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

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Table of Contents

I. Introduction.....3

II. Background.....5

III. Overview of AOAC Official Method 960.48 – Protein Efficiency Ratio (PER), Rat Bioassay.....6

IV. "Appropriate Modifications" of AOAC Official Method 960.48.....8

A. Need for "Appropriate Modifications" to Update the AOAC Method and for Use of Infant Formulas in PER Bioassays.....8

B. Conduct and Analysis of a PER Study with "Appropriate Modifications" (Matching the Casein Reference (Control) and Test Diets).....9

V. Miscellaneous.....30

A. Protocols and Reports.....30

B. Reference Guidelines.....31

VI. Paperwork Reduction Act of 1995.....32

VII. References.....32

VIII. Appendices.....36

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Protein Efficiency Ratio (PER) Rat Bioassay Studies to Demonstrate That a New Infant Formula Supports the Quality Factor of Sufficient Biological Quality of Protein: Guidance for Industry[1]

This guidance represents the current thinking of the Food and Drug Administration (FDA or we) on this topic. It does not establish any rights for any person and is not binding on FDA or the public. You can use an alternative approach if it satisfies the requirements of the applicable statutes and regulations. To discuss an alternative approach, contact the FDA staff responsible for this guidance as listed on the title page.

I. Introduction

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

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

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[1] This guidance has been prepared by the Office of Critical Foods, Infant Formula Premarket Review Staff in cooperation with the Office of Policy, Regulations, and Information in the Human Foods Program at the U.S. Food and Drug Administration.

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

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

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

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

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

II. Background

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

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

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

[5] 79 FR 7934 at 8023.

similar content of nitrogen, fat, ash (i.e., inorganic residue remaining after water and organic matter have been removed by heating), moisture, and crude fiber.

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

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

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

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

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

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[6] See 21 CFR 106.96(f).

[7] As used in this document, the terms "match," "matched," and "matching" do not mean identical. Section IV.B.1.a explains what we consider to be "matching."

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

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

The AOAC Method provides a procedure by which the quality of a protein can be evaluated and compared with those of other proteins. Protein "quality" can be defined as the ability of a protein to meet the essential amino acid needs of an animal, including humans. The AOAC Method is a standardized bioassay, and its collaborative study data have been published[8] (Ref. 9, 10).

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

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

While the AOAC Method is flexible in many respects, ensuring consistency between the test and casein control groups with regard to diet composition is critical to ensuring that the assay is specific for protein quality and the comparison is not influenced by other uncontrolled differences. To ensure the test and reference diets are matched except for protein source, adjustments are needed to the casein reference diet specified in the AOAC Method. In conducting a PER assay, the performance of the contemporaneous reference group[9] is as critical to the evaluation of the results as is the performance of the test group because the results are reported as relative rather than absolute values (Ref. 4).

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

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

AOAC Method Diet Formulation and Adjustments

Ingredient	Dietary level, %	Dietary adjustment
Protein	10	Protein from test sample
Cottonseed oil	8	Minus fat content of test sample
Salt mixture	5	Minus ash content of test sample
Vitamin mixture	1	No adjustment described
Cellulose	1	Minus fiber content of test sample
Water	5	Minus moisture content of test sample
Sucrose or cornstarch	To 100	100 minus weight of all other constituents

According to the AOAC Method, proximate analysis[10] is needed to adjust the diets so that all comparisons between the test protein ingredient and the reference casein protein are made with diets having similar overall contents of nitrogen, fat, ash, moisture, and crude fiber (Ref. 1). Thus, the minimum specifications for chemical analyses for PER study diets include the determination of nitrogen, fat, ash, moisture, and crude fiber. Additional analyses can be performed if desired, and many methods are available (e.g., Official Methods of Analysis of AOAC INTERNATIONAL, AOAC INTERNATIONAL, Rockville, MD; Official Methods and Recommended Practices of the American Oil Chemists Society, American Oil Chemists Society, Urbana, IL; and Approved Methods of Analysis of the Cereals and Grains Association, Cereals and Grains Association, St. Paul, MN).

