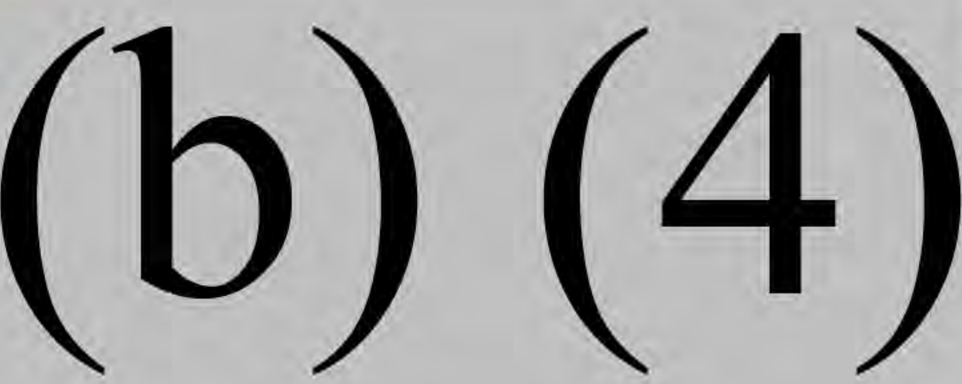
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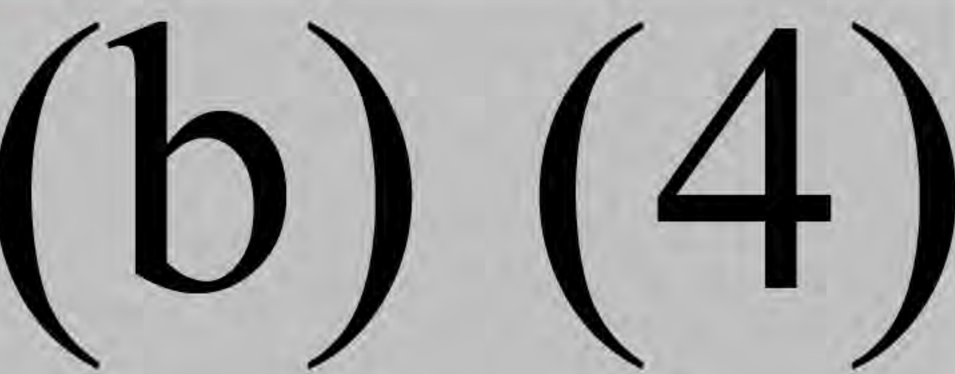
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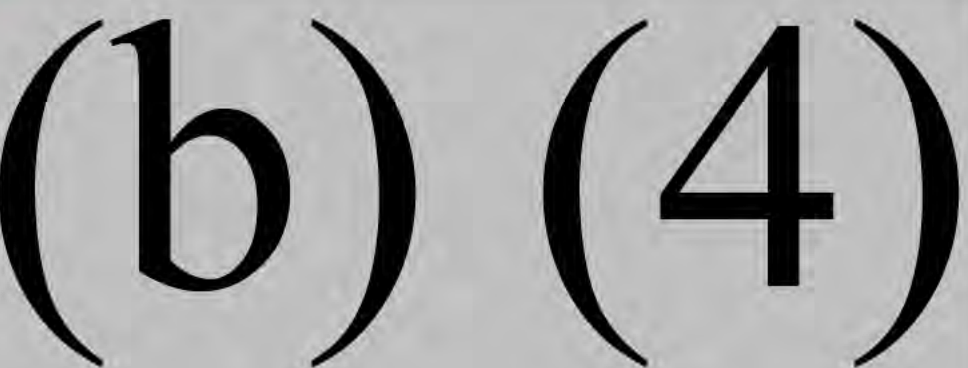
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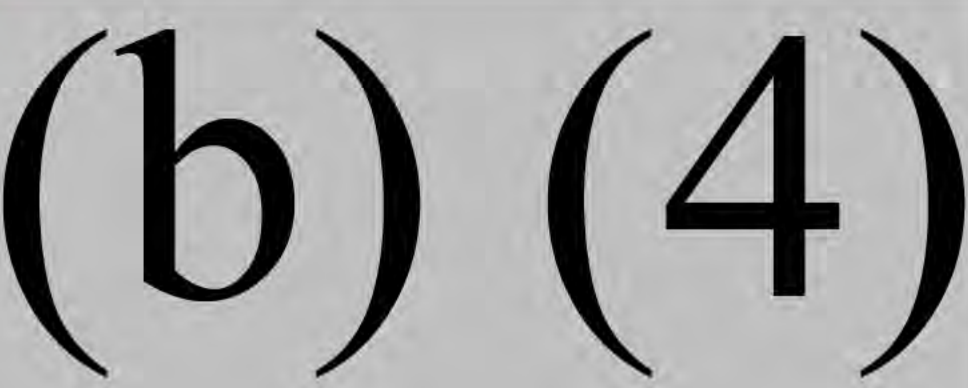
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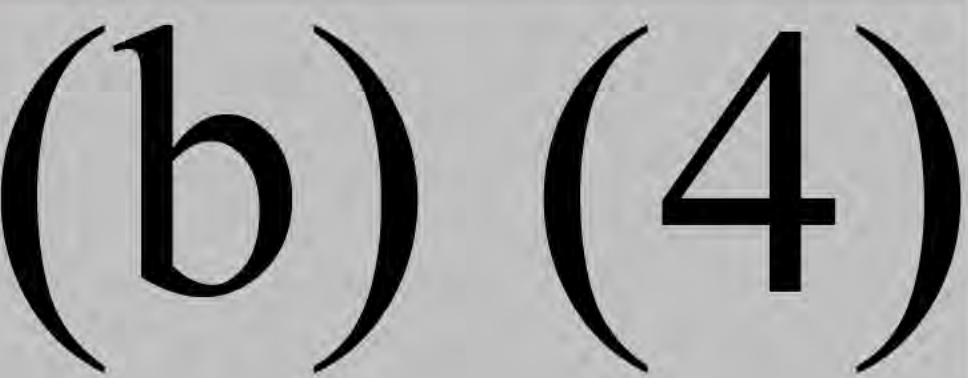
