DIETARY SUPPLEMENTS

Status of Methodology for the Determination of Fat-Soluble Vitamins in Foods, Dietary Supplements, and Vitamin Premixes

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Fat-soluble vitamins (FSVs) include vitamin A, carotenoids, vitamins D, E, and K. New legislation is being introduced in many countries to reinforce regulatory compliance of declared concentrations of vitamins and other micronutrients in food products and dietary supplements. The levels of FSVs are likely to be more closely scrutinized due to their potential health risks associated with overdosing, in particular of vitamin D. However, a proviso of stricter regulatory compliance is that analytical methods must be fit-for-purpose, providing adequate accuracy and precision. Official methods have been published by organizations such as AOAC INTERNATIONAL, European Committee for Standardization, International Dairy Federation, U.S. Pharmacopeia, and International Organization for Standardization. The methods available for foods, dietary supplements, and vitamin premixes are evaluated in this review. In general, these methods show adequate precision for regulatory compliance; however, the field of application has not often been evaluated for a sufficiently large range of food matrixes. Gaps have been noted in the range of published official procedures, particularly for carotenoids and vitamin premixes. The potential of some recent developments in sample preparation and chromatographic techniques were evaluated to provide improved procedures for FSV analysis in the future.

Fat-soluble vitamins (FSVs) include vitamin A, provitamin A, carotenoids, vitamins D, E, and K. Knowledge about their roles in human metabolism and health has rapidly grown in recent years particularly in the field of carotenoids. Intakes of FSVs from supplements and fortified foods have become a cause for concern particularly for vitamins A and D (1–3); the acute and chronic effects of these vitamins at high doses are well documented. The UK Food Standards Agency (4) made a comprehensive review and reported risk assessments for FSVs: vitamin A, β-carotene, vitamin D, vitamin E, and vitamin K. Guidance levels were established for vitamins A, D, and K and safe upper levels were established for β-carotene and vitamin E. The Codex Alimentarius (5) is expected to introduce shortly maximum levels of vitamins with Upper Safe Limits for infant formula.

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Increasingly tough legislation is being enacted, or being discussed, for regulatory compliance of micronutrients in fortified food products and dietary supplements. The European Union (EU) is moving towards harmonized rules for addition of vitamins and minerals in food products and definition of acceptable tolerances for declared nutrients. Some countries, like Belgium, Brazil, China, and Mexico, have already placed strict tolerances (±) around the declared values for some nutritional parameters. Other countries may also introduce similar legislation. This places more importance on the quality of the analytical procedures which have to be fit for the purpose of enforcing or monitoring regulatory compliance. The levels of FSVs will surely be closely scrutinized due to the potential health risks associated with overdosing, in particular of vitamin D.

The objective of the present study is not to review the numerous publications describing analytical methods for FSVs which are already well covered elsewhere (6–10), but rather to review the status of official international methods published by organizations such as AOAC INTERNATIONAL (11), European Committee for Standardization (CEN; 12–16), and International Organization for Standardization (ISO; 17–22), and some national organizations for analysis of FSVs in food products, dietary supplements, and vitamin premixes. The second objective is to examine the potential of recent developments published in the scientific literature to provide improved procedures for analysis of FSVs in the future.

Determination of Fat-Soluble Vitamins in Food Products

Background

A list of the official AOAC, CEN, and ISO methods available for determining FSVs has been reported (23). These procedures involve mostly liquid chromatography (LC), but also include spectrophotometric and gas chromatographic (GC) techniques.

A number of points impact on the fitness of purpose of these various methods, including size of test portion, efficiency of extraction procedures and losses,

chromatographic techniques (calibration standards, methods of calibration, internal and external standards, and specificity of detection), use of reference materials as check samples, and adequacy of method validation.