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

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

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

IV. "Appropriate Modifications" of AOAC Official Method 960.48

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

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

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

Studies that have evaluated variables that might be encountered when determining the PER of infant formulas have found that modifications of the levels and sources of fat and carbohydrate in casein reference diets to match those in various infant formulas led to significant changes in the resultant PER study values (Ref. 7). For example, PER values (g body weight gain/g protein consumed for the 28-day study period; mean ± SEM[11]; 10 rats/group) varied from 3.2 ± 0.13 in an unmatched casein reference diet to 2.3 ± 0.24 and 2.1 ± 0.16 in two matched, milk-based formula test diets (Ref. 7). The PER value of 2.1 is 66% of the value measured in the unmatched reference group and shows the magnitude of the difference that may result when reference and test diets are not well-matched. In these data, the PER values of the milk-based infant formulas appear to be significantly underestimated in comparison to the PER value of the casein reference group. The protein quality evaluation of infant formulas using the PER bioassay warrants the use of matched casein reference diets for each type of formula (Ref. 7).

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

B. Conduct and Analysis of a PER Study with "Appropriate Modifications" (Matching the Casein Reference (Control) and Test Diets)

1. Preparation of Experimental Diets

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

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

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

79 FR 7934, 8023 (Feb. 10, 2014).[12] The Infant Formula Interim Final Rule also stated, "Although the method has limitations with respect to assessment of the quality of protein sources for infant formulas, the limitations are greatly reduced by modification of the test and control diets" (79 FR 7934 at 8023). The final rule also provided details on "[t]hree dietary adjustments commonly required for evaluation of the protein quality of infant formulas": (1) matching of the fat content of the reference and test diets; (2) matching of the carbohydrate composition of the reference and test diets; and (3) removal of water from liquid infant formula to achieve the lower limit of nitrogen (1.8% by weight) specified by the PER bioassay (79 FR 7934 at 8023 to 8024). FDA also has considered other areas in which modifications may be appropriate, including the matching of minerals and vitamins in the casein reference and test diets, acclimation diets and length of acclimation, and record-keeping.

The sub-sections below describe FDA's current thinking with respect to matching the PER study casein reference diet and test diet; preparation of PER study casein reference diets; protein source and conversion of nitrogen to protein; fats and carbohydrates (including a discussion of vitamin E as it relates to content of polyunsaturated fatty acids (PUFA)); removal of water from liquid infant formulas and determination of moisture in PER study diets; mineral content;

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

vitamin content; fiber; sulfur amino acids (SAA) (methionine, cystine); other constituents (including vitamin C); developing estimates of compositions of PER study reference diets (minerals and vitamins) to within 20% (above or below) test diets; and chemical analysis (demonstrating the appropriateness of modifications).

a. Matching of Nutrients in PER Study Casein Reference Diets to Test Diets

We recommend the matching of nutrients in casein reference diets, including minerals and vitamins, to within 20% of the corresponding levels in the test diets. For example, if the amount of nutrient X in the test diet is 10%, we recommend that the amount of nutrient X in the casein reference diet be between 8% and 12%. Although closer matching would be ideal from the perspective of minimizing confounding variables, closer matching (e.g., within 10%) is difficult to accomplish as a practical matter and may not yield commensurate benefits. In contrast, differences of a greater magnitude (e.g., within 30%) would result in excessive variability, particularly with respect to essential trace minerals and vitamins. Thus, our recommendation to match nutrients to within 20% represents a balance between a too-restrictive recommendation and one that might lead to deficits of essential vitamins or trace minerals. We provide Appendix 6 in this Guidance to show how such matchings might be accomplished.

b. Preparation of PER Study Casein Reference Diets

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

c. Protein Source, Conversion of Nitrogen to Protein

In the AOAC Method, the protein content of a product is calculated from its nitrogen content by applying a factor considered suitable for converting nitrogen to protein in the food. Such factors are based on the nitrogen content of the predominating protein present in various foods. The AOAC Method specifies the use of the factor 6.25 as the nitrogen conversion factor for setting protein levels. FDA considers this conversion factor appropriate (i.e., there is no need for modifications) for the purpose of setting protein levels in a PER study.