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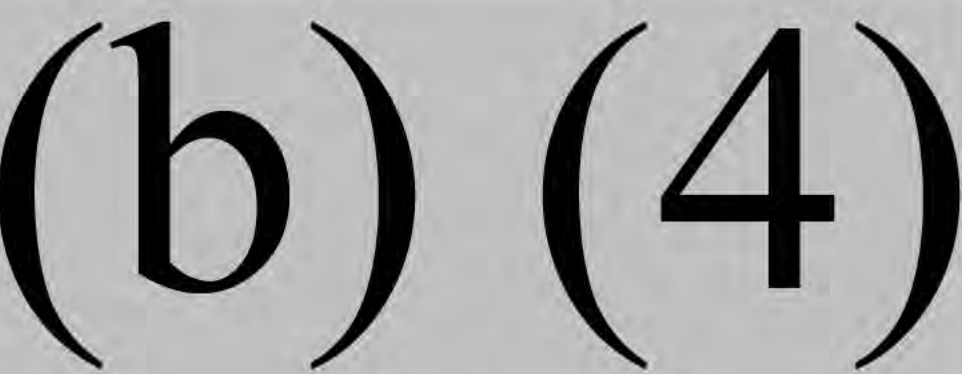
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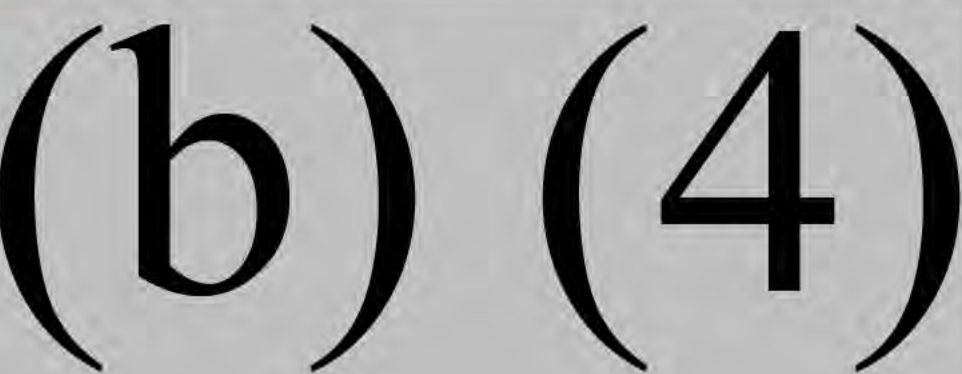
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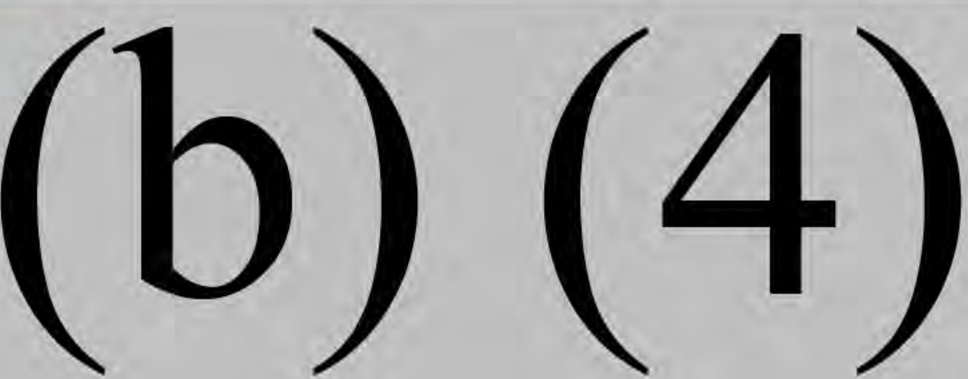
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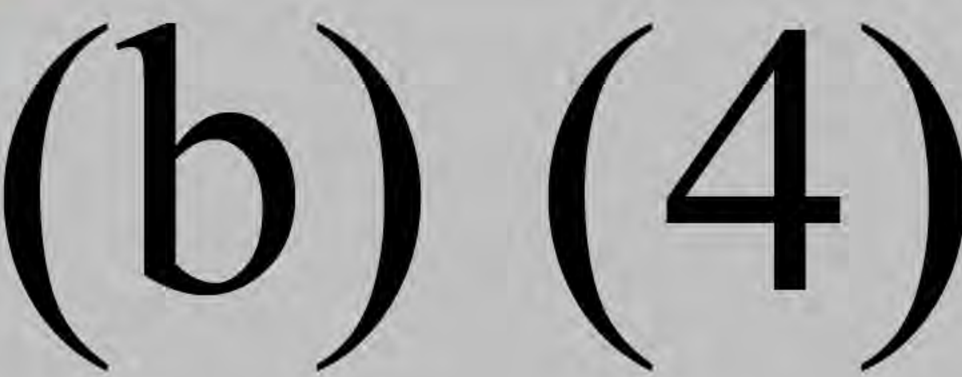
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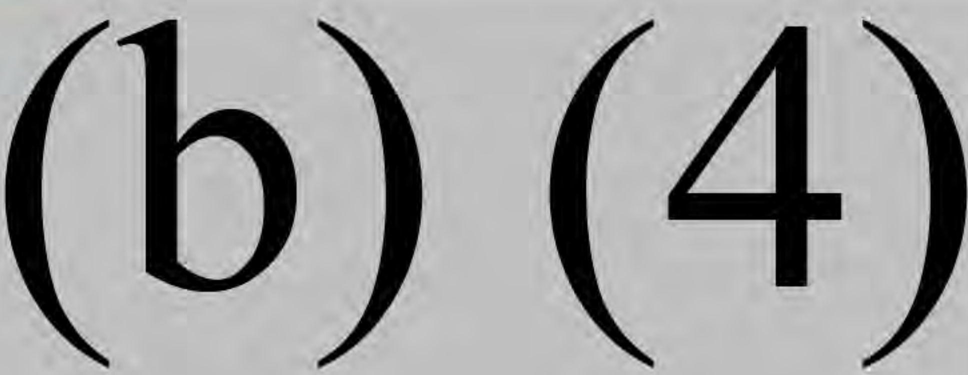
