

<b>FOOD AND DRUG ADMINISTRATION</b> <b>OFFICE OF REGULATORY AFFAIRS</b> <b>ORA Laboratory Manual Volume IV Section 11</b>	<b>Document Number:</b> IV-11	<b>Revision #: 02</b> <b>Revision Date:</b> 05/27/2020
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## 1. Purpose

This training chapter introduces the trainee to the four areas of nutrient analysis: water-soluble vitamins, fat-soluble vitamins, proximate analysis, and metals. The training presented in this section is basic training for an analyst in the Southeast Food and Feed Laboratory Nutrient Analysis Branch (NAB). The Office of Training, Education and Development (OTED) offers New Hire Laboratory Analyst Training curriculum (Bingo card) which specifies sections of the ORA Lab Manual as part of the New Hire Curriculum. Described are nutrient analyses for new hire training and suggested on-the-job training samples of selected techniques. Additional techniques and training exercises are included that are frequently used in nutrient analysis. This document does not include the entire range of methodology performed in NAB.

The trainer will follow the preset unit for introduction of a particular assay. The trainer will demonstrate the method with the trainee. After the method has been demonstrated, the trainee will run the method with trainer assistance. The final test will be the trainee's ability to run the method independently.

## 2. Scope

The incumbent is a regulatory scientist in an FDA/ORA field laboratory and is expected to bring full professional competence in his/her discipline to bear in carrying out analyses and interpreting the significance of test results. The incumbent is assigned scientific analyses on a wide range of samples including those that are difficult, complex, or unusual.

## 3. Responsibility

### A. Trainer

1. Coordinates training with Supervisor(s) and Trainee.
2. Works with trainees to ensure completion of all training needed to meet their regulatory responsibilities.

### B. Trainee

1. Completes required training within specified timeframes.
2. Reports training received and submits documentation for training to supervisor.

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- C. Supervisor
  - 1. Implements and reviews training records.
- D. Quality Management
  - 1. Maintains employee competencies matrix records for staff.

#### 4. Background

In 1973, the Food and Drug Administration (FDA) issued regulations requiring nutritional labeling on food for the following: food containing one or more added nutrients, food with labeling or advertising claims about the food's nutritional properties, or its usefulness in the daily diet. Nutritional labeling was voluntary for almost all other foods. In 1975, voluntary nutritional labeling went into effect. In 1984, sodium was added to the list of required nutrients, and potassium to the list of optional nutrients, on the nutrition panel. Effective in 1985, the new regulation also defined terms such as "low sodium." In 1990, FDA proposed extensive food labeling changes due to growing concern over diet, health, and labeling uniformity. These changes, now known as the Nutritional Labeling and Education Act (NLEA), included mandatory nutrition labeling for most foods, standardized serving sizes, and uniform use of health claims. Final regulations and health claims became effective in 1993; nutrient content claims became effective in 1994. In 1994, The Dietary Supplement Health and Education Act (DSHEA) was passed requiring mandatory nutritional labeling of dietary supplements. The Food Safety Modernization Act (FSMA) of 2016 updated the requirements for Nutrition and Supplement Facts Labels.

The Southeast Food and Feed Laboratory Nutrient Analysis Branch (NAB), formerly the Atlanta Center for Nutrient Analysis (ACNA) established in 1976, performs nutrient profiles on all human and animal food products and dietary supplements. NAB was established to analyze the products that had voluntary nutritional labeling. The mission of the branch has changed to accommodate the changes in regulations over time. In addition to voluntary (then later mandatory) nutritional labeling for foods and supplements, the Infant Formula Act of 1980 impacted NAB as well. In 1978 and 1979, two formulas were found to be deficient in chloride. The manufacturer wanted to reduce the amount of sodium in the formula and reduced the levels of chloride below the levels found in human milk. After that incident, the Act was passed and placed strict quality standards on these products. NAB analyzes all infant formula to determine adherence to the Act.

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NAB is the national servicing laboratory for nutritional analysis and performs all regulatory work for all FDA districts. The compliance program areas covered include 7321.002 Medical Foods, 7321.006 Infant Formulas, 7321.005 Domestic NLEA, 7321.007 Import NLEA, and 7321.008 Dietary Supplements.

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## 5. References

- A. Federal Food, Drug, and Cosmetic Act, Washington, DC: U.S. Government Printing Office
  - B. Title 21 of the Code of Federal Regulations, Parts 101 to 169
  - C. Official Methods of Analysis of AOAC International (current ed.)
  - D. United States Pharmacopeia and National Formulary (current ed.)
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## 6. Procedure

### 6.1. Sources of Methodology

Samples are analyzed using compendium methods as applicable. Compendium methods take precedence over use of other methods. Official methods designated as such by organizations such as AOAC International (AOAC), United States Pharmacopeia (USP), or National Formulary (NF) are considered compendia methods. If a compendium method is not found, the Center for Food Safety and Applied Nutrition (CFSAN) is consulted. Whether a method is compendia or non- compendia, it is to be validated or verified with recovery and reproducibility studies, use of positive and negative controls, use of Standard Reference Material, or in-house quality assurance/quality control materials as per the Guidelines for the Validation of Chemical Methods. If a product is labeled as USP, the correct USP analytical method is used for analysis.

NAB routinely encounters products that have no official method for the matrix. Use of AOAC procedures with modifications, memos of analysis (in-house methods), and standard operating procedures with proper controls and CFSAN approval have been used over the years.

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## 6.2. Water Soluble Vitamins (Chemical Analysis)

### 6.2.1. Thiamine (B<sub>1</sub>)

#### 6.2.1.1. Objective

The analyst will become familiar with Thiamine (B<sub>1</sub>) through thiamine reading materials and exercise questions. The analyst will be able to perform thiamine procedure as written in the AOAC.

#### 6.2.1.2. References

1. *Methods of vitamin assay* (3rd ed., chap. 6, pp. 123-142). Interscience Publishers.
2. Sebrell and Harris. *The vitamins* (Vol. V, chap. 15 on Thiamine).
3. Combs, G. F., Jr. *The vitamins* (chaps. 1, 3, 10, 19).
4. Eitenmiller, R. R., Ph.D., and Landen, W. O. (1999). *Vitamin analysis for the health and food sciences*. Boca Raton, FL: CRC Press.
5. *AOAC official methods of analysis* (current ed.) Gaithersburg, MD: AOAC International.

#### 6.2.1.3. Pre-Assay Questions

1. What is a vitamin?
2. Name examples of fat-soluble and water-soluble vitamins.
3. What are the recommended daily allowances of thiamine in adults, infants, and lactating and pregnant women?
4. What are other names for thiamine?
5. List some natural food products that are considered rich sources of thiamine.
6. What form of thiamine is present in animal and plant products?
7. What is the function of thiamine in the body?
8. What are the effects of thiamine deficiency?
9. Are there any toxicity effects when high dosages of thiamine are taken? If so what are the effects?
10. Discuss the solubility of thiamine in water, alcohol, and organic solvents.
11. Discuss the stability of thiamine with respect to light, pH, oxidizing and reducing agents.

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12. Draw a structure of the thiamine molecule.

#### 6.2.1.4. Exercise

Analyst will read pertinent information pertaining to thiamine and answer questions on thiamine. The analyst will perform thiamine procedure on a current Standard Reference Material and a practice sample.

#### 6.2.1.5. Post Assay Questions

1. In the procedure for thiamine what are possible sources of error? Could substances within the sample matrix interfere with the thiamine determination?
2. What precautions should one take during the procedure to protect the analyte and analyst?
3. What is the final concentration of the sample extract in the procedure?
4. What are some stopping points where the procedure can be interrupted and stored overnight without affecting the results?

### 6.2.2. Riboflavin (B<sub>2</sub>)

#### 6.2.2.1. Objective

The analyst will become familiar with riboflavin (Vitamin B<sub>2</sub>) through reading materials and exercise questions. The analyst will be able to perform the riboflavin method as written in the AOAC.

#### 6.2.2.2. References

1. *Methods of vitamin assay* (3rd ed., chap 7, pp. 147-166). Interscience Publishers.
2. Sebrell and Harris. *The vitamins* (Vol. V, chap. 14 on Riboflavin).
3. Combs, G. F., Jr. *The vitamins* (chaps. 1, 3, 10, 19).
4. Eitenmiller, R. R., Ph.D., and Landen, W. O. (1999). *Vitamin analysis for the health and food sciences*. Boca Raton, FL: CRC Press.
5. *AOAC official methods of analysis* (current edition) Gaithersburg, MD: AOAC International.