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

d. Fats and Carbohydrates

Fat content: The original protein evaluation basal diet described in the AOAC Method contained 8% cottonseed oil, which was readily available in the past, but is no longer readily available.[13] With respect to infant formula, FDA stated the following in the Infant Formula Interim Final Rule:

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

(79 FR 7934 at 8024.)

Infant formulas contain a variety of fat sources, and FDA considers it important to match the fat content and fatty acid compositions of the PER study casein reference diet both quantitatively and qualitatively to those of the infant formula-based test diet. We recommend that the matchings of fat (and carbohydrate) between the test and casein reference diets be made to within 20%.

That said, we recognize that some leeway may be needed to achieve the appropriate texture and palatability of the casein reference diet. If matching to within 20% of the total fat and saturated fat levels in the test diet fails to yield a casein reference diet of suitable texture/palatability, the level of saturated fat in the casein reference diet may be increased and the level of unsaturated fat decreased proportionally, while maintaining the total fat percentage in the casein reference diet to within 20% of the total fat level in the test diet. If matching to within 20% of the total fat level in the test diet fails to yield a casein reference diet of suitable texture/palatability, then we recommend an adjustment to the fat composition of the casein reference diet by adding saturated fat in the minimum amount necessary to achieve suitable texture/palatability. Note that use of a

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

defatted test sample is incompatible with the requirements of 21 CFR 106.96(f) because 21 CFR 106.96(f) requires testing of an infant formula product, not a modified form of the product.

The matching of fat and fatty acid compositions is important because, in both humans and animals, the body's need for vitamin E increases with an increase in consumption of PUFAs and with the degree of unsaturation of dietary PUFAs. The rat requirement for vitamin E (*RRR-α*-tocopherol; formerly called d-α-tocopherol) is 18.0 milligrams per kilogram (mg/kg) diet (equivalent to 27 international units (IU)/kg) when lipids comprise less than 10% of the diet (e.g., a diet containing 50.0 grams (g) fat/kg, 6.0 g linoleic acid (n-6)/kg, and an estimated requirement of 2 g/kg linolenic acid (n-3), which can be substituted with other long-chain n-3 PUFA)[14] (Ref. 11). Higher concentrations of vitamin E may be required if high-fat diets are fed (Ref. 11). Harris and Embree reviewed studies in which rats were fed diets containing varying ratios of vitamin E:PUFA (expressed as mg of *d-α*-tocopherol to grams of PUFA), which were sufficient either to induce vitamin E deficiency or to relieve it (Ref. 13).

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

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

We suggest that the total PUFA contents of the PER study test and casein reference diets be estimated from the Certificates of Analysis (CoA) or other information and used with dietary concentrations of vitamin E to calculate the ratio of vitamin E:PUFA as mg vitamin E/g PUFA for each diet. We note that vitamin E concentrations in most appropriately modified casein control and infant formula-based test diets are at least double the National Research Council (NRC) requirement of 27 IU/kg (Ref. 11). Thus, the minimum ratio of 0.48 ± 0.28 mg of d-α-tocopherol to grams of PUFA (described above) may be met in many PER studies with infant formulas without any adjustment. We recommend that sponsors speak with diet formulators and those performing PER studies to review available data to confirm whether an adjustment is needed.

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

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

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

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

(79 FR 7934 at 8024.)

