Fortification Basics

Principles of Assay Procedures

Micronutrients added to foods are analyzed using various procedures depending on their nature and properties. Some micronutrients can be detected using relatively simple colorimetric methods. Where resources are available, more sophisticated methods such as high pressure liquid chromatography (HPLC) (Fig. 1), which separates the compound of interest in a pre-treated food sample, followed by spectrophotometric or fluorometric detection can also be used.

Before starting a program for micronutrient analyses, some essential elements need to be put in place:

- A quality assurance system must be set up to ensure that the manufactured food is safe, unadulterated, properly labeled, and meets all the company's specifications and government regulations (Table 1).
- Food samples must be representative and selected at random, with an adequate and reproducible sampling procedure.
- Personnel must be trained in the assay method(s), that should have been previously identified or set-up.
- Equipment required must be available on-site in working condition.

Figure 1
HPLC Equipment

Table 1
Developing a Quality Assurance System

An effective quality assurance system includes:
• *Ingredient inspection and control* - testing all ingredients against reference standards.
• *Manufacturing control* - identifying quality criteria and chemical, microbiological, and physical hazards; establishing and monitoring the critical control points involved in manufacturing fortified food.
• *Distribution control* - ensuring that the fortified food is unadulterated, properly labeled, and packaged to minimize micronutrient losses.

Vitamin A assays

Vitamin A is one of the most unstable micronutrients. Industrially produced vitamin A, like retinyl palmitate, is more stable than naturally occurring vitamin A, although it remains sensitive to air, light, moisture, heat, and acid conditions.

Vitamin A levels have been determined using colorimetric and spectrophotometric methods for a long time. Currently, HPLC is the method of choice (Table 2). The use of HPLC is preferred when samples have a significant amount of interfering substances such as other vitamins, minerals, proteins, and carbohydrates.

Semi-quantitative method
Colorimetric method

The colorimetric method involves adding a chromogenic reagent to a volume of solubilized fortified food sample. The reagent reacts with retinol to produce a blue color, whose intensity is proportional to the amount of retinol in the sample. The intensity of the blue color is measured against a set of known standards (Fig. 2). The formed blue color is very unstable and necessitates a fast and skillful worker. Because this assay method is inexpensive, and does not need sophisticated equipment, it is used in many countries.

Quantitative method
Spectrophotometric method

The sample is irradiated with UV light and its absorbance is measured. The absorbance is proportional to the vitamin A content in the sample. The spectrophotometric method can be used to monitor vitamin A levels in fortified products at the production level.

Table 2
Vitamin A Assays and their Advantages and Limitations

Assay	Advantages	Limitations
Colorimetric	Simple Rapid Inexpensive	Semi - quantitative Sample pretreatment Not applicable for field
Spectro-photometric	Sensitive Rapid Inexpensive	Needs UV apparatus Sample pretreatment Not applicable for field
HPLC	Reliable High resolution No interferences Accurate	Expensive Training of personnel Sample pretreatment Not applicable for field