#### 6.2.2.3. Pre-Assay Questions

1. Is Riboflavin a water-soluble or fat-soluble vitamin?
2. What are the recommended daily allowances of riboflavin in adults, infants, and lactating and pregnant women?

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3. What are other names for riboflavin?
4. List some natural food products that are considered rich sources of riboflavin.
5. What form of riboflavin is present in animal and plant products?
6. What is the function of riboflavin in the body?
7. What are the effects of riboflavin deficiency in man?
8. Are there any toxicity effects when high dosages of riboflavin are taken? If so, what are the effects?
9. Discuss the solubility of riboflavin in water, alcohol, and organic solvents.
10. Discuss the stability of riboflavin with respect to light, pH, oxidizing and reducing agents.
11. Draw a structure of the riboflavin molecule.

#### **6.2.2.4. Exercise**

The analyst will read pertinent information pertaining to riboflavin and answer questions. Analyst will perform riboflavin procedure on a current Standard Reference Material and a practice sample.

#### **6.2.2.5. Post Assay Questions**

1. In the procedure for riboflavin what are possible sources of error? Could substances within the sample matrix interfere with the riboflavin determination?
2. What precautions should one take during the procedure to protect the analyte and analyst?
3. What is the final concentration of the sample extract in the procedure?
4. What are some stopping points where the procedure can be interrupted and stored overnight without affecting the results?

### **6.2.3. Vitamin C**

#### **6.2.3.1. Objective**

This training module is designed to train the employee to analyze vitamin C (ascorbic acid). This module covers three methods. These methods include the following:

1. 2,6-dichloroindophenoldichloroindophenol titrimetric method

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2. Total vitamin C microfluorometric method
3. L -Ascorbic acid determination by Liquid chromatography with UV detection

The employee will read background material on vitamin C, answer questions, and receive the methodology training.

#### **6.2.3.2. References**

1. Deutsch, M. J., and Weeks, C. E. (1965). Microfluorometric assay for vitamin C. *Journal Association of Official Analytical Chemists*, 48, 1248.
2. Deutsch, M. J. (1967). Assay for vitamin C: a collaborative study. *Journal of Association of Official Analytical Chemists*, 50, 798.
3. AOAC Official Methods (current ed.) 985.33, 967.22 and 2012.22 Gaithersburg, MD: AOAC International.
4. Eitenmiller, R. R., Ph.D., and Landen, W. O. (1999). *Vitamin analysis for the health and food sciences* (chap. 6, p. 223). Boca Raton: CRC Press.
5. Combs, G.F. (1992). *The vitamins* (chap. 9, p. 225). New York: Academic Press Inc.

#### **6.2.3.3. Pre-Assay Questions**

1. What is the recommended daily intake for vitamin C in adults, children, and infants?
2. What is disease related to vitamin C deficiency?
3. What is the common name for vitamin C?
4. What is the definition of vitamin C?
5. What are the primary dietary sources of vitamin C?
6. What are the 2 main biologically active forms of vitamin C?
7. What is the stability of L-ascorbic acid in solution?

#### **6.2.3.4. Exercise**

There are three commonly used methods for vitamin C in NAB:

1. AOAC, (current ed.) 985.33 by titration for infant formulas and medical foods
2. AOAC, (current ed.) 967.22 by microfluorometric assay for all other matrixes

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3. AOAC, (current ed.) 2012.22 by HPLC in Infant Formula and Adult/Pediatric Nutritional Formula

The methods differ at the quantitation step and the forms of ascorbic acid that is measured. The trainee will read and make an outline of each procedure. The trainer will demonstrate each method. The trainer and trainee will perform each of the methods with various sample matrixes and controls. In house controls such as infant formula, medical food, dietary supplement, and current SRM are usable, as well as, samples received in the laboratory that has been previously analyzed. These samples or controls may be spiked at different levels unknown to the trainee. The trainee will perform the method and meet the criteria of the method. When proficiency is demonstrated, the trainee will run official samples.

**6.2.3.5. Post-Assay Questions**

1. What are the three main analytical procedures for vitamin C in NAB? What form of vitamin C does each method measure?
2. What is the purpose of Norit?
3. What is the purpose of phenylenediamine?
4. What is the purpose of EDTA?
5. What are the stopping points in each method?
6. What are the sources of interference in each method, and how does an analyst avoid them?

**6.2.4. Multianalyte Water Soluble Vitamins**

**6.2.4.1. Objective**

The analyst will become familiar with analysis of multianalyte water soluble vitamins by ID-LC-MS/MS through reading materials and exercise questions. The analyst will be able to perform the analysis procedure as written in the AOAC.

**6.2.4.2. References**

1. In house validated Multianalyte WSV method
2. AOAC Official method (current ed.) 2015.14

**6.2.4.3. Exercise**

The analyst will read pertinent information pertaining to the analysis of multiple water soluble vitamins by LC-MS/MS and answer questions. Analyst will

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perform the extraction and analysis procedure on a current Standard Reference Material and a practice sample

#### **6.2.4.4. Post-Assay Questions**

1. Which ionization method is used for either of these methods?
2. What is the importance of the internal standard?
3. Explain the reason why the mobile phase requires the use of 0.1% formic acid in water?
4. To what temperature is the Extraction solution heated? Why is this important?
5. What is the target concentration for the sample preparation?

### **6.3. Water Soluble Vitamins (Microbiological Analysis)**

#### **6.3.1. Folic Acid**

##### **6.3.1.1. Objective**

This module is designed to train the employee to analyze folic acid. This module covers three methods. These methods include the following:

1. a single enzyme digestion for the analysis of medical foods and infant formula,
2. a trienzyme digestion for all other food products, and
3. an alkaline digestion for dietary supplements.

The analyst will read background material on folic acid, answer questions, and receive the methodology training using a current Standard Reference Material and a practice sample.

##### **6.3.1.2. References**

1. U.S. Food & Drug Administration, Southeast Food and Feed Laboratory. Memo of analysis for total folate analysis (internal document).
2. *AOAC Official Methods* (current ed.), 992.05, 944.12, 960.46, 2004.05, and 2011.06. Gaithersburg, MD: AOAC International.
3. U.S. Food & Drug Administration, Southeast Regional Laboratory. (2000, August 2). Total diet program: folate in foods. Standard Operating Procedure N/AM/4/1/94.

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4. Martin, J., Landen, W. O., Soliman, A., and Eitenmiller R. R., Ph.D. (1990). Application of trienzyme extraction for total folate determination in foods. *Journal of Association of Official Analytical Chemists*, 73, 805.
5. Rader, J., Weaver, C., and Angyal, G. (1998). Use of microbiological assay with trienzyme extraction for measurement of pre-fortification levels of folates in enriched cereal-grain products. *Food Chemistry*, 62, 451.
6. Souza, S. and Eitenmiller, R. R., Ph.D. (1990). Effects of different enzyme treatments on extraction of total folate from various foods prior to microbiological assay and radioassay. *Journal of Micronutrient Analysis*, 7, 37.
7. Aiso, K. and Tamura, T. (1998). Trienzyme treatment for food folate analysis: optimal pH and incubation time for alpha-amylase and protease treatments. *Journal Nutr. Sci. Vitaminol.*, 44, 361.
8. Handout: Structures and Properties of Folic Acid.
9. Handout: Microbiological Determination of Vitamins.
10. Mills, J. (2000). Fortification of foods with folic acid – how much is enough? *New England Journal of Medicine*, 342, 1442.
11. Williams, R. (1994, May). FDA proposes folic acid fortification. *FDA Consumer*.
12. U.S. Food and Drug Administration. (1999, March-April). Folic acid awareness. *FDA Consumer*.
13. Eitenmiller, R. R., Ph.D. and Landen W. O., W. 1999. *Vitamin Analysis for the Health and Food Sciences*. Boca Raton: CRC Press.
14. Combs, G. (1992). *The vitamins*. San Diego, CA: Academic Press Inc.
15. Spallholz, J., Boylan, L., and Driskell, J. *Nutrition chemistry and biology* (2nd ed.). Boca Raton, FL: CRC Press
16. Glenn, C. (1997). Putting folates to work. *Food Formulating*, p. 47.
17. Rader, J., Weaver, C., and Angyal, G. (2000). Total folate in enriched cereal-grain products in the United States following fortification. *Food Chemistry*, 70, 275.
18. U.S. Food & Drug Administration. (2000). Letter regarding *dietary supplement health claim for folic acid with respect to neural tube defects*. Docket No.: 91N-100H.
19. U.S. Food and Drug Administration, Office of Public Affairs. (2000). *History of FDA's total diet study*.