FDA's current thinking is that it is critical to the conduct of a well-designed PER study that the casein reference diet contain a level of lactose comparable to the amount present in the infant formula test diet.[15] However, we recognize that, in formulating the infant formula test diet, a high rate of addition[16] of a high-lactose infant formula to a test diet will limit the amounts of other carbohydrates, such as cornstarch, that can be added to the casein control diet. As noted above with respect to fat, some flexibility can be gained by working within the 20% range recommended for matching nutrients in the test and casein reference diets. The flexibility of such a 20% range could be used, for example, to add somewhat lower amounts of lactose and somewhat greater amounts of cornstarch to the casein reference diet. For example, if a casein reference diet is formulated to match an infant formula-based test diet containing 40 g lactose/100 g and 5 g cornstarch/100 g, then matching to within 20% of the infant formula test values would mean that the reference diet would contain between 32 g to 48 g lactose/100 g and between 4 g to 6 g cornstarch/100 g. As is the case with fat, we recognize that there may be a need to exceed a 20% matching range for lactose to achieve suitable palatability or texture of the casein reference diet.

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

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

[15] The need for inclusion of lactose in acclimation diets fed prior to a PER study with a high-lactose test diet is considered in Section IV.B.3.a.

[16] The "rate of addition" refers to how much of the infant formula must be added to the PER study test diet.

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

(79 FR 7934 at 8024.)

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

Determination of Moisture in PER Study Diets: The appropriate modifications that FDA discussed in the Infant Formula Interim Final Rule did not address the issue of water in the PER diets (a separate issue from the removal of water from liquid infant formulas with the goal of bringing the nitrogen level to the concentration specified in the AOAC Method). Furthermore, the issue of the water content of the PER study diets is distinct from the issue of providing drinking water to the animals during the study,[17] and one cannot substitute for the other. The water content of the diets needs to be considered in formulating the PER study diets because adjustment for water (expressed as "moisture" in the formula shown in the AOAC Method; Appendix 1) is one of the primary specifications in the development of the PER study diets. PER study casein reference and test diets should both contain equal moisture contents because the level of hydration may influence PER study results (Ref. 4). The AOAC Method provides a formula for adjusting the water content of PER diets to about 5% based on the moisture content of the test material. Earlier studies have shown that differences in water content may influence PER study results, with PER values for casein increasing with the percent of water added to the diets (Ref. 4 and references therein). A second effect of water that should be considered is the point at which water is added to the diets. In earlier studies, higher PER values were obtained when water was added to casein at the beginning of the diet mixing process than when water was added to the final diet after mixing other ingredients (Ref. 15). FDA recommends that the issue of water in PER study diets be discussed with diet formulators with experience in blending diet ingredients such as oils and carbohydrates to avoid problems with diet consistency or palatability. Increased flexibility in the water content of the diets can be gained by working within a 20% range for matching. We recommend that the water content in the diets be matched as closely as possible, but acknowledge that there may be a need to go beyond 20% to ensure that the casein reference diets are of suitable consistency and palatability.

f. Mineral Content

The following paragraphs describe FDA's recommendations regarding the mineral content of PER study casein reference diets. We consider the use of the AOAC Method mineral mixture as well as other mineral mixtures, taking into consideration the sulfur/sulfate content of the final

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

diets. We also consider whether ash content is an appropriate proxy for the mineral content of PER study diets.

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

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

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

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

We also consider the use of the Bernhart-Tomarelli mineral mixture (Ref. 17) to be appropriate when 0.010 g Na_2SeO_4 and 0.0070 g $(NH_4)_6Mo_7O_{24}$ $4H_2O$ are added per kg of the mixture. When used at 4% of diets, this Se- and Mo-supplemented mixture will provide 0.00017 g Se/kg diet and 0.00016 g Mo/kg diet and will meet the specifications in the NRC's Nutrient Requirements of Laboratory Animals (Ref. 11). This mixture contains 0.501 g sulfate/kg when used at 4%.