Influence of Test Portion and Encapsulation

Some procedures for sampling and mixing products are described in various ISO documents (24–27). A point rarely observed in the literature is that the size of the test portion, particularly for heterogeneous products, may influence both the precision of analytical results for FSVs and their measurement uncertainty. A summary of the test portions recommended in various official methods is given in Table 1 (12–19, 21, 22, 28–40). AOAC Method **985.30** (41) clearly defines the sampling procedure for ready-to-feed (RTF) infant formula: mix 12 cans and then take a 10 mL test portion. However, the instructions for powdered infant formula vary from method to method and there is no uniform approach; the test portion may vary from 1 to 140 g depending on the procedure (Table 1). In contrast, the European CEN procedures (12–16), which are intended to be horizontal over a wide range of food products, define test portions between 5 and 10 g for vitamins A, E, D, and β-carotene although larger test portions are admitted. Small test portions of 1 g powder or 10 mL liquid are used for extraction of vitamin K_1 (16). Larger sample sizes (20–50 g) are required by ISO procedures for skim milk powders (17–19) and 50 g for animal feeding stuffs (21, 22).

It would be useful to adopt a common approach and to standardize the weight of the test sample as well as the size of the test portion to be analyzed. For infant formula, cereals, other fortified foods, and beverages, one approach could be to define the test sample as a single serving portion or even a daily portion. The serving size should be mixed with a suitable volume of water and homogenized with a laboratory mixer. For example, 25 g product could be blended with 50 g water, and then a suitable aliquot taken for analysis. If the extraction procedure requires 10 g test sample, expressed as dry matter, then 30 g diluted product (1 + 2 water, w/w) could be taken from the diluted homogenized test portion. The use of a precision test, such as a test for robustness or repeatability, should be used to validate the sampling/subsampling technique.

Another factor influencing homogeneity is that FSVs may be encapsulated (e.g., vitamins A and D) in fortified products to improve their stability during the duration of the product shelf life. The encapsulated particles tend to be heterogeneously distributed throughout food products, and this may also negatively influence the precision of the analytical procedure. The science of encapsulation is developing rapidly via technologies like micro- and nano-encapsulation. However, encapsulation of materials is constantly being adapted; for example, high melting point triglycerides and various gums or proteins may be used as coating materials which may make extraction of FSVs more difficult.

Extraction Procedures

In the various official procedures for FSVs, extractions are usually made either by saponification/solvent extraction or by direct solvent extraction. Supercritical fluid extraction (SFE) has been reported as an alternative procedure but has not yet been accepted as official. Several general reviews of extraction and cleanup procedures have been published (6, 8, 10).

In all of these procedures, it is important to avoid losses by oxidation. Low-actinic glassware should be used to reduce these losses. It may also be useful to install fluorescent lighting fitted with an appropriate UV filter in the vitamins laboratory. Castanheira et al. (43) showed that significant errors may result from poor use of volumetric glassware in vitamins analysis.

Saponification and Solvent Extraction

A proportion of the indigenous FSV content of a food are bound up with a lipoprotein complex, and hence the protein-fat bonds must be broken to release the vitamins (6). Saponification is commonly used to liberate bound or esterified forms of vitamins A, E, D, and carotenoids. A wide variety of saponification and extraction conditions are used. Saponification is generally performed under reflux conditions with addition of antioxidants such as ascorbic acid, pyrogallol, butylated hydroxy toluene (BHT), or hydroquinone to reduce oxidation losses, along with nitrogen flushing. It is important to adapt the saponification conditions, concentration of sodium hydroxide or potassium, ethanol, and sample weight (related to fat content) to obtain optimum extraction, and minimum degradation losses. The various international methods propose a variety of saponification conditions depending on the matrix type, size of test portion, and fat content. An example is shown in Table 2. High-starch samples such as breakfast cereals are usually hydrolyzed with Taka-diastase or similar enzyme preparations before saponification to avoid the formation of intractable aggregates and to improve the extraction.

The intrinsic variability occurring during analyses of FSVs was investigated (44). It was concluded that overnight saponification in either methanol or ethanol media at room temperature provided the best conditions for simultaneous extraction of FSVs. An overnight cold saponification procedure was reported by ISO (20) for extraction of products containing tocopherols and tocotrienols and was also suggested by the CEN as an alternative procedure for vitamins A and D (12, 15). For carotenoids, saponification is often used to release carotenol esters and other bound forms. Conditions need to be optimized for particular matrixes (9) but a side effect is loss of xanthophylls.