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20. U.S. Food and Drug Administration. (1996). Folic acid to fortify U.S. food products to prevent birth defects. *HHS News*, P96-3.
21. U.S. Food and Drug Administration. (1997). FDA announces name changes for lower-fat milks and folic acid fortification for bakery products. *HHS News*, P97-47.
22. U.S. Food and Drug Administration, Office of Public Affairs. (1996). *Folic acid fortification*.

### 6.3.1.3. Pre-Assay Questions

1. Why did the Food and Drug Administration decide to fortify cereal-grain products with folic acid?
2. What is the recommended daily intake of folic acid for adults, infants, and children?
3. What are the four predominant forms of naturally occurring folates?
4. What is the stability of folic acid?
5. What are the symptoms/consequences of folic acid deficiency?
6. What are the consequences of too much folic acid in the diet?
7. What are good sources of folic acid in foods?

### 6.3.1.4. Exercise

There are four commonly used methods for folic acid in NAB:

1. Memo of Analysis: total folate analysis based on AOAC Official Method (current ed.) 992.05
2. Folic acid in vitamin preparations: AOAC Official Method (current ed.) 944.12
3. Folate in cereals AOAC Official Method (current ed), 2004.05
4. Folate in Infant Formula and Adult/Pediatric Nutritionals by LC-MS/MS AOAC Official Method (current ed), 2011.06

The methods differ in the digestion procedures, the microbiology portion of the assays are similar for methods 1, 2, and 3. The digestion of methods 3 and 4 are similar but, while method 3 makes use of microbiological assay, method 4 uses LC-MS/MS for quantitation. The analyst will read and make an outline of the three different procedures. The trainer will then demonstrate each method. The trainee and trainer will perform each of the methods with various sample matrixes and controls. In-house controls such as an infant formula, dietary supplements, and current SRM are usable, as well as, samples received in the

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laboratory that have been previously analyzed. The trainee will then demonstrate proficiency in the methods by performing them independently with proper samples and controls. The trainer will also demonstrate operation of the pipettors and the Autoturb II reader.

#### **6.3.1.5. Post-Assay Questions**

1. What are the analytical procedures used in NAB for determining folic acid? How do they differ?
2. What are the stopping points in each method?
3. What are the precautions used in each method to preserve the integrity of the samples/vitamin?
4. What are the hazardous materials and dangers in each method?

#### **6.3.2. Niacin, Biotin, Vitamin B<sub>6</sub> (Pyridoxine), Vitamin B<sub>12</sub> (Cyanocobalamin) and Pantothenic Acid**

##### **6.3.2.1. Objective**

The purpose of this training is to provide the analyst training to analyze water-soluble vitamins by AOAC microbiological methods and memo of analysis. Trainee will familiarize with Media (Broth and Agar) preparation, inoculum preparation, and instruments, reagents, and supplies used in the procedures.

##### **6.3.2.2. Assignment**

1. Review references and answer questions.
2. Review laboratory chemical hygiene plan, hazardous waste plan, and SRL Quality Assurance Plan.
3. Perform each assay using a current SRM along with training sample [see Exercises below].
4. Measure at least one recovery by spiking the sample with known amount of standard.
5. Read samples on Autoturb and Spectronic 30.
6. Calculate results using computer program.
7. Familiarize with weekly microorganism transfers to maintain cell line, Agar to Agar, and Agar to broth.

##### **6.3.2.3. References**

1. Combs, G. F. The vitamins: fundamental aspects. *Nutrition & Health*.

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2. Eitenmiller, R. R., Ph.D., and Landen, W. O., Jr. Vitamin analysis for the health & food sciences.
3. Angyal, G. Method for the microbiological analysis of selected nutrients.
4. Horowitz, W. (Ed.). (2000). *AOAC official methods of analysis* (current ed.). Gaithersburg, MD: AOAC International.

#### **6.3.2.4. Questions**

1. Discuss the stability and solubility of each vitamin with respect to light, pH, oxidation, reduction, water, and organic solvents.
2. Name the diseases caused by the deficiency of each vitamin. Discuss symptoms of the diseases caused by each vitamin.
3. List some natural food products that are considered rich sources for each vitamin.
4. What is the recommended concentration of each vitamin in the final sample assay extract?
5. Indicate the stopping points, where an analyst can interrupt the procedure for overnight storage without affecting the results.
6. Identify the test organisms listed below and indicate the vitamins assayed by each. (a) L.I. (b) S. f. (c) L.p. (d) L.c. (e) S.u.
7. How are the test organisms kept viable?
8. What are an inoculated and an uninoculated blank?
9. Define heat treatment and sterilization?
10. Why is lactic acid bacteria widely used in microbiological assays?
11. Why it is possible to use a test organism to assay more than one vitamin?
12. Why transfer bacteria weekly?
13. What is Lactobacillus agar, Lactobacillus broth, agar slants, agar stabs, 0.9% Saline, vitamin B<sub>6</sub> broth, and rinse and define the purpose of each.
14. How long do stab cultures stay active?
15. How often does one transfer stab cultures?
16. How long does one grow most bacteria for an inoculum?
17. What is the incubating temperature range for most assays? Is temperature critical to an assay?

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18. What is the growth time for microbiological assays? Is the growth time critical to an assay?
19. What is the recommended temperature for storing agar, slants stabs?
20. Discuss the purpose of basal media in the assay.
21. What are the criteria of an official assay?
22. What is the advantage of an eight point standard curve versus a five point standard curve?
23. What precautions should be taken when performing microbiological assays to avoid contamination and invalidating the assays?
24. What are the differences between titrimetric and turbidimetric assays?
25. How does one determine if a sterilization cycle is complete and has rendered the items inside as sterile?
26. What are the three major factors that affect the growth of microorganisms?
27. How does one determine when the standard has reached its maximum growth?

#### **6.3.2.5. Exercise**

The trainee will read each method and make an outline of procedure. The trainer will demonstrate and discuss different aspects of each method with trainee. The trainee will perform each method with or without trainer help, but under his/her supervision. The trainee will run a current SRM and a training sample. Upon satisfactory completion of training, trainee will run official sample.

### **6.4. Fat Soluble Vitamins (HPLC-UV, LC-MS/MS)Vitamin D**

#### **6.4.1.1. Objective**

This training module is designed to train the employee to analyze Vitamin D. The employee will read background material on Vitamin D, answer questions, and receive the methodology training using a current Standard Reference Material and a practice sample.

#### **6.4.1.2. References**

1. AOAC (Current Ed.) Official Method 2011.11 and 2016.05.
2. <https://health.gov/our-work/food-nutrition/2015-2020-dietary-guidelines/guidelines/appendix-12/>

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3. <https://ods.od.nih.gov/factsheets/VitaminD-Consumer/>

**6.4.1.3. Pre-Assay Questions**

1. What is PTAD, and what is it used for?
2. How do you calculate the concentration of the SILD<sub>2</sub>SS or SILD<sub>3</sub>SS?
3. What is the amount vitamin D in your samples and standards solutions? (at time in injection)
4. What are some of the side effects of low vitamin D in children and adults?
5. What are some food sources of vitamin D?
6. What is the daily recommended intake of vitamin D in infants, children, and adults?

**6.4.1.4. Exercise**

The trainee will read each method and make an outline of procedure. The trainer will demonstrate and discuss different aspects of the method with trainee. The trainee will perform each method with or without trainer help, but under his/her supervision. The trainee will run current SRM and a training sample. Upon satisfactory completion of training, trainee will run official sample.

**6.4.2. Vitamin K**

**6.4.2.1. Objective**

This training module is designed to train the employee to analyze Vitamin K. This method covers the AOAC 999.15 procedure. The employee will read background material on Vitamin K, answer questions, and receive the methodology training.