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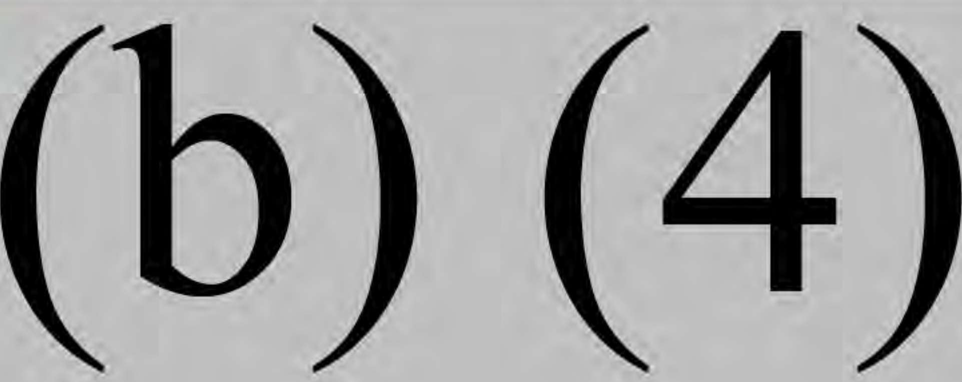
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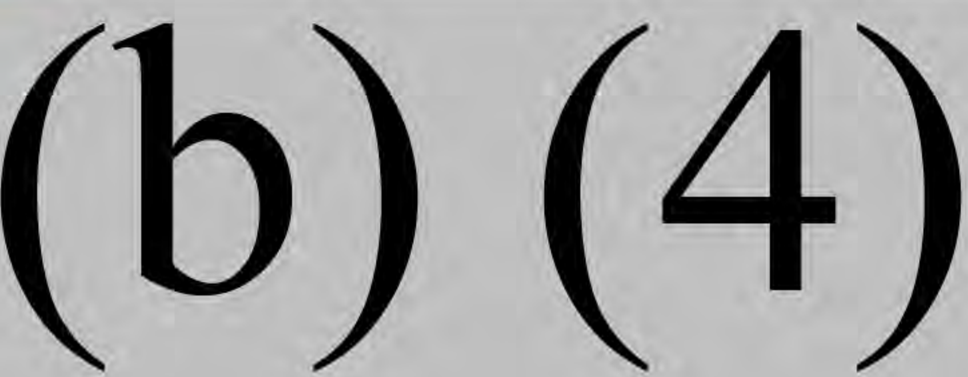
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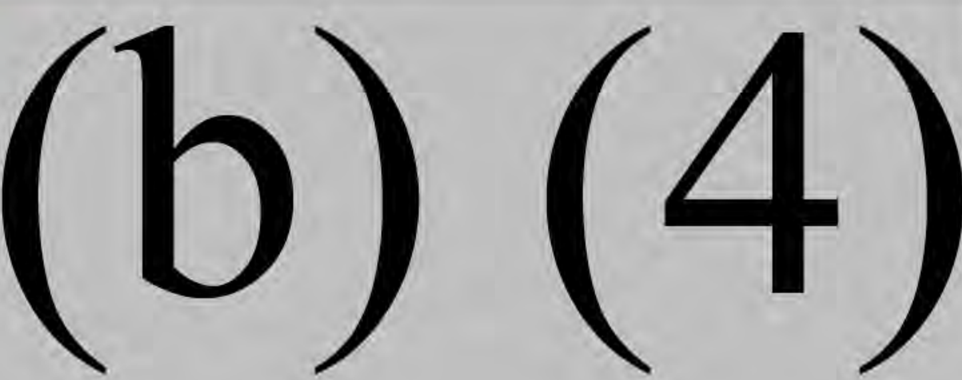
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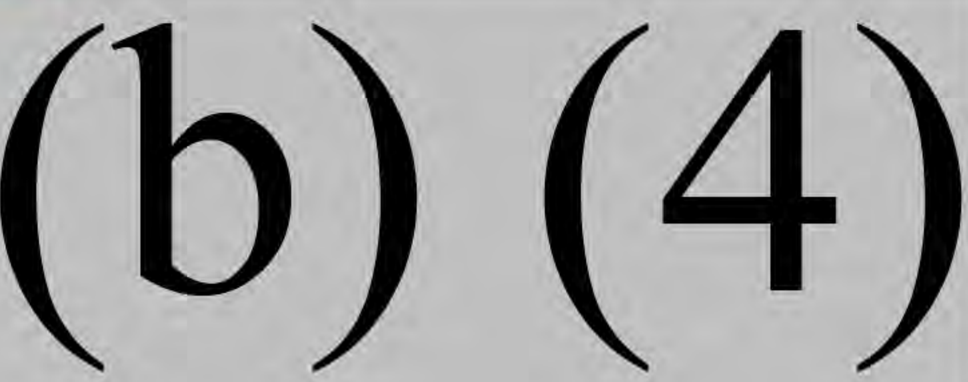
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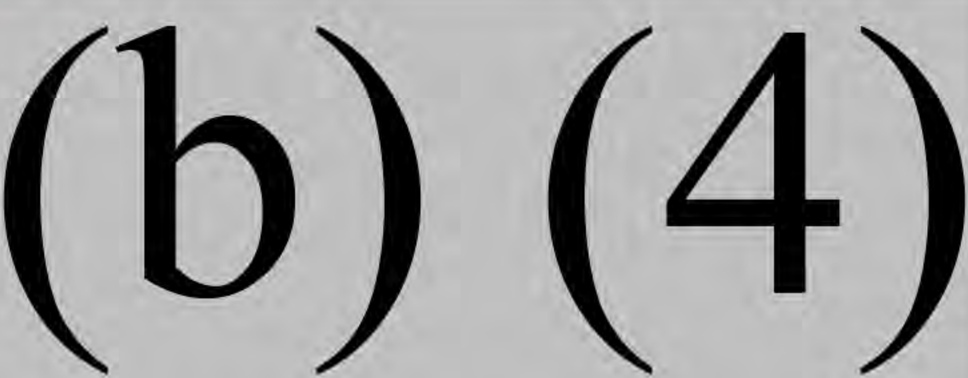