After saponification, FSVs are usually separated by multiple liquid–liquid extractions (LLE) with nonpolar organic solvents (e.g., petroleum ether, hexane, heptane). The organic phases are pooled and then concentrated by evaporation or evaporated to dryness and redissolved in the LC mobile phase. This process creates considerable quantities

Table 1. Comparison of test portions used in various official analytical procedures

Vitamin	Method reference	Title	Test portion	Ref.
Vitamin A	AOAC **992.06**	Vitamin A (retinol) in milk-based infant formula—liquid chromatographic method	RTF: Mix 12 cans and then 10 mL test portion or test portion containing 20 IU vitamin A	28
	AOAC **992.04**	Vitamin A (retinol isomers) in milk and milk-based infant formula—liquid chromatographic method	Powders: Reconst 140 g/L, then 40 mL test portion; RTF: Mix 12 cans, take 40 mL; milk 40 mL; concentrates 20 mL	29
	AOAC **2001.13**	Vitamin A (retinol) in foods	Low-fat products (<40% fat) 5 g, high-fat products (2 g)	30
	AOAC **2002.06**	Retinyl palmitate (vitamin A) in fortified fluid milk—liquid chromatography	2 mL liquid milk	31
	AOAC **974.29**	Vitamin A in mixed feeds, premixes, and human and pet foods	10–40 g depending on product type	32
	AACC 86.06	Vitamins A and E in foods by HPLC	Low-fat products (<40% fat) 5 g; high-fat products (2 g)	33
	ISO 12080-1:2000	Dried skimmed milk—determination of vitamin A content Part 1—colorimetric method	Weigh 20 g test sample and 50 g water; dissolve at >80°C; dilute to 100 mL with water; take 25 mL of suspension as test portion	17
	ISO 12080-2:2000	Dried skimmed milk—determination of vitamin A content Part 1—method using HPLC	Weigh 20 g test sample and 50 g water; dissolve at >80°C; dilute to 100 mL with water; take 25 mL of suspension as test portion	18
	EN 12823-1:2000	Foodstuffs—determination of vitamin A by HPLC—Part 1—measurement of *all-trans*-retinol and 13-*cis* retinol	Test portion 2–10 g (up to 20 g permitted)	12
	ISO 14565:2000	Animal feeding stuffs—determination of vitamin A content—method using HPLC	Test portion 50 g	22
β-carotene	EN 12823-2:2000	Foodstuffs—determination of vitamin A by HPLC—Part 2: measurement of β-carotene	Test portion 2–10 g	13
Vitamin E	AOAC **992.03**	Vitamin E activity (*all-rac*-alpha-tocopherol) in milk-based infant formula—liquid chromatographic method	RTF: Mix 12 cans and then 10 mL test portion; or about 0.095 IU vitamin E activity	34
	EN 12822:2000	Foodstuffs—determination of vitamin E by HPLC—measurement of α-, β-, γ-, and δ-tocopherols	Test portion 10–30 g	14
	AACC 86.06	Vitamins A and E in foods by HPLC	Low-fat products (<40% fat) 5 g; high-fat products (2 g)	33
	ISO 6867:2000	Animal feeding stuffs—determination of vitamin E content—method using HPLC	Test portion 50 g	21
Vitamin D	AOAC **981.17**	Vitamin D in fortified milk and milk powder—liquid chromatographic method	50 g powder or 200 mL liquid milk	35
	AOAC **992.26**	Vitamin D_3 (cholecalciferol) in RTF milk-based infant formula—liquid chromatographic method	RTF: Mix 12 cans and then weigh test portion equivalent to 12 IU vitamin D_3	36
	AOAC **995.05**	Vitamin D in infant formulas and enteral products—liquid chromatographic method	Prepare product according to label directions, weigh 15 mL test portion	37
	AOAC **2002.05**	Cholecalciferol (vitamin D_3) in selected foods	Oils and margarine, 5–8 g; milk powders and solid products, 10 g; milk and liquid products, 50 g	38
	ISO 14892:2002 IDF 177	Dried skimmed milk—determination of vitamin D content using HPLC	Weigh 50 g test sample and 50 g hot water (60–80°C); dissolve; add another 50 g hot water; cool to RT; take 20 g of suspension as test portion	19
	EN 12821:2000	Foodstuffs—determination of vitamin D by HPLC—measurement of cholecalciferol (D_3) and ergocalciferol (D_2)	Oil and fat 2 g; margarine butter: extract fat with *n*-hexane, quantities not specified; other products 2–10 g	15
Vitamin K	AOAC **999.15**	Vitamin K in milk and infant formulas—liquid chromatographic method	Powders, 1 g; liquids or RTF, 10 g	39
	AOAC **992.27**	*trans*-Vitamin K_1 (phylloquinone) in RTF milk-based infant formula—liquid chromatographic method	RTF: Mix 12 cans and then weigh test portion equivalent to <175 mcg/L or 20 mL	40
	EN 14148:2003	Foodstuffs—determination of vitamin K_1 by HPLC	Powders, 1 g; liquids or RTF, 10 g	16