**6.4.2.2. References**

1. *AOAC official methods of analysis* (current ed.) 999.15 Gaithersburg, MD: AOAC International.
2. Modifications of AOAC Official Method 999.15 to Improve the Quantitation of Vitamin K1 in Complex Formulated Nutritional Products Pierluigi Delmonte, Steven Barrientos, and Jeanne I. Rader U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Regulatory Science, College Park, MD 2074

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3. Indyk, H. E., and Woollard, D.C. (2000). Determination of vitamin K in milk and infant formula by liquid chromatography: collaborative study. *Journal of Association of Official Analytical Chemists International*, 83(1), 121-130
4. Indyk, H. E., Woodllard, D. C. (1997, May). Vitamin K in milk and infant formulas: determination and distribution of phylloquinone and menaquinone-4. *Analyst*, 122, 465- 469.
5. Eitenmiller, R. R., Ph.D., and Landen, W. O. (1999). *Vitamin analysis for the health and food sciences*. Boca Raton: CRC Press.
6. Combs, G. (1992). *The vitamins*. San Diego, CA: Academic Press Inc.

#### **6.4.2.3. Pre-Assay Questions**

1. What is the recommended daily intake (Vitamin K) for adults, infants, and children?
2. What are the forms of vitamin K and where do they come from (source)?
3. What is the stability of Vitamin K?
4. What are the symptoms/consequences of too much or too little vitamin K in the diet for adults and children or infants?
5. What are good sources of vitamin K in foods?

#### **6.4.2.4. Exercise**

The method to be used is the AOAC 999.15 along with modifications per CFSAN publication noted in reference 2. The trainer will demonstrate the assay procedure, then the trainee and the trainer will perform the assay including the High Performance Liquid Chromatography (HPLC) set-up and run. (In house controls such as the current SRM and a blank are included, as well as samples received in the laboratory that have been previously analyzed.) The trainee will then demonstrate proficiency by performing the assay independently with proper samples and controls.

#### **6.4.2.5. Post-Assay Questions**

1. What are the differences in the new and old procedures?
2. What is the purpose of the buffer, the Lipase, the alcohol solution, the K<sub>2</sub>CO<sub>3</sub>, and the hexane?
3. Explain the post column reductor. What precautions are needed in the use of this piece of equipment?

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4. What is the purity factor and how is it determined?
5. Why would an analyst use a slightly elevated column temperature instead of ambient temperature?
6. Explain the system suitability test.
7. What does using a “forced zero” in the linear regression curve mean?
8. If a sample calculates to have a fluorescence value above the highest standard point value, how can one get it on the curve?
9. What are the stopping points in the assay?
10. What are the precautions used in the method to preserve the integrity of the sample/vitamin?
11. What are the hazardous materials and dangers in the method?

## **6.5. Proximate Analysis**

### **6.5.1. Fats (GC-FID)**

#### **6.5.1.1. Objective**

The trainee will read references, answer questions, and receive methodology training. The trainee will demonstrate proficiency by running standard reference sample and a practice sample. Upon completion of the training, the trainee will demonstrate proficiency by analyzing official samples.

#### **6.5.1.2. References**

1. AOAC Official Method (current ed.) Gaithersburg, MD, AOAC International.
2. [www.medline.gov/ency/patientinstructions.000104.htm](http://www.medline.gov/ency/patientinstructions.000104.htm)
3. [www.Britannica.com/topic/fat](http://www.Britannica.com/topic/fat) and [www.webmd.com/diet/guide/types-fat-in-foods#1](http://www.webmd.com/diet/guide/types-fat-in-foods#1)

#### **6.5.1.3. Pre-assay Questions**

1. What are fats?
2. What are some important functions of fat?
3. What are forms of fat?
4. What are examples of “good” fats?
5. Give two examples of fats that come from vegetable?
6. Are fats hydrophobic or hydrophilic?

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7. What are fat molecules made from?

#### **6.5.1.4. Exercise**

The Analyst will read information pertaining to fat analysis and answer questions. Analyst will perform fat analysis on a Standard Reference Material and a practice sample.

#### **6.5.1.5. Post Assay Questions**

1. Where in the method would be the best stopping points?
2. The fat extraction requires use of a peroxide former.
3. What is this peroxide former?
4. What causes the analytes to separate in a gas chromatography system?
5. Linoleic acid analysis is required on what food products?

### **6.5.2. Protein**

#### **A. Objective**

This training module is designed to train the employee to analyze protein. This module covers two methods. These methods include the following:

1. Kjeldahl method
2. Dumas method

The employee will read background material on protein analysis, answer questions, and receive the methodology training

#### **B. References**

1. AOAC Official Method of Analysis (current ed.) Gaithersburg, MD, AOAC International.
2. Britannica.com
3. Ghr.nlm.nih.gov
4. Hsph.harvard.edu/nutritionsource/what-you-should-eat
5. [www.webmd.com/men/features/benefits](http://www.webmd.com/men/features/benefits-protein) -protein

#### **C. Pre-Assay Questions**

1. What is Protein?
2. What is the consistence of proteins?
3. What do proteins do?
4. What are the three types of proteins?

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5. What foods are a good source of protein?

#### **D. Exercise**

The analyst will read information pertaining to Protein and answer questions. Analyst will perform protein analysis on a Standard Reference Material and a practice sample.

##### **6.5.2.1. Post-assay Questions**

###### *Kjeldahl Protein analysis*

1. Before starting the Kjeldahl digestion what reagents are needed?
2. Before you start analyzing the protein sample and placing it on the Kjeltec 8400 what reagents are needed for analyzation?
3. At the end of the analysis what numbers are we looking for from the Kjeltec analyzer?

###### *Dumas Protein analysis with the Leco Trumac instrument:*

4. Why do you perform maintenance on the primary filter tube, before beginning the analysis?
5. Why do you perform diagnostic tests, such as the leak test and system check before starting the analysis?
6. Why is EDTA run in triplicate before beginning analysis, but after running the blanks?

#### **6.6. Metals Analysis Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES), and Inductively Coupled Plasma – Mass Spectrometer (ICP-MS)**

##### **6.6.1. Iron, Calcium, Phosphorus, Manganese, Magnesium, Zinc, Copper, Sodium, Potassium, Selenium, Chromium, Molybdenum**

###### **6.6.1.1. Objective**

This module provides training in the several metal analysis methods. These methods include the analysis of different metal elements using the ICP-OES technique, and ICP-MS. The employee will read background material on metals, answer questions, and receive the methodology training.

###### **6.6.1.2. References**

1. Instruments Manual for the Perkin-Elmer, and Agilent.
2. National Research Council. (1989). *Recommended dietary allowance* (10th ed.). Washington, DC: National Academy Press.

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3. Stewart and Whitaker. (1984). *Modern methods of food analysis*. CT: AVI Publishing Co., Inc.
4. Willard, Merritt, Dean, and Settle. *Instrumental methods of analysis* (6th ed., pp.154- 176).
5. SOP's on elemental analysis using ICP-OES and ICPMS.
6. *AOAC Official Method (current ed.) 2011.14 Minerals and Trace Elements in Milk and Milk Products, Infant Formula, and Adult Nutritionals*.
7. *AOAC Official Method of Analysis* references listed after elemental analysis of infant formula.
8. Elemental Analysis Manual (EAM) 4.7 Inductively Coupled Plasma-Mass Spectrometric Determination of Arsenic, Cadmium, Chromium, Lead, Mercury, and other Elements in Food using Microwave Assisted Digestion.
9. U.S. Food & Drug Administration, Center for Food Safety and Applied Nutrition.
10. *Compliance program guidance manual*. Compliance Program 7321.006 Infant Formula and 7321.005 Domestic/Import NLEA, Nutrient Sample/Analysis & General Food Labeling
11. Code of Federal Regulation. (2003). Title 21, Pt. 101.9-Nutrition labeling of food (c) and (g). Washington DC: Office of Federal Register National Archives and Records Administration.
12. Code of Federal Regulation. (2003). Title 21, Pts. 136-Bakery Products, 139-Macaroni and Noodle Products. Washington DC: Office of Federal Register National Archives and Records Administration.
13. *United States Pharmacopoeia*.

### **6.6.1.3. Questions**

#### *Background*

1. How is sensitivity, sensitivity check and detection limits defined?
2. How are limits of detection (LOD) and limits of quantitation (LOQ) defined?
3. How critical is temperature in the digestion procedures?
4. What are good stopping places during toxic elements and nutritional analysis procedures?