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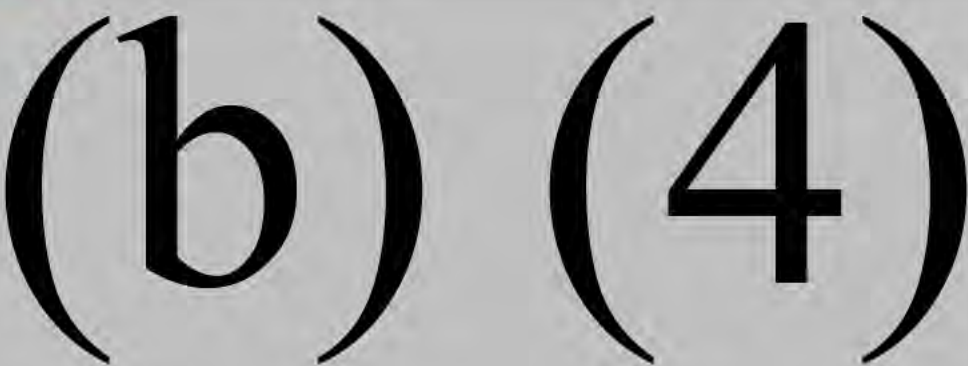
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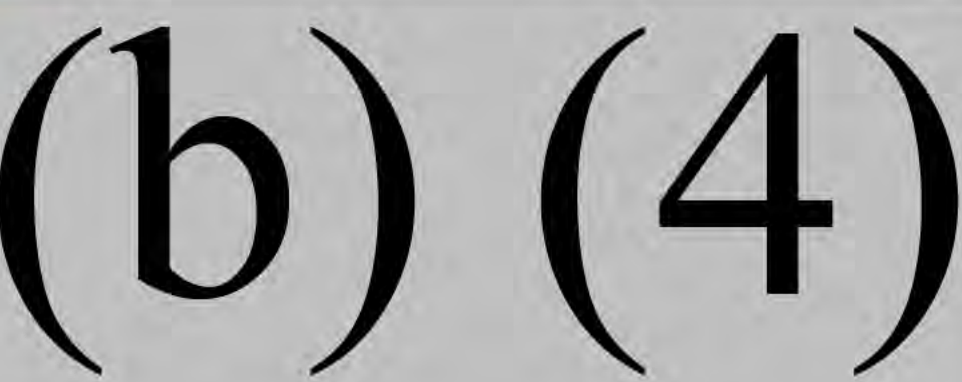
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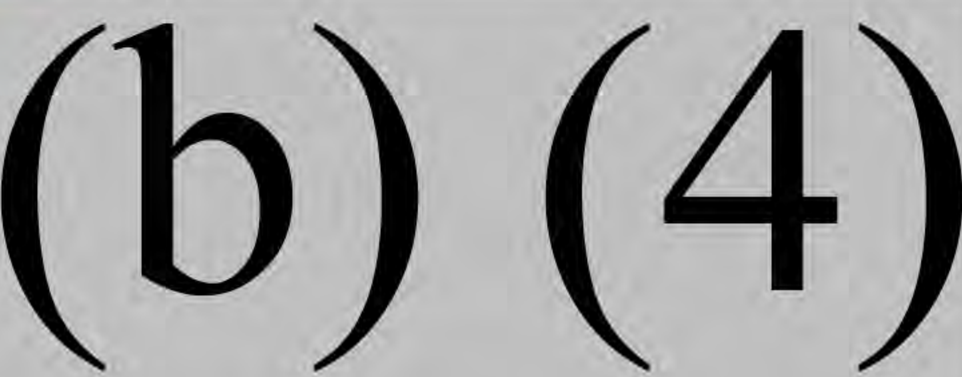
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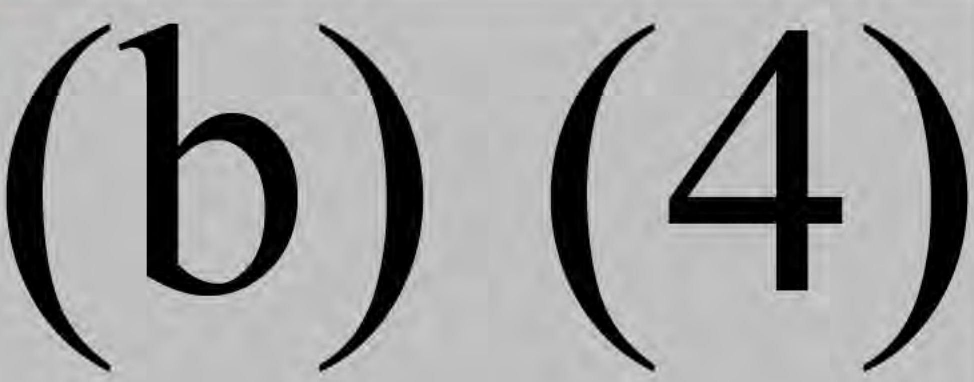
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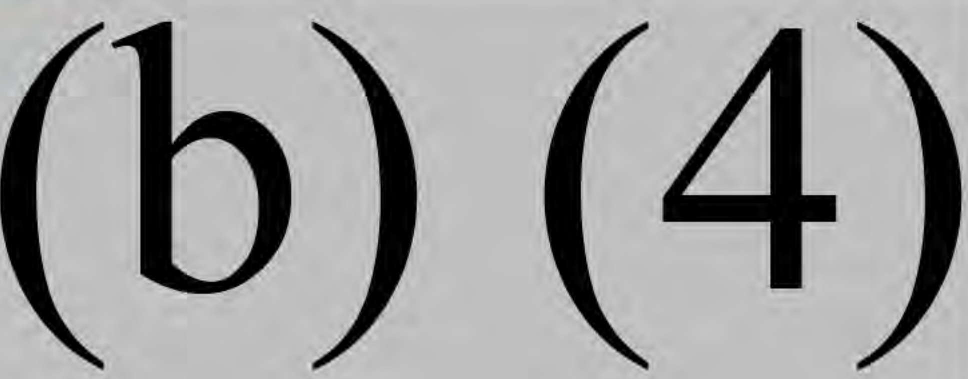