Table 2. Examples of saponification conditions for vitamin A from EN method (ref. 12)

Sample mass, g	Alcohol	Antioxidants	Potassium hydroxide
2–5	50 mL methanol	0.25 g ascorbic acid	5 mL of a 50 g/100 mL solution
5–10	100 mL ethanol	1 g ascorbic acid + 0.04 g sodium sulfide	20 mL of a 60 g/100 mL solution
10–20	150 mL ethanol	1 g ascorbic acid	50 mL of a 60 g/100 mL solution

of waste solvent and is very time consuming. The use of hexane is not recommended in some countries and the use of mixed ethers may pose a safety problem in the laboratory environment.

The LLE step is considered to be one of the major bottlenecks. For vitamin A, at least 3 extractions are required, and even then the vitamin may not be completely recovered. One way to improve this situation is to use solid-phase extraction (SPE). The use of Oasis® columns was described (45) as an alternative to LLE for determining vitamins A and E in animal feeds. It was able to double the throughput of analyses per day. Results for both vitamins were similar to those from the EU approved methods (46, 47). Chromabond® XTR was used (48) for simultaneous extraction of vitamins A, D, and E from fortified food products. Extrelut® may also be used as an alternative to Chromabond XTR, but the particle size is less uniform, which makes it less robust. This latter SPE technique is also mentioned in the CEN methods as an alternative to solvent extraction (12, 14, 15) and is used routinely in many laboratories. A similar approach (49) using a disposable Kieselguhr cartridge was used to extract vitamin D_3 and provitamin D_3 from saponified fish.

The use of SPE as an alternative to LLE needs further evaluation by means of a collaborative test since it offers significant advantages: considerably reduced solvent consumption, faster sample preparation, and high recoveries of vitamins A, D, and E.

Other approaches have been evaluated. For example a rapid microwave technique was used for saponification, followed by cyclohexane extraction of vitamins A and E from beverages (50). This could be evaluated on other matrixes. Another approach was developed, applicable to all 9 food sectors identified by AOAC (51, 52). Samples are saponified in basic aqueous ethanol, neutralized, and diluted, in tetrahydrofuran (THF)–ethanol before direct LC analysis. This procedure, which avoids LLE partition, was collaboratively studied and accepted as an official procedure (Method 86.06) by the American Association of Cereal Chemists (AACC; 33) for vitamins A and E. This procedure has also been accepted by AOAC (30) as a First Action Method for vitamin A, but not for vitamin E, pending a further collaborative study.

Enzymatic Hydrolysis

Because vitamin K is not stable at alkaline pH, a lipase is used in place of saponification to hydrolyze lipids (53), followed by a single extraction into hexane. This procedure for vitamin K_1 in infant formula (milk- and soya-based) was collaboratively studied and adopted by both the CEN and AOAC (16, 39). The procedure can also be used to determine menaquinone-4 (MK_4) and 2′,3′-dihydroK_1 (53). The recommended test portion is rather small (1 g powder or 10 mL liquid), which is adequate for homogeneous infant formula powders but may be problematic for other fortified food products that are heterogeneous. If larger test portions