There are other mineral mixtures commercially available from manufacturers of animal feeds, including American Institute of Nutrition (AIN) "AIN-76A" and "AIN-93G," which are constituents of, respectively, rodent diets AIN-76A and AIN-93G. These mineral mixtures

provide sulfate, as potassium sulfate, at 0.301–0.337 g/kg diet (Appendix 3). Our current thinking is that inorganic sulfur should be included in reference casein diets at concentrations no lower than 0.30 g/kg, the concentration that NRC (1995) identifies as potentially beneficial (Ref. 11).[18] We recommend that infant formula manufacturers consult diet formulators regarding use of mineral mixtures in PER studies with infant formulas. The composition of each of the above mineral mixtures is listed in Appendix 3.

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

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

Thus, while our current thinking is that the ash content alone is not an appropriate surrogate when matching minerals in test and casein reference diets in a PER study with infant formulas,

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

we recommend that this issue be resolved by developing estimates of compositions of projected PER study test and casein reference diets prior to initiation of the PER study. In Section IV.B.1.k and Appendix 6, FDA provides a discussion and a table that shows how the compositions of PER study test and casein reference diets can be developed from the composition of the infant formula.

g. Vitamin Content

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

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

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

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

Because most infant formulas provide significant quantities of vitamins as well as minerals, careful calculations should be made before diet formulation begins to determine the contributions of vitamins anticipated from the test formula and from the vitamin mixture(s). In some cases, the infant formula itself may provide most or perhaps all vitamins, as per the NRC's Nutrient Requirements of Laboratory Animals (Ref. 11). Adjustments in amounts of vitamin mixture added to the infant formula-based test diet or preparation and use of a specific vitamin mixture to adjust for such a finding may be needed. We recommend that manufacturers or sponsors discuss this with diet formulators to identify commercially available or custom vitamin mixtures whose use will reduce the number of individual additions of vitamins that need to be made to match casein reference and infant formula-based test diets.

Specific recommendations for several vitamins follow:

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

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

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

iv. **Vitamin A**: The estimates of vitamin requirements for rats have changed over the years. This is most obvious in the concentrations of vitamin A included in vitamin mixtures and diets. The AOAC Method vitamin mixture as listed in the AOAC Method and the AOAC vitamin mixture each provide 20,000 IU vitamin A per kilogram diet versus the current NRC (Ref. 11) specification of 2,300 IU per kilogram diet. Infant formula typically has a high concentration of vitamin A. Because of the importance of matching the casein reference and test diets, for infant formula, the concentration of vitamin A in the reference diet should be matched to that of the test diet even if such matching results in reference diets of high vitamin A content.[19]

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

vi. **Choline**: Choline is an essential nutrient and serves as a methyl-donor in many physiological reactions. While choline is not a vitamin, it is frequently listed with the vitamin component of diets (Ref. 11, 14). Choline (form unspecified) is included as a part of the AOAC Method vitamin mixture (Appendix 1). Its dihydrogen citrate salt is used in the commercially available AOAC vitamin mixture (Appendix 4). Choline is not included in the AIN-76A and AIN-93G vitamin mixtures (Appendix 4). Rather, the AIN-

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

76A and AIN-93G diet formulations include choline bitartrate as separate additions to the final diets. The consequence of this change is that if the AIN-76A or AIN-93G vitamin mixtures are used to replace the AOAC Method vitamin mixture, and the need for the separate addition of choline is overlooked, the resulting finished casein reference diet will be deficient in choline. The situation with respect to choline in the PER study test diet will need to be determined by evaluation of the choline content of the infant formula and its rate of addition in the test diet. The levels of choline in the casein reference diet and in the infant formula test diet should be matched. FDA's current thinking is that choline may need to be included as a separate addition to both diets.

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

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

h. Crude Fiber and Non-Digestible Carbohydrates

The PER study diets described in the AOAC Method (Section III) include 1% fiber as cellulose. For the test diet, this ingredient is calculated as 1% cellulose minus the fiber content of the test sample and is measured as crude fiber in the proximate analysis. A review of CoA submitted with recent PER studies showed that AOAC Official Method 962.09, a method for crude fiber in animal feed and pet food, is often used for determination of crude fiber.