Figure 2
Semi - Quantitative Colorimetric Determination of Vitamin A in Sugar

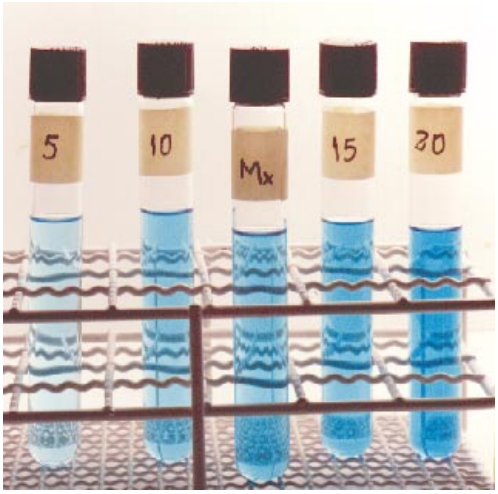


Figure 3
Vitamin A Determination by HPLC

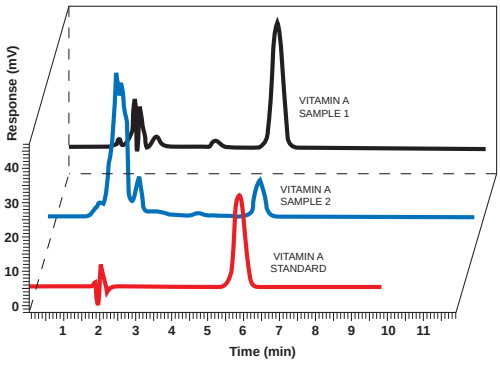
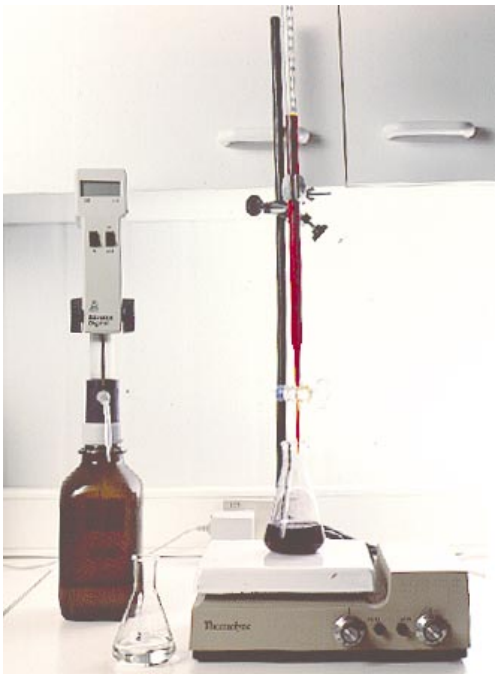


Figure 4
Titrimetric Determination of Vitamin C



HPLC method

In this method, retinol is separated from other substances, which absorb radiant energy at equal or similar wavelengths to retinol, using hexane. Retinol is then detected using spectrophotometric or fluorometric techniques. A typical HPLC chromatograph of retinol analysis is presented in Figure 3. The HPLC method is very reliable mainly because of its ability to effect rapid separation and the high resolution achieved. High costs of equipment, and time required, do not permit several measurements per shift. Highly trained personnel are also required.

Vitamin B-complex assays

Thiamin (vitamin B_1) is analyzed quantitatively by fluorometric methods. The method of choice is the thiochrome procedure, which involves treatment of thiamin with an oxidizing agent (ferricyanide or hydrogen peroxide) to form a fluorescent compound (thiochrome). The intensity of fluorescence is proportional to the thiamin concentration.

Riboflavin (vitamin B_2) is usually assayed fluorometrically by measuring its characteristic yellowish green fluorescence. It can also be assessed microbiologically, using *Lactobacillus casei*, where the growth of this riboflavin-dependent microorganism correlates with the amount of vitamin in the sample. The growth response of the organism is measured either by titration or by measuring turbidity.

Niacin is assayed semi-quantitatively with sulfanilic acid to yield a yellow color. The intensity of the yellow color correlates with the amount of niacin present, which is measured against a set of standards. Niacin can be quantitatively determined using microbiological assays (*Lactobacillus plantarum*) or colorimetric methods (cyanogen bromide as the color reagent). Microbiological assays are preferred over colorimetric methods for foods containing high levels of Maillard browning products (for example, cocoa products), in order to minimize color interference.

Microbiological assays for quantifying pyridoxine (vitamin B_6) and its isomers, pyridoxal and pyridoxamine, rely on the growth response of *Saccharomyces uvarum*.

Microbiological assays are also used for quantitative determination of folic acid, pantothenic acid, and vitamin B_{12} in foods. The test organisms used in folate assays are *Streptococcus faecalis* or *Lactobacillus casei*. *Saccharomyces carlbergensis* and *Lactobacillus plantarum* are common test organisms used in determining pantothenic acid because they do not grow in the absence of pantothenic acid. Similarly, vitamin B_{12} can be determined using microbiological assays with the test organism, *Lactobacillus leichmannii*.

High pressure liquid chromatography (HPLC) methods to determine most B-complex vitamins have been considered and evaluated, but have not yet been validated as official methods by the Association of Official Analytical Chemists (AOAC). There is ongoing interest in developing and validating these methods. Techniques also exist for simultaneous determination of all water-soluble vitamins by HPLC using UV/visible spectrophotometric detection.