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5. What is the biochemical function of iron and sodium in the body?

*21 CFR and Compliance Program*

6. What are the RDI values for iron, calcium, sodium, and selenium in adults? What are the Infant Formula Act requirements for these same elements?
7. List three foods that have standard requirements for minerals. Cite the requirements with the respective references.
8. Distinguish/define: sodium-free, low sodium, and reduced sodium.
9. A sample of crackers is labeled as low sodium with the sodium content declared at 35 milligrams per serving. The serving size is 30 grams. Is this product labeled correctly for sodium? The sample is found to contain 70 milligrams sodium per serving. Is the sample a violation for sodium according to the CP 7321.005 for NLEA?

*Elemental Analysis*

10. What precaution is to be taken for nitric acid during digestions?
11. What is the purpose of HCl during digestion process?
12. What is the primary difference between ICP-OES and ICP-MS technology?
13. What are some advantages of using the ICP-OES technology versus the ICP-MS technology for elemental analysis?

**6.6.1.4. Exercise**

This training exercise will consist of performing all determinations in duplicate. The employee is to assay the Infant Formula SRM (current version), any other assigned samples, and 3 method blanks.

1. Assay the samples for Calcium, Phosphorous, Magnesium, Iron, Copper, Manganese, Zinc, Sodium, and Potassium using the ICP-OES technique. (Method: AOAC 2011.14)
2. Assay the samples for Selenium using the ICP-MS technique. (Method: EAM 4.7)
3. Assay a medical food sample for Chromium and Molybdenum using the ICP-MS technique. (Method: EAM 4.7)

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## 7. Glossary/Definitions

None

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## 8. Records

None

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## 9. Supporting Documents

None

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## 10. Document History

Revision #	Status* (D, I, R)	Date	Author Name and Title	Approving Official Name and Title
1.2	R		LMEB	LMEB
1.3	R	02/14/13	LMEB	LMEB
02	R	05/27/2020	LMEB	LMEB

\* - D: Draft, I: Initial, R: Revision

## 11. Change History

Revision #	Change
1.2	Sections 11.4.3, 11.4.4 expanded and answers added. Table of Contents – 11.5 title changed; 11.6 added.
1.3	Header – Division of Field Science changed to Office of Regulatory Science
02	Revised into new format and made corrections throughout: Created sections 1-3 and 7-9. Updated methods, removed outdated methods, and corrected references. Added Section 6.2.4 Multianalyte Water Soluble Vitamins, Section 6.4.1 Vitamin D, Section 6.5.1 Fats (GC-FID), Section 6.5.2 Protein. Removed Section 11.4.4.I Cholesterol (HPLC).

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## 12. Attachments

### List of Attachments

Attachment A - Answer Key

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## Attachment A - Answer Key

### 1.2. Water Soluble Vitamins (Chemical Analysis)

#### 12.1.1. Thiamine (B<sub>1</sub>)

##### C. Pre-Assay Questions

1. **What is a vitamin?** A vitamin is an organic compound that is a natural component of foods and in minute amounts is essential for normal physiological function. Absence or inadequate amounts in the diet can cause specific known deficiency syndromes.
2. **Name examples of fat-soluble and water-soluble vitamins.** Fat-soluble: vitamins A, E, D, and K. Water-soluble: thiamine, riboflavin, niacin, folic acid, B<sub>6</sub>, B<sub>12</sub>, pantothenic acid, and vitamin C.
3. **What are the recommended daily allowances of thiamine in adults, infants, and lactating and pregnant women?** Adults: 1.2 mg, infants: 0.35 mg, pregnant and lactating women: 1.47 mg.
4. **What are other names for thiamine?** Vitamin B<sub>1</sub>, Aneurin.
5. **List some natural food products that are considered rich sources of thiamine.** Brewers and bakers yeast, liver, cereal grains
6. **What form of thiamine is present in animal and plant products?** Plants: free thiamine. Animals: present in the phosphorylated forms with the predominant form being thiamine diphosphate.
7. **What is the function of thiamine in the body?** Thiamine acts as a coenzyme in the body, it serves as an essential co-factor in multienzyme alpha-ketoacid dehydrogenase complexes.
8. **What are the effects of thiamine deficiency?** The effects of deficiency are known as beriberi which can be followed by mental confusion, muscular weakness, enlarged heart, and death.
9. **Are there any toxicity effects when high dosages of thiamine are taken? Is so, what are the effects?** No toxic effects associated with high dosages but can cause gastric upset.
10. **Discuss the solubility of thiamine in water, alcohol, and organic solvents.** Water, 100g/100mL; 95% Ethanol, 1g/100mL; 100% Ethanol, 0.3g/100mL; insoluble in acetone, benzene, hexane, and chloroform.
11. **Discuss the stability of thiamine with respect to light, pH, oxidizing and reducing agents.** In pH solutions of 3.5, the vitamin will withstand sterilization temperatures. In the dry form of the vitamin, it is stable and

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not sensitive to atmospheric oxidation. In solution, thiamine is sensitive to reduction and oxidation. The oxidation of the vitamin will produce its inactive form called thiochrome.

12. **Draw a structure of the thiamine molecule.** See the Merck Index.

**E. Post Assay Questions**

1. **In the procedure for thiamine what are possible sources of error? Could substances within the sample matrix interfere with the thiamine determination?** The sources of error could be calculation errors; errors in making reagents, instrument errors, and components in the sample matrix that could inhibit accurate determination.
2. **What precautions should one take during the procedure to protect the analyte and analyst?** Keep the sample away from direct sunlight, make sure pH is correct, work under a hood, and wear gloves, safety glasses, and lab coat
3. **What is the final concentration of the sample extract in the procedure?** AOAC 17<sup>th</sup> ed. 50.1.08 (986.27) states 15 ug; AOAC; AOAC current ed. 45.1.06 (953.17) states 0.2 ug.
4. **What are some stopping points where the procedure can be interrupted and stored overnight without affecting the results?** AOAC 17<sup>th</sup> ed. 50.1.08 (986.27) states: after the 3 hour enzyme hydrolysis, after filtration, and after collection from column, that the sample extracts can be placed in refrigerator overnight. AOAC 17<sup>th</sup> ed. 45.1.06 (953.17) states that after the filtration step samples can be placed in refrigerator overnight.

**12.1.2. Riboflavin (B<sub>2</sub>)**

**C. Pre-Assay Questions**

1. **Is Riboflavin a water-soluble or fat-soluble vitamin?** Riboflavin is a water-soluble vitamin.
2. **What are the recommended daily allowances of riboflavin in adults, infants, and lactating and pregnant women?** Riboflavin for adults: 1.3 mg; infants 0.4 mg; lactating and pregnant women 12.0mg.
3. **What are other names for riboflavin?** Vitamin B<sub>2</sub>, ovoflavin, lactoflavin, riboflavine.
4. **List some natural food products that are considered rich sources of riboflavin.** Bakers' yeast, broccoli, spinach, breads, and cereals.

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5. **What form of riboflavin is present in animal and plant products?** In animals, riboflavin is present in fungi cells. It is the in the form ovoflavin in eggs, in human milk it is FAD, and in other living cells and tissues it is present as FMN.
6. **What is the function of riboflavin in the body?** Riboflavin participates in oxidation– reduction functions, and it aids in electron transport, catabolism of amino acids and the production of uric acid.
7. **What are the effects of riboflavin deficiency in man?** Riboflavin deficiency results in lacrimation, seborrheic dermatitis, purple swollen tongue, burning and itching of eyes.
8. **Are there any toxicity effects when high dosages of riboflavin are taken? Is so, what are the effects?** No toxicity reported in humans.
9. **Discuss the solubility of riboflavin in water, alcohol, and organic solvents.** Riboflavin is soluble in water 0.12g per 100ml, in ethanol at .0045g per 100ml, and it is insoluble in acetone, benzene, hexane, and chloroform.
10. **Discuss the stability of riboflavin with respect to light, pH, oxidizing and reducing agents.** It is stable in strong mineral acids, is oxidized by chromic acid, and is destroyed by  $\text{KMnO}_4$ . It is unstable in alkaline solution. It is sensitive to both visible and ultraviolet light. It becomes fluorescent in neutral solution of pH 6.7 to 6.8.
11. **Draw a structure of the riboflavin molecule.** Refer to the Merck Index.