Infant formulas do not contain cellulose or other sources of crude fiber, but they frequently contain non-digestible, fiber-like carbohydrates such as fructooligosaccharides (FOS) or galactooligosaccharides (GOS). Because the original AOAC Method includes 1% fiber as cellulose, and fiber is not included in infant formula, we considered whether the addition of cellulose is beneficial in conducting an infant formula PER study.[20]

The NRC's Nutrient Requirements of Laboratory Animals notes that although fiber has not been shown to be required by rats, inclusion of fiber may be potentially beneficial to rats (Ref. 11). The NRC's Nutrient Requirements of Laboratory Animals states that feeding rats fiber increases fecal bulk and the weight of the cecum and colon and decreases gastrointestinal time. Thus, the inclusion of fiber helps normalize rat digestion. According to a laboratory that has conducted

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

numerous PER studies with infant formula, even in cases in which nondigestible carbohydrates such as FOS and GOS are present, without added cellulose, diarrhea is worse through acclimation and tends to persist longer. In contrast, in diets with even low levels of cellulose (< 0.3%), the duration of diarrhea is mostly resolved in the first week. With 1% cellulose, diarrhea tends to be fully resolved in both groups by the end of the first week. These observations suggest that FOS and GOS are not substitutes for cellulose in rat diets.

Therefore, we recommend that both the test and casein control diets contain 1% cellulose regardless of the presence of indigestible carbohydrates such as FOS and/or GOS in the infant formula. We recommend that FOS and GOS, if present in the infant formula, be added to the casein control diet as appropriate.

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

i. Inorganic Sulfur/Sulfate and Sulfur Amino Acids (SAAs) (Methionine, Cystine)

While we do not have data that would assist in making a precise evaluation regarding additions of inorganic sulfur/sulfate in the PER study casein reference control diets in studies with infant formulas, our current thinking is that inorganic sulfur/sulfate should be included in reference diets at concentrations no lower than 0.30 g/kg, the concentration that NRC identifies as potentially beneficial (Ref. 11).

Casein, the reference protein used in the AOAC Method, has a low concentration of cystine in relation to methionine. The AOAC Method does not include the addition of cystine, despite this low concentration of cystine in the reference protein casein. We think that, when the sulfate concentration is maintained at a minimum of 0.30 g/kg in the casein reference diet, the addition of cystine to the casein reference diet is unnecessary.

The following section provides background information and analysis to support these recommendations.

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

The rat requirement for SAAs is expressed as the sum of methionine plus cystine (i.e., methionine + cystine). This value is 9.8 g/kg (0.98 g/100 g diet; 0.98%) for rats fed diets

containing 15% protein (Ref. 11). While the corresponding value for rats fed 10% casein protein diets has not been determined, Peace *et al.* found that optimal feed-to-weight-gain ratio, relative net protein ratio, and plasma amino acid parameters were obtained when weanling rats were fed 8% casein plus amino acid diets containing 0.44% (0.44 g/100 g diet; 4.4 g/kg diet) total SAAs with cystine replacing 33% to 60% by weight of dietary methionine (Ref. 20). The inclusion of cystine at the expense of methionine in 8% protein diets improved overall rat performance and utilization of dietary methionine (Ref. 20). Provision of cystine may decrease the requirement for enzymatic conversion of methionine to cystine and its derivatives, permitting more efficient utilization of methionine for protein synthesis (Ref. 20). The ability of cystine to substitute for a portion of the methionine requirement is important because cystine is present in limited quantities in 10% casein diets. Thus, the contribution that cystine can make to overall methionine metabolism is reduced in rats fed 10% casein protein diets.

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

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

While we do not have data that would assist in making a precise evaluation regarding additions of inorganic sulfate in the PER study casein reference control diets in studies with infant formulas, our current thinking is that inorganic sulfur should be included in the casein reference diet at concentrations no lower than 0.30 g/kg, the concentration that NRC identifies as potentially beneficial (Ref. 11).