Vitamin C assays

Vitamin C can be quantitatively analyzed by either titrimetric or fluorometric methods. The titrimetric method (Fig. 4) involves the measurement of decolorization of 2,6-dichloroindophenol dye by ascorbic acid. This method is not suitable for highly colored products (for example, colored fruit juices) because of the difficulty of determining the endpoint during titration. The fluorometric method involves oxidation of ascorbic acid to dehydroascorbic acid, which reacts with phenylene diamine

to produce a fluorescent compound whose intensity is proportional to the vitamin C concentration.

Vitamin D assays

Vitamin D is quantitatively determined using liquid chromatography. After saponification and extraction of the sample, purification is achieved by sequentially using alumina and silica columns.

Vitamin E assays

Vitamin E levels can be determined spectrophotometrically, although the HPLC method with fluorescence detection is preferred, as it permits the measurement of different forms of vitamin E; thus, total vitamin E activity. However, it is a sophisticated technique and requires trained personnel to execute the analysis.

Iron assays

Qualitative method

This method is applicable for qualitative determination (presence or absence) of iron in enriched or iron-fortified flour. Ferric iron added to flour reacts with a thiocyanate (KSCN) reagent to form a red colored complex. A deeper red color will be formed with enriched and fortified flour compared with the untreated flour.

Quantitative methods

Quantitative iron assays involve extraction and detection.

Iron extraction: Iron extraction can be done by dry or wet ashing.

Dry ashing

The sample is dried overnight in a muffle furnace at 500 to 600° C, followed by acid hydrolysis in the presence of hydrochloric acid.

Wet ashing

The sample is hydrolyzed with concentrated sulfuric acid at high temperature and/or pressure. Iron extraction is more complete using wet ashing, but there are risks in handling hot, concentrated acids.

Iron detection: Once the iron has been extracted, it is detected using colorimetric or atomic absorption spectrophotometric (AAS) methods.

Colorimetric methods

Reagents that produce changes in color depending on the level of iron in the food are utilized. For this method, iron is reduced to the ferrous form with a suitable agent (hydroxylamine hydrochloride or ascorbic acid), after which the reduced iron is reacted with an appropriate color agent (α-dipyridyl or orthophenanthroline). Orthophenanthroline does not react with most organic constituents (carbohydrates, lipids, and proteins); hence is regarded as the best color reagent for analyzing samples with high organic matter.

AAS method

This method can be used to detect and quantify iron (and other minerals), from a single extraction, using an atomic absorption spectrophotometer. Iron in solution is atomized and the absorbance is measured at a wavelength specific to iron (248 nm). It is an expensive method and requires skilled

Table 3
Iron Assays and their Advantages and Limitations

Assay	Advantages	Limitations
Spectro-photometric	Sensitive Simple Inexpensive Rapid detection	Needs UV apparatus Needs overnight ashing Not applicable for field
AAS	Reliable Sensitive Accurate Rapid detection Ideal for large sample	Expensive equipment Training of personnel Needs overnight ashing Not applicable for field

Figure 5
Positive and Negative Spot Tests for Iodine in Salt

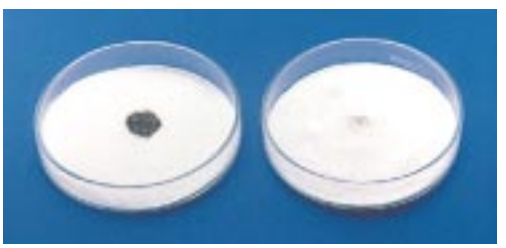


Table 4
Iodine Assays and their Advantages and Limitations

Assay	Advantages	Limitations
Spot tests	Simple Rapid Inexpensive Field-friendly	Not quantitative
Titrations	Accurate Simpler than LC Rapid Inexpensive	Training of personnel Not applicable for field
LC	Sensitive Accurate Reliable No interferences	Expensive Training of personnel Sample pretreatment Not applicable for field