#### **E. Post Assay Questions**

1. **In the procedure for riboflavin what are possible sources of error? Could substances within the sample matrix interfere with the riboflavin determination?** Sources of error could be calculations of sample weights, error in making reagent, dilution errors. There could also be other substances present in the matrix of the sample that could hinder accurate results.
2. **What precautions should one take during the procedure to protect the analyte and analyst?** To protect the analyte, protect the analyte from unnecessary light, make sure solutions are made properly and that pH is at proper level. Use proper equipment such as flask, filter paper. To protect the analyst, work under a hood, wear protective equipment such as gloves, goggles, and lab coat.
3. **What is the final concentration of the sample extract in the procedure?** 0.1ug/ml.

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4. **What are some stopping points where the procedure can be interrupted and stored overnight without affecting the results?**  
After 30-minute digestion, after the first pH step and first dilution; after filtration of extract; and after the final dilution step the solutions can be place in a refrigerator overnight.

### 12.1.3. Vitamin C

#### C. Pre-Assay Questions

1. **What is the recommended daily intake for vitamin C in adults, children, and infants?** Adults 90mg; children 15 mg; infants 50mg.
2. **What is disease related to vitamin C deficiency?** Scurvy.
3. **What is the common name for vitamin C?** L-ascorbic acid
4. **What is the definition of vitamin C?** Refers to compounds exhibiting full or partial biological activity of L-ascorbic acid
5. **What are the primary dietary sources of vitamin C?** Citrus fruits, potatoes, tomatoes, fortified foods
6. **What are the two main biologically active forms of vitamin C?** D-hydroascorbic acid, L-ascorbic acid.
7. **What is the stability of L-ascorbic acid in solution?** Very unstable.

#### 12.1.3.1. Post-Assay Questions

1. **What are the three main analytical procedures for vitamin C in NAB? What form of vitamin C does each method measure?** (1.) 2, 6-Dichloroindophenol titrimetric method AOAC (985.33). This method measures L-ascorbic acid. (2.) Vitamin C (total) microfluorometric method AOAC (967.22). This method measures L-ascorbic acid and dehydroascorbic acid. (3.) L -Ascorbic acid determination by Liquid chromatography with UV detection. This method measures L-ascorbic acid.
2. **What is the purpose of Norit?** Norit oxidizes L-ascorbic acid to dehydroascorbic acid.
3. **What is the purpose of o-phenylenediamine?** O-phenylenediamine is used to produce a highly fluorescent and easily measurable compound.
4. **What is the purpose of EDTA?** EDTA works as a chelating agent to remove interfering molecules from the derivatization reaction.

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5. **What are the stopping points in each method?** The major stopping point of method 985.33 and 967.22 is after the addition of meta-phosphoric acid. The solution is stable up to 10 days.
6. **What are the sources of interference in each method, and how does an analyst avoid them?** Microfluorometric method: high starch products (the way to avoid the starch is to extract it with cold ethanol). Titration: minerals interfere (the way to avoid this is the addition of EDTA).

#### 12.1.4. Multianalyte Water Soluble Vitamin Analysis by LC-MS/MS

##### E. Post-Assay Questions

1. **Which ionization method is used for either of these methods?** The electrospray ionization method (ESI) is used for ionizing compounds with relatively-high polarity, by spraying the mobile phase into a strong electric field to form a fine aerosol of charged droplets.
2. **What is the importance of the internal standard?** An internal standard is a chemical substance that is added in a constant amount to samples, the blank and calibration standards in a chemical analysis. Used to correct for the loss of analyte during sample preparation, injection and ionization. Isotopically labeled analyte specific internal standard helps account for analyte suppression which is a major factor with small molecule quantitation using LC-QQQ.
3. **Explain the reason why the mobile phase requires the use of 0.1% formic acid in water?** Solvents are typically chosen based on a compound of interest's solubility and compatibility with various ionization techniques used in LC/MS. Volatility and the solvent's ability to donate a proton are important in ESI and other atmospheric ionization techniques.
4. **To what temperature is the Extraction solution heated? Why is this important?** About 70 mL of Extraction solution is pre-heated (ca. 60 deg. C). A heated extraction is important to help with extraction of the any encapsulated vitamins.
5. **What is the target concentration for the sample preparation? A** general target of 30-50 ppb at time of analysis.

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## 12.2. Water Soluble Vitamins (Microbiological Assays)

### 12.2.1. Folic Acid

#### C. Pre-Assay Questions

1. **Why did the Food and Drug Administration decide to fortify cereal-grain products with folic acid?** To ensure that women of childbearing age would get the desired 400 ug/day of folic acid in their diet.
2. **What is the recommended daily intake of folic acid for adults, infants, and children?** 0.081 mg infants DFE, 0.15 mg DFE children, 0.4 mg DFE adults
3. **What are the four predominant forms of naturally occurring folates?** 5-methyl- H<sub>4</sub>PteGlu<sub>5</sub>, tetrahydrofolate pentaglutamate, pteroylglutamic acid, 5, 10-methenyl-H<sub>4</sub>PteGlu.
4. **What is the stability of folic acid?** Slightly soluble in acid form, very soluble in salt form in solutions of dilute alkali, hydroxides, sulfuric and hydrochloric acid. Folic acid is stable in alkali solutions.
5. **What are the symptoms/consequences of folic acid deficiency?** Neural tube defects.
6. **What are the consequences of too much folic acid in the diet?** Masking of pernicious anemia in the elderly.
7. **What are good sources of folic acid in foods?** Liver, fortified cereals.

#### E. Post-Assay Questions

1. **What are the analytical procedures used in NAB for determining folic acid? How do they differ?** AOAC methods for infant formula and dietary supplements as well as the tri- enzyme method for foods. The three methods differ in sample preparation and digestion time. These differences help to liberate the different forms of folic acid.
2. **What are the stopping points in each method?** The stopping points are after the various dilutions.
3. **What are the precautions used in each method to preserve the integrity of the samples/vitamin?** Minimizing exposure to light, air, and oxygen.
4. **What are the hazardous materials and dangers in each method?** The use of autoclaves, acids, and alkaline solutions.

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## **12.2.2. Niacin, Biotin, Vitamin B<sub>6</sub> (Pyridoxine), Vitamin B<sub>12</sub> (Cyanocobalamin) and Pantothenic Acid**

### **12.2.2.1. Questions**

**1. Discuss the stability and solubility of each vitamin with respect to light, pH, oxidation, reduction, water, and organic solvents.**

Niacin: Soluble in water, insoluble in ethyl ether. Stable in its dry form and aqueous solutions, unaffected by light or pH.

Vitamin B<sub>6</sub>: Soluble in water, acetone, and alcohol, slightly soluble in ether and chloroform. Stable in heat, concentrated alkali and hydrochloric acid. Destroyed by light. Vitamin B<sub>12</sub>: Soluble in water, alcohol, and phenol, insoluble in most organic solvents. Stable at low pH.

Biotin: Soluble in water, ethyl alcohol, insoluble in petroleum ether, chloroform and ether. Stable in strong acids, but unstable in alkali.

Pantothenic acid: soluble in water, ethyl acetate, glacial acetic acid, slightly soluble in ether, amyl alcohol, and insoluble in benzene and chloroform. Stable in calcium and sodium salt forms.

**2. Name the diseases caused by the deficiency of each vitamin. Discuss symptoms of the diseases caused by each vitamin.**

Niacin: Pellegra symptoms--insomnia, anorexia, anemia.

Vitamin B<sub>6</sub>: Symptoms--dermatitis, microcytic anemia, irritability, depression, convulsions

Vitamin B<sub>12</sub>: Symptoms--weakness, nervous system degeneration, confusion, depression.

Biotin: Symptoms--anorexia, nausea, depression.

Pantothenic acid: "Tingling Foot Syndrome," other symptoms include gastric distress, nausea, insomnia, emotional instability, muscular weakness.

**3. List some natural food products that are considered rich sources for each vitamin.**

Niacin: organ meats, poultry, fish, nuts, cereals, grains.

Vitamin B<sub>6</sub>: bananas, seeds, rice, beef, pork, poultry.

Vitamin B<sub>12</sub>: beef, pork, lamb, seafood, eggs.

Biotin: organ meats, egg yolks, soybeans.

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Pantothenic acid: all meats, egg yolks, whole grains, molasses.