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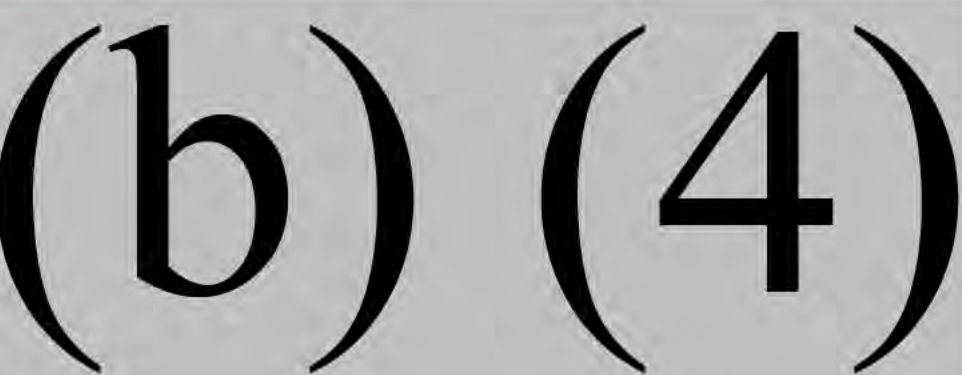
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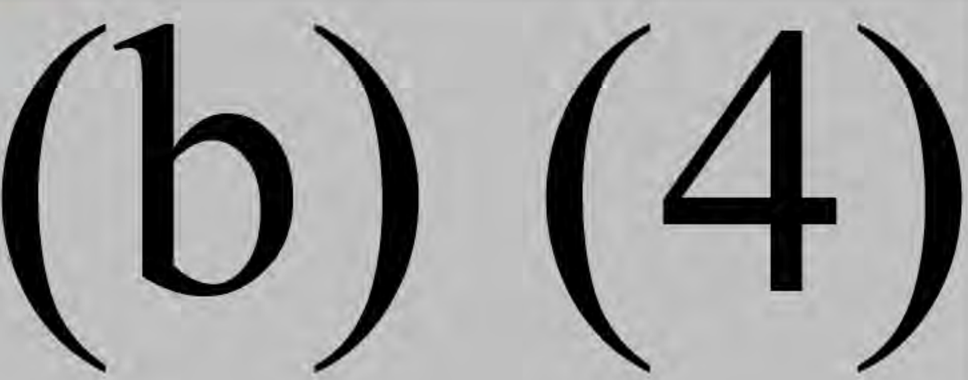
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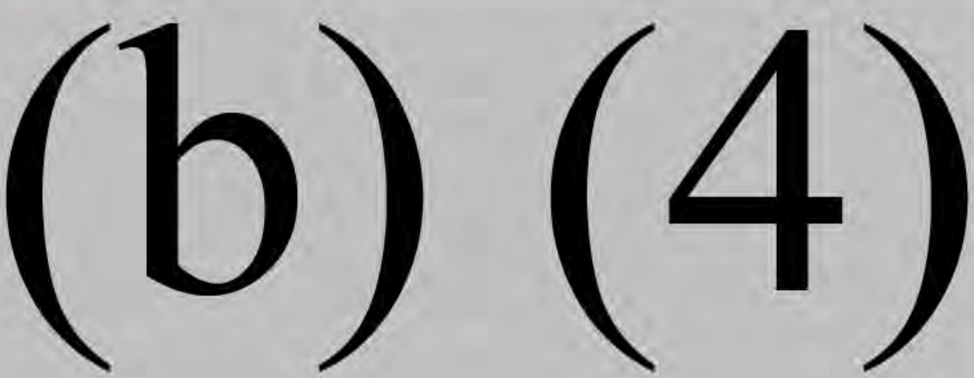
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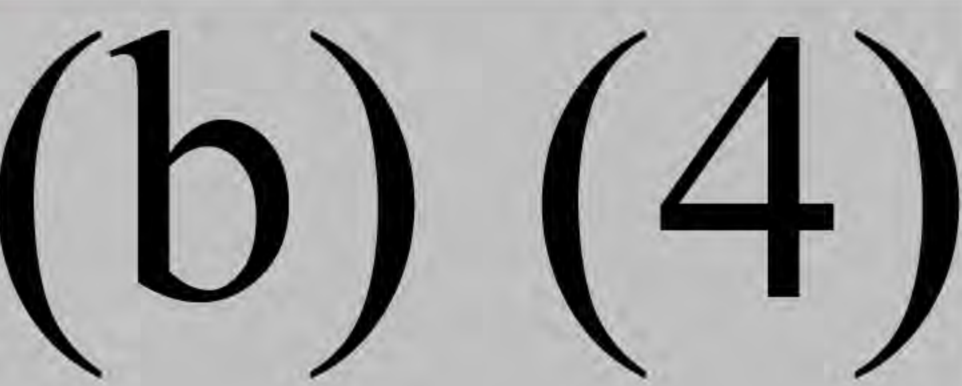
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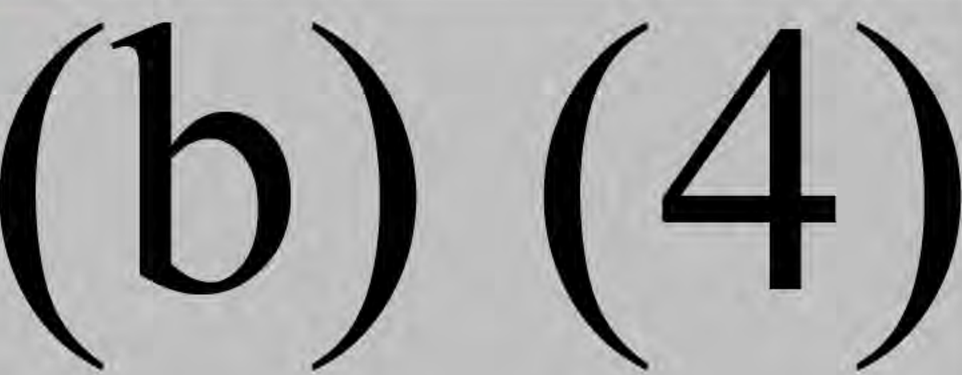