Table 5
List of Suppliers for Laboratory Equipment, Chemicals, and Fortificants

Aldrich P.O. Box 355 Milwaukee, WI 53201-9358	Tel:(414) 273 3852 Fax:(414) 273 4979	Chemicals, Glassware, Lab. equipment
BASF 6700 Ludwigfhafen-Rhein Ludwigfhafen, Germany	Tel:(049) 621 600 Fax:(049) 622 525	Fortificants, Premixes
Beckman Instruments, Inc. 2500 Harbor Blvd. Fullerton, CA 92634	Tel:(714) 871 4848 Fax:(714) 521 3700	Spectrophotometer, Chromatography equipment
Fisher Scientific 711 Forbes Ave. Pittsburgh, PA 15219-4785	Tel:(412) 490 8300 Fax:(201) 379 7638	Chemicals Glassware
F. Hoffmann-La Roche Ltd. CH-4002, Basel Switzerland	Tel:(061) 688 1111 Fax:(061) 691 9600	Fortificants, Premixes
Millipore Intertech P.O. Box 10 Marlborough, MA 01752	Tel:(508) 624 8400 Fax:(508) 624 8630	Centrifuge, Filtration devices
NIST[a] Bldg. 202, Room 204 Gaithersburg, MD 20899	Tel:(301) 975 6776 Fax:(301) 948 3730	Standards, Reference materials
Perkin Elmer 761 Main Ave. Norwalk, CT 06859-0012	Tel:(203) 762 1000 Fax:(203) 762 6000	AAS instrument, Chromatography equipment
Sarstedt P.O. Box 468 Newton, NC 28658-0468	Tel:(704) 465 4000 Fax:(704) 465 4003	Biological assays
Sigma Chemical Co. P.O. Box 14508 St. Louis, MO 63178-9916	Tel:(314) 771 5750 Fax:(314) 771 5757	Chemicals, Test kits
VARIAN[b] 220 Humboldt Court Sunnyvale, CA 94089	Tel:(408) 734 5370 Fax:(408) 744 0261	Spectrophotometer, Chromatography equipment
UNICEF Supply Division UNICEF Plads, Free port DK-2100, Copenhagen Denmark	Tel:(45) 3 527 3527 Fax:(45) 3 526 9421	Iodine spot test kits

[a] National Institute of Standards and Technology
[b] Parts and supplies center
Modification of the original table by Dary, O., G. Arroyave, H. Flores, F.A.C.S. Campos, M.H.C.B. Lins. 1996. Sugar Fortification with Vitamin A. Part 3. Analytical methods for the control and evaluation of sugar fortification of vitamin A. USAID/INCAP.

personnel to optimize operating parameters. AAS can also be used to simultaneously determine the content of other minerals, including, calcium, copper, magnesium, manganese, and zinc.

The advantages and limitations of iron assays are shown in Table 3.

Iodine assays

Qualitative method
Spot tests

Spot tests can be used in qualitative determinations of iodine in salt. Qualitative iodine tests indicate only the presence or absence, not the amount, of iodine in salt (Fig. 5). Spot tests are specific to the form of iodine in salt. In the case of samples fortified with *iodide*, salt iodide is oxidized with an acidic solution to liberate free iodine which then turns starch blue. Salt fortified with *iodate* is analyzed with iodate spot tests where iodate in salt oxidizes an iodide reagent in the presence of hydrogen ions to form free iodine which turns starch blue.

Quantitative methods
Titration method

Like spot tests, titration procedures also are specific to the form of iodine in salt. In samples fortified with *iodate*, addition of an acidic solution liberates free iodine from salt iodate. Free iodine is then titrated with thiosulfate and the amount of thiosulfate used is proportional to the amount of iodine in salt. In salt fortified with *iodide*, bromine oxidizes iodide ions to free iodine, which is titrated with thiosulfate solution. It is a fairly simple and rapid technique compared with the liquid chromatography method. However, it requires personnel with good laboratory skills.

Liquid chromatographic method

Iodine can be quantitatively determined using liquid chromatography (LC). The sample is pretreated by passing it through a membrane filter to remove protein and other insoluble materials. Iodide in the filtrate is separated by ion pair liquid chromatography and detected electrochemically at 0 to 50 mV. It is a quick and sensitive method ideal for analyzing a large number of samples. However, it is an expensive method and requires skilled personnel to perform the analyses.

The advantages and limitations of iodine assays are presented in Table 4.

References

For details on any of the methods above, please refer to:
1. AACC. 1994. Approved methods of the American Association of Cereal Chemists. Eighth edition. American Association of Cereal Chemists, Inc. Minnesota, USA.
2. AOAC. 1993. Methods of analysis for nutrition labeling. Edited by D.M. Sullivan and D.E. Carpenter. Association of Official Analytical Chemists International, Arlington, Virginia, USA.
3. Dustin, J.P. and Ecoffey, J.P. 1978. A field test for detecting iodine-enriched salt. Bulletin of the World Health Organization. 56(4):657-658.

50995