4. **What is the recommended concentration of each vitamin in final sample assay extract?** Niacin: 0.01-0.02 ug/mL. Vitamin B<sub>6</sub>: 1.0 ng/mL. Vitamin B<sub>12</sub>: 0.014 ng/mL. Biotin: 0.1-0.04 ug/mL. Pantothenic acid: 0.01ug/mL.
5. **Indicate the stopping points, where an analyst can interrupt the procedure for overnight storage without affecting the results.** With all assays, one can stop after the digestion step and subsequent dilutions.
6. **Identify the test organisms listed below and indicate the vitamins assayed by each.**
  - L.l.: *Lactobacillus (leichmannii) delbrueckii*: vitamin B<sub>12</sub>.
  - S. f.: *Streptococcus faecalis*: folic acid, riboflavin.
  - L.p.: *Lactobacillus plantarum* : niacin, biotin, pantothenic acid.
  - L.c.: *Lactobacillus casei*: folic acid.
  - S.u.: *Saccharomyces pastorianus (uvarum)*: vitamin B<sub>6</sub>.
7. **How are the test organisms kept viable?** Growth on slants and stabs, -70 °C storage.
8. **What is an inoculated and an uninoculated blank?** Inoculated blank: broth or media, no vitamin and inoculated with test organism. Uninoculated blank: broth or media, no vitamin, no test organism.
9. **Define heat treatment and sterilization?** Sterilization is the treatment of samples at high temperature and pressure to kill most microorganisms. Heat treatment is not sterilization, will only kill a few organisms.
10. **Why is lactic acid bacteria widely used in microbiological assays?** The acids produced by the organisms are a good measure of growth.
11. **Why it is possible to use a test organism to assay more than one vitamin?** Some organisms have more than one selectable marker.
12. **Why transfer bacteria weekly?** To keep the culture viable.
13. **What is Lactobacillus agar, Lactobacillus broth, agar slants, agar stabs, 0.9% Saline, vitamin B<sub>6</sub> broth and rinse, and define the purpose of each.**

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Lactobacillus agar: sterile culture media (solid) containing specific nutrients at concentrations ideal for *Lactobacillus* type organisms to grow.

Lactobacillus broth: sterile liquid culture media with same criteria as above.

Agar slants: same as Lactobacillus agar except when prepared while media is still hot and in a liquid state, the culture tubes containing the media are placed at an angle to create a larger surface area for organisms to grow.

Agar stabs: same as Lactobacillus agar prepared in culture tubes, the tubes are allowed to remain upright when they solidify at room temperature post sterilization.

0.9% saline: this solution is equivalent to natural saline, used to wash any nutrients from growth media out of cells.

Vitamin B<sub>6</sub> Broth: this broth is the sterile culture media containing specific nutrients to support *Saccharomyces pastorianus*.

Rinse: used in B<sub>6</sub> assay to wash cells.

14. **How long do stab cultures stay active?** 1-2 months.
15. **How often does one transfer stab cultures?** 1 month (approx. 30 days).
16. **How long does one grow most bacteria for an inoculum?** 12-24 hours.
17. **What is the incubating temperature range for most assays? Is temperature critical to an assay?** 37 ± 0.5° C for bacteria and 30 ± 0.5 °C for yeasts.
18. **What is the growth time for microbiological assays? Is the growth time critical to an assay?** 12-24 hours. Yes, growth time is very critical.
19. **What is the recommended temperature for storing agar, slants stabs** 4°C
20. **Discuss the purpose of basal media in the assay.** Provides the necessary, non-selective nutrients for growth.
21. **What are the criteria of an official assay?** Eight out of 12 tubes.
22. **What is the advantage of an eight point standard curve versus a five point standard curve?** More points allow for less estimation between definitive points on the standard curve.

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23. **What precautions should be taken when performing microbiological assays to avoid contamination and invalidating the assays?** Use aseptic techniques when making transfers or inoculating. Clean dilutor between samples. Make sure the water bath is clean. Autoclave tubes at the correct temperature and pressure.
24. **What are the differences between titrimetric and turbidimetric assays?** A titrimetric assay is an experiment using acid producing bacteria to determine the response of bacteria in the designated growth media to a selective growth required substance such as a vitamin. Turbidimetric assays serve the same purpose except the measurement is of cell density.
25. **How does one determine if a sterilization cycle has been is complete and has rendered the items inside as sterile?** Add a sterilization indicator in with the samples or media during an autoclave cycle. The uninoculated blank is also an indicator.
26. **What are the three major factors that affect the growth of microorganisms?** Oxygen source, temperature, nutrient source.
27. **How does one determine when the standard has reached its maximum growth?** After overnight growth (usually 16 hrs.) take a percent (%) transmittance reading at 550nm using the standard with the highest concentration (the uninoculated blank is used to zero the instrument). Once every hour afterwards take the same reading, a plateau of growth is reached when the successive percent (%) transmittance readings change by less than 2%.

### 12.3. Fat Soluble Vitamins (HPLC-UV, LC-MS/MS)

#### 12.3.1. Vitamin D

##### C. Pre-Assay Questions

1. **What is PTAD, and what is it used for?** 4-Phenyl-1,2,4-triazole-3,5-dione which is used as a derivatizing agent to modify the Vitamin D molecule to make it able to be detected using ESI mass spectrometry.
2. **How do you calculate the concentration of the SILD2SS or SILD3SS?**
3. **What is the amount vitamin D in your samples and standards solutions? (at time in injection)**
4. **What are some of the side effects of low vitamin D in children and adults?** Bone loss, muscle pain, fatigue, depression, cognitive impairment.

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5. **What are some food sources of vitamin D?** Some fish, fish liver oil, egg yolks, fortified dairy and grain products.
6. **What is the Daily Recommended Intake of vitamin D in infants, children, and adults?** 400-800 IU/day or 10-20 ug/day

### 12.3.2. Vitamin K

#### C. Pre-Assay Questions

1. **What is the recommended daily intake (Vitamin K) for adults, infants, and children?** Infants 25ug/day, adults 12080 ug/day.
2. **What are the forms of vitamin K and where do they come from (source)?** Phylloquinone K1 is the most common natural form produced by plants. Menadione K3 is a synthetic form.
3. **What is the stability of Vitamin K?** Stable with most food processing procedures, slightly unstable if heated at frying temperatures, exposure to light and alkaline conditions can destroy it.
4. **What are the symptoms/consequences of too much or too little vitamin K in the diet for adults and children or infants?** Liver disease.
5. **What are good sources of vitamin K in foods?** Green leafy vegetables, legumes, and vegetable oil.

#### E. Post-Assay Questions

1. **What are the differences in the new and old procedures?** Old procedures used UV, newer procedures utilize fluorescence. This presents a problem because most matrices have UV interfering compounds. The new procedures use post-column reduction columns packed with zinc metal reducing quinones to hydroquinones, which are detected through fluorescence.
2. **What is the purpose of the buffer, the Lipase, the alcohol solution, the K<sub>2</sub>CO<sub>3</sub>, and the hexane?**

Buffer: make conditions favorable for lipase activity

Lipase: hydrolyzes fat and fatty acids

Reagent alcohol: stops enzyme activity

Potassium carbonate: denatures protein, destroys enzyme

Hexane: used to extract the vitamin

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3. **Explain the post column reductor. What precautions are necessary in the use of this piece of equipment?** A post-column reductor is a solid phase reductive column with metallic zinc particles. It reduces non-specific fluorescence compounds.
4. **What is the purity factor and how is it determined?** It is the measured absorbance of working standard at 248nm, determined by calculating purity divided by the theoretical absorbance at 248nm, of standard at the same concentration for purity.
5. **Why would one use a slightly elevated column temperature instead of ambient temperature?** To keep temperature stable.
6. **Explain the system suitability Test.** Relative Standard Deviation <2.0. See United States Pharmacopoeia for further definition.
7. **What does using a “forced zero” in the linear regression curve mean?** Linear regression line should touch zero in order to calculate linear regression.
8. **If a sample calculates to have a fluorescence value above the highest standard point value, how can one get it on the curve?** 1:1 dilution.
9. **What are the stopping points in the assay?** After the hexane extraction, take an aliquot, evaporate to dryness, and blanket with nitrogen. It can be kept for three days in refrigerator.
10. **What are the precautions used in the method to preserve the integrity of the sample/vitamin?** UV/HPLC grade solvents should be used, pH meter calibration should be accurate, zinc column should be packed properly, and water should not touch zinc column.
11. **What are the hazardous materials and dangers in the method?** Hexane, dichloromethane, and methanol are carcinogenic solvents. Gloves should be worn and work done in a hood.