Lack of justification for addition of methionine:

We note that *DL*-methionine was the recommended supplement to the AIN-76A diet. During the formulation of the AIN-93 diets, the AIN noted that compared with other milk proteins, *L*-cysteine or *L*-cystine and not *L*-methionine were the amino acids found in small amounts in casein. For this reason, the AIN recommended *L*-cystine instead of *L*-methionine as the supplement to the AIN-93 diets (Ref. 22). We are unaware of a justification for adding methionine alone to raise the (methionine + cystine) concentration in the control diet because there is insufficient information from which to derive an estimate of the concentrations of total SAA or of methionine alone that are needed for 10% protein diets.

j. Other Constituents (Including Vitamin C)

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

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

k. Developing Estimates of Compositions of PER Study Casein Reference and Test Diets (Minerals and Vitamins)

In Appendix 6, we recommend an approach that can be used to develop the compositions of PER study test and casein reference diets from the composition of the infant formula that is the subject of the submission. The first step using this approach is to list all components of the infant formula in units/100 g or units/kg. The rate of addition of the formula to the test diet will provide the starting point for formulation of mineral and vitamin premixes. The sum of nutrients provided by the infant formula at its rate of addition and contributions from mineral and vitamin mixtures will provide information to formulate the reference diet. We recommend matching the mineral and vitamin compositions of the PER study reference diet within 20% of the mineral and vitamin compositions of the PER study test diet.

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

l. Chemical Analyses (Demonstrating the Appropriateness of Modifications)

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

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

2. PER Values for Casein Reference Control Diets

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

Casein reference control data — Original conditions of the AOAC Method: The available data show that when the AOAC Method is performed as described (including, among other components, 10% protein, 0% lactose, 8% fat, and 5% AOAC-specified mineral mixture), sustained growth can be achieved in the control group over the 28-day bioassay period. FDA considers that PER values for the casein control group between 2.62 and 3.09 g body weight gain per g protein consumed over the 28 days are attainable under the original conditions of the AOAC Method, which includes use of a mineral mixture providing 0.50–0.96 g sulfur/kg diet (see Footnote 6 for data and Appendix 3 for compositional information).

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

Casein reference control data — AOAC Method modified for infant formulas — High-lactose formulas: We have also identified attainable PER values when the AOAC Method is appropriately modified for use with infant formulas and high-lactose infant formulas are studied. The limited data available were obtained from studies in which the AOAC Method was performed with infant formulas with diets including, among other components, 10% protein, 43% lactose, 24% fat, and 4% of the Bernhart and Tomarelli mineral mixture (Appendix 3; Ref. 7) for a 28-day bioassay period. FDA considers that PER values for the casein control group of (mean ± SD; n=2) 2.2 ± 0.14 g body weight gain per g protein consumed over the 28-day bioassay period (range 2.06-2.36) are attainable under dietary conditions of high lactose and high fat when mineral mixtures are used that provide 0.50–0.96 g sulfur/kg diet (Appendix 3).

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

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

3. Experimental Animals

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

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

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

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

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

Housing. Housing animals individually during acclimation, while not specified in the AOAC Method, will facilitate the transfer to individual housing and individual feeding that is required during the 28-day PER study experimental period (Ref. 1). Housing in wire-bottom cages throughout will reduce coprophagy (i.e., eating of feces).[21]

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

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

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

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

DeAngelis *et al.* conducted studies in which weanling rats (21 days old) were fed diets containing 10% protein and varying levels of lactose (0, 1, 2, 5, 10, 20, and 50%) for 15 days (Ref. 25). They observed that PER values (at 15 days) at 10% and 20% lactose were not

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

different from those obtained under control conditions. They noted that growth of weanling rats was normal when lactose in diets was present at levels < 20%. When dietary lactose was 50%, PER values (at 15 days) decreased to 0.92 ± 0.45 (mean, standard deviation) (g weight gain/protein ingested) from PER values of 3.51 ± 0.15 for 0% lactose and 3.04 ± 0.19 for 20% lactose. No acclimation period was reported in this 15-day study. While these data suggest that rats tolerate lactose at levels ≤ 20%, they do not address the need for lactose in acclimation diets.