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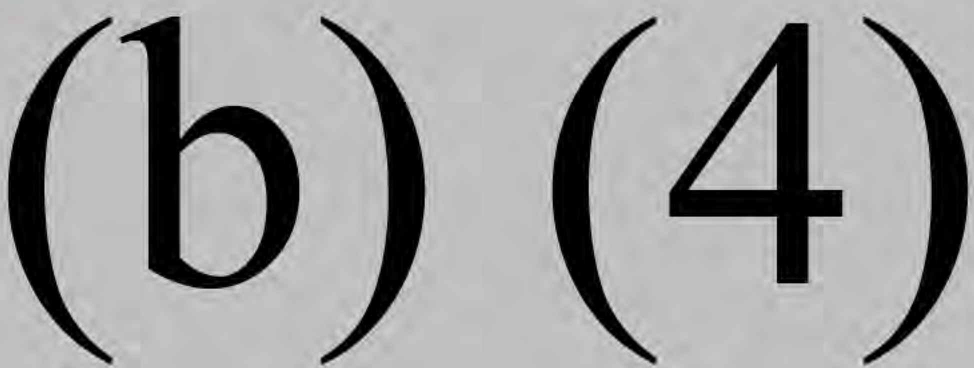
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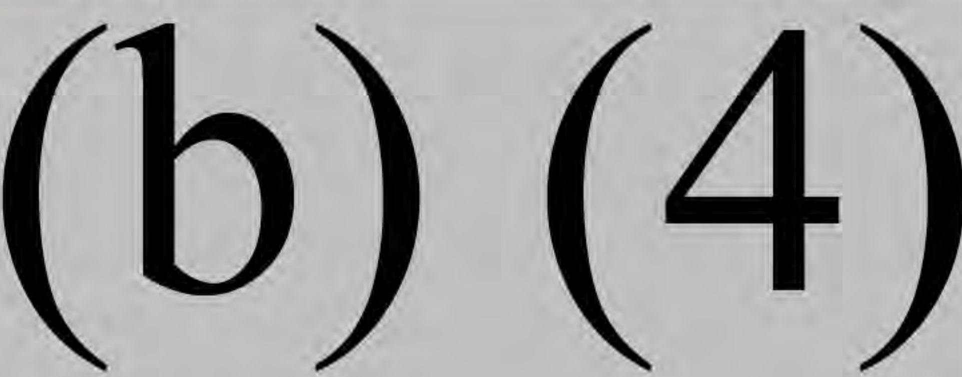
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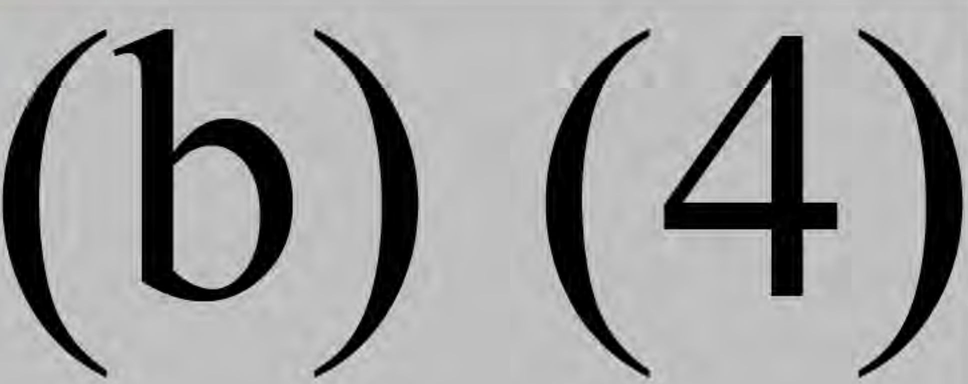
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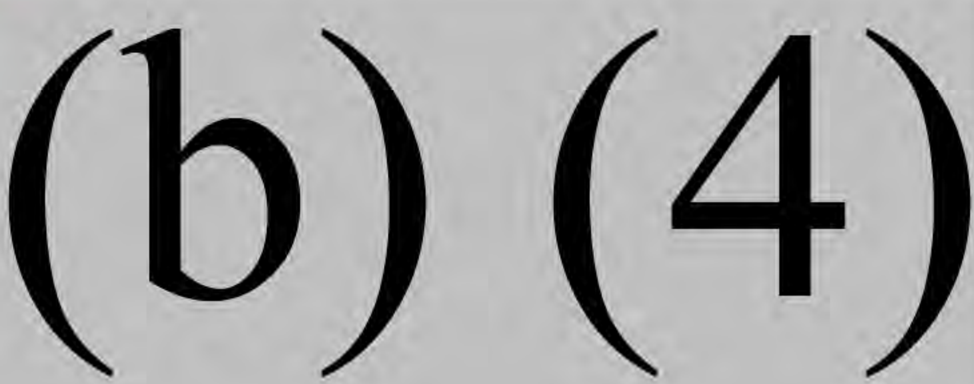
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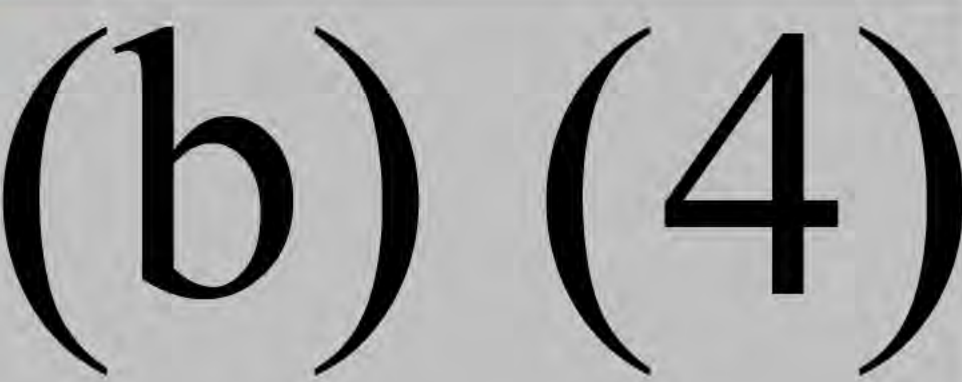
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Full Genome Sequence Analysis of

Corynebacterium glutamicum KCCM80058

(CONFIDENTIAL)

REPORT DATE: July 15, 2020

CJ BLOSSOM PARK

TITLE: Full Genome Sequence analysis of *Corynebacterium glutamicum* KCCM80058

OBJECTIVE OF THE STUDY

This study was conducted to analyze of the genome sequence and the open reading frame of *Corynebacterium glutamicum* KCCM80058 to evaluate the effect of the genetic modification on the genome of production strain.

SCCHEDULE OF THE STUDY

Data of Receipt: July 08, 2020

Data of Test: July 09, 2020

Data of Final report: July 15, 2020

TESTING FACILITY

Name: CJ Blossom Park, BIO) R&D system

Address: 42nd street 55, Gwanggyo-ro, Yeongton-gu, Suwon-si, Gyeonggi-do, CJ Blossom Park 16495, Republic of Korea

RESPONSIBLE STAFFS

Study Director Sang Jun Kim

Quality Assurance Manager Sung Gun Lee

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