## 12.4. Proximate Analysis (Various Methods)

### 12.4.1. Fats

#### C. Pre-Assay Questions

1. **What are fats?** Fats are lipid macronutrients used as one of the energy sources of the body.
2. **What are some important functions of fat?** give you energy, keep your body warm, protect organs, help your body absorb vitamins, and produce hormones that the body needs.

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3. **What are forms of fat?** Saturated, monounsaturated, trans fat, and polyunsaturated fat.
4. **What are examples of good fats?** Monounsaturated and polyunsaturated fats are good fats because they are food for your heart, and your cholesterol.
5. **Give two examples of fats that come from vegetable?** Coconut oil, olive oil, corn oil and palm oil are some examples.
6. **Are fats hydrophobic or hydrophilic?** Fat are lipids and are not soluble in water therefore, they are hydrophobic.
7. **What are fat molecules made from?** Fat molecules are glycerides, which are esters formed by the reaction of there molecules of fatty acids with one molecule of glycerol.

**E. Post-Assay Questions**

1. **Where in the method would be the best stopping points?** After the removal of samples from the water bath (80 degrees for 40 minutes) and the addition of 10mL of ethanol, and after the fat extractions using PET and Ethyl Ethers (before the dry down). Samples after the water bath can be kept in the refrigerator and the ethers extraction can be kept in the freezer (to limit vaporization).
2. **The fat extraction requires use of a peroxide former. What is this peroxide former? What is an added ingredient to this peroxide former that slows the formation of peroxides? What are the precautions that must be taken? What are some potential dangers to working with a peroxide former?** The peroxide former is called Ethyl Ether. Ethyl Ether tends to absorb and react with oxygen to form dangerous peroxides that can become violently explosive if subjected to shock, heat, or some other form of physical friction. BHT is added to the Ethyl Ether to help delay the formation of these dangerous peroxides. Work utilizing Ethyl Ether must be performed in a hood, bottles must be dated with arrival and open dates (as part of the lab controlled peroxide inventory in LIMS), bottles "in use" must be used before opening another bottle, and all bottles aired out in hood when fully emptied.
3. **What causes the analytes to separate in a gas chromatography system?** Interaction of the analytes that are in the gas mobile phase with the column's stationary phase retards the passage of the analytes through the column. Analytes, depending on their affinity for the stationary phase, are separated as they move through the column and

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exit to the detector at different times. The Retention Time is a measure of the time it takes the analyte to move through the column and into the detector.

4. **Linoleic acid analysis is required on what food products?** Infant formula and medical food if listed require linoleic acid analysis.

#### 12.4.2. Protein

##### C. Pre-Assay Questions

1. **What is Protein?** An essential macro nutrient that is the basic component of living cells.
2. **What is the consistence of proteins?** Proteins are made from carbon, hydrogen, oxygen, nitrogen, and sometime sulfur which form amino acids.
3. **What do proteins do?** Some of the uses of Protein in the body are a fuel source, and to build and repair tissues.
4. **What are the three types of proteins?** Three types of proteins are fibrous, globular, and membrane.
5. **What foods are a good source of protein?** Animal products, nuts, and legumes

##### E. Post-Assay Questions

###### *Kjeldahl Protein analysis*

1. **Before starting the Kjeldahl digestion what reagents are needed?** Sulfuric acid and Kjeltabs
2. **Before you start analyzing the protein sample and placing it on the Kjeltec 8400 what reagents are needed for analyzation?** Sodium hydroxide (45% NaOH solution), HCl (hydrochloric acid) and Kjeltec solution.
3. **At the end of the analysis what numbers are we looking for from the Kjeltec analyzer?** The percent of Nitrogen the is in the sample.

###### *Dumas Protein analysis with the Leco Trumac instrument*

4. **Why do you perform maintenance on the primary filter tube, before beginning the analysis?** The Leco Primary Filter tube contains steel wool as the packing material. During the analysis, the steel wool gets oxidized and turns the color of rust, brown/orange looking color. The reason this needs to be serviced is because during analysis, your data is affected by this oxidation and your numbers will begin to fluctuate.

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You need to service this before the analysis with the addition of new steel wool and glass wool. This will prevent any fluctuation in the numbers.

5. **Why do you perform diagnostic tests, such as the leak test and system check before starting the analysis?** The Leco Primary Filter tube contains steel wool as the packing material. During the analysis, the steel wool gets oxidized and turns the color of rust, brown/orange looking color. The reason this needs to be serviced is because during analysis, your data is affected by this oxidation and your numbers will begin to fluctuate. You need to service this before the analysis with the addition of new steel wool and glass wool. This will prevent any fluctuation in the numbers.
6. **Why is EDTA run in triplicate before beginning analysis, but after running the blanks?** EDTA is a calibration standard. Initially, a standard curve is generated with different concentrations of EDTA. The standard is run in triplicate for each analysis because you have to correct for drift from the calibration curve. If values are off by 10%, you have to perform the drift analysis again.

## 12.5. Metals Analysis Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES), and Inductively Coupled Plasma – Mass Spectrometer (ICP-MS)

### 12.5.1. Questions:

#### *Background*

1. **How is sensitivity, sensitivity check and detection limits defined?**  
Sensitivity: Concentration of an element that is needed to produce a signal of 1% absorption (0.0044 absorbance units).

Sensitivity check: concentration of element that will produce signal approximately 0.2 absorbance units under optimum conditions of the wavelength listed.

2. **How are limits of detection (LOD) and limits of quantitation (LOQ) defined?**

$$\text{LOD} = \text{S/N} = 3$$

$$\text{LOQ} = \text{S/N} = 10$$

S= signal output that is measured as difference between sample and blank (avg.)

N= noise standard deviation of the fluctuations of the instrument output with a blank

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3. **How critical is temperature during the digestion procedure?** The temperature is critical to destroy the organic matter such a carbohydrate.
4. **What are good stopping places during toxic elements and nutritional analysis procedure?** In both cases, can stop after acid addition or leave the digestion vessels in the oven overnight, and after sample dilutions are made.
5. **What is the biochemical function of iron and sodium in the body?** *Iron* is used to make hemoglobin, acts as an oxygen carrier in the blood and muscle tissues and in enzyme catalysis. *Sodium* is absorbed by using ATP from the intestinal lumen. It counteracts calcium in muscular contraction and plays a role in osmotic balance.

*21 CFR and Compliance Program*

6. **What are the recommended daily values for iron, calcium, sodium, and selenium in adults? What are the Infant Formula Act requirements for these same elements?**
7. **List three foods that have standard requirements for minerals. Cite the requirements with the respective references.** Infant formula: ratio of calcium to phosphorous can be >1.1 and <2.0 21CFR 107.100(e). Iron-enriched macaroni products: 21CFR 139.117(b) (1) (2) (3). Iron-enriched rolls: 21CFR 136.115(a) (1) (2).
8. **Distinguish/define sodium-free, low sodium, and reduced sodium.** *Sodium free*: when a food contains less than 5mg of sodium per reference value serving. *Low sodium*: a food that has less than 140mg sodium/reference amount. *Reduced sodium*: food that contains less than 25% sodium per reference amount.
9. **A sample of crackers is labeled as low sodium with the sodium content declared at 35 milligrams per serving. The serving size is 30 grams. Is this product labeled correctly for sodium? The sample is found to contain 70 milligrams sodium per serving. Is the sample a violation for sodium according to the CP 7321.005 for NLEA? The sample is labeled correctly.**

*Elemental Analysis*

10. **What precaution is to be taken for nitric acid digestions?** The digestion is not to proceed too rapidly, or sample may char. Nitric acid vapor may cause harm if digestion vessels aren't opened carefully.
11. **What is the purpose of HCl during digestion process?** To prevent interferences.

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12. **What is the primary difference between ICP-OES and ICP-MS technology?** ICP-MS has lower detection limits and is more accurate for low concentration determinations.
13. **What are some advantages of using ICP-OES technology versus ICP-MS technology for elemental analysis?** ICP-OES technology is more robust since it's tolerates higher acid concentrations than ICPMS technology