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

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

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

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

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

b. Assay Period, Type, and Frequency of Measurements

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

c. Monitoring Attrition and Adverse Effects of Diets

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

4. Tabulation and Calculation of Results (e.g., Statistical Analyses; Reference Data; Record-keeping)

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

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

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

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

V. Miscellaneous

A. Protocols and Reports

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

While not required, we recommend that the protocol be reviewed by us before initiation of the PER study. Manufacturers that are interested in sending a protocol for review may contact the Infant Formula Premarket Review Staff (IFPRS) at (240) 402-1450. After protocols are provided to the IFPRS, reviewers may analyze aspects of the protocol, including the plans for diet preparation, plans for matching the nutrient contents of test and control diets, proposals for acclimation diets, proposals for data collection, and records to be maintained. In the review, IFPRS may identify areas that the manufacturer or laboratory may want to reconsider, such as lack of appropriate matching between control and test diets, proposed use of acclimation diets that do not seem to match either the test or control diets, or situations in which we cannot identify the origin of specific nutrients in the diets. Instances may be identified in which specific pieces of information are missing or where calculations appear to be inaccurate. IFPRS will then provide feedback to the requestor.

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

1. The full specification for the infant formula under consideration, with nutrient composition expressed as units/100g or units/kg (i.e., quantitative formulation and nutrient content);

2. Information related to calculation of compositions of PER study acclimation, casein reference, and test diets; and

3. Information on the conduct of the live animal phase of the study, including selection criteria for rats upon arrival (e.g., by random numbers), selection criteria for rats when assigned to study groups (e.g., stratified randomization procedures), and descriptions of animal husbandry practices (e.g., type of caging, feeders and water bottles or watering system, animal room temperature and humidity).

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

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

B. Reference Guidelines

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

We recognize that there is limited information on the specifications for all nutrients in 10% protein diets. As an example of how NRC's Nutrient Requirements of Laboratory Animals specifications may differ in diets of low protein content, Peace *et al.* reported that the sulfur-amino acid requirements for rats fed 8% protein were 0.33% of the diet or 4.1% of dietary protein, rather than the value of 0.98% (*DL*-methionine + cystine) of the NRC diet (Ref. 4, 14, 20). We consider that most nutrient levels would be significantly lower for the 10% protein PER study diets than NRC values for 15% protein diets, but we are not able to determine the reductions that would be appropriate for each nutrient. Thus, we are unable to provide guidance on how best to use the levels provided in NRC's Nutrient Requirements of Laboratory Animals in such circumstances (Ref. 11). These levels should be used with caution and should not be used to adjust the overall compositions of the PER study diets (Ref. 11). Rather, they should be used to identify and correct a PER study diet that may be deficient in a specific vitamin or mineral and to identify and correct a PER study diet that may be formulated to contain a potentially toxic level of a specific mineral or vitamin (see, e.g., Section IV.B.1.f–g).

VI. Paperwork Reduction Act of 1995

This guidance contains information collection provisions that are subject to review by the Office of Management and Budget (OMB) under the Paperwork Reduction Act of 1995 (44 U.S.C. 3501-3521).

The time required to complete this information collection is estimated to average 56 hours per response, including the time to review instructions, search existing data sources, gather the data needed, and complete and review the information collection. Send comments regarding this burden estimate or suggestions for reducing this burden to:

Office of Critical Foods, HFS-850
Human Foods Program
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740
(Tel) 240-402-1700

An Agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number. The OMB control number for this information collection is 0910-0256. To find the current expiration date, search for this OMB control number at https://www.reginfo.gov/public/do/PRAMain.

VII. References

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

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VIII. Appendices

Appendix 1. AOAC Official Method 960.48

Appendix 2. Adjustments required by the Association of Official Analytical Chemists AOAC Official Method 960.48.

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

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

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

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

Appendix 1 is a PDF file of the AOAC Method.

Document History:

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