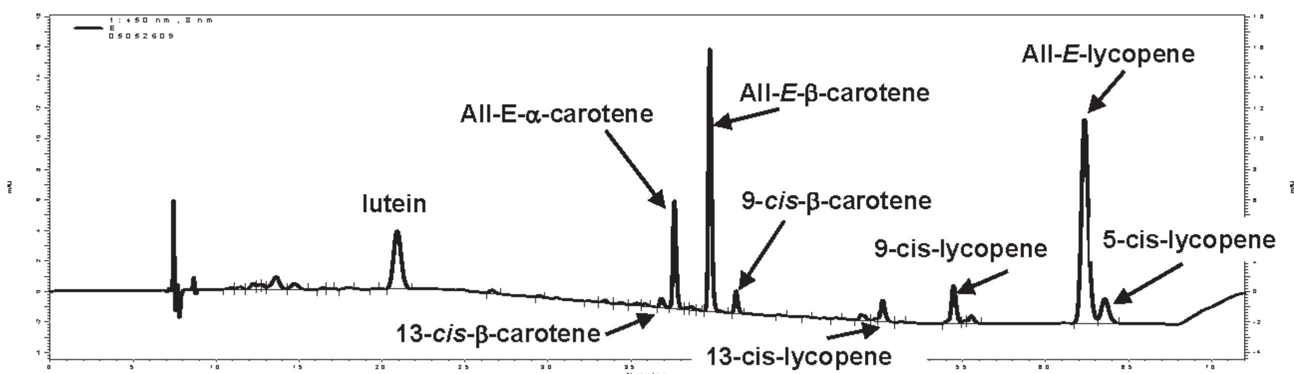

Figure 1. Example of an LC separation of carotenoids in a health care product, using a C_{30} column.

Table 3. Comparison of calibration procedures and matrixes validated in the official procedures

Vitamin	Method	Vitamin forms used for calculation	Calibration procedures	Matrixes validated	Ref.
A	EN 12823-1 (LC)	*All-trans* retinol and 13-*cis* retinol	External calibration using *all-trans* retinol and 13-*cis* standards; procedure also permits internal standards, but none specified	Margarine, milk powder	12
	ISO 12080-2 (LC)	*All-trans* retinol	External calibration using *all-trans* retinyl acetate which is saponified and extracted as per sample	Skimmed milk powder	18
	ISO 12080-1 (colorimetry)	*All-trans* retinol	External calibration using *all-trans* retinyl acetate which is saponified and extracted as per sample	Skimmed milk powder	17
	ISO 14565 (LC)	*All-trans* retinol and 13-*cis* retinol	External calibration using *all-trans* retinyl acetate which is saponified and extracted as per sample; chromatographic conditions give a single peak for all retinol isomers	Animal feeding stuffs and semi-moist pet food	22
	AOAC **2001.13** (LC) and AACC 86.06 (LC)	*All-trans* retinol and 13-*cis* retinol	External calibration using *all-trans* retinol to measure both *all-trans* and 13-*cis* retinols; factor of 1.08 used to correct for lower absorbance of 13-*cis* peak	Nonfat milk powder, corn cereal, margarine, butter, multigrain cereal, infant formula, cottage cheese, NIST SRM 1846, canned tuna in oil, cheese sauce, chicken gravy, whole egg powder	30, 33
	AOAC **992.04** (LC)	*All-trans* retinol and 13-*cis* retinol	External calibration using *all-trans* retinol and 13-*cis* standards	Milk-based infant formula	29
	AOAC **992.06** (LC)	*All-trans* retinol and 13-*cis* retinol	External calibration using *all-trans* retinyl palmitate which is saponified and extracted as per sample; 13-*cis* retinol concn is multiplied by 0.75 to account for its different vitamin A activity	Milk-based infant formula	28
	AOAC **2002.06** (LC)	Retinyl palmitate	External calibration using *all-trans* retinyl palmitate, with retinyl acetate as an internal standard	Fortified fluid milk and chocolate milk	31
E	AOAC **992.03** (LC)	*All-rac*-α-tocopherol	External calibration	Milk-based infant formula	34
	ISO 6867 (LC)	DL-α-tocopherol	External calibration with DL-α-tocopherol	Animal feeding stuffs and semi-moist pet food	21
	EN 12822 (LC)	Vitamin E activity (sum of DL-α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol)	External calibration with DL-α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol, applying appropriate factors for each tocopherol	Margarine (CRM 122), milk powder (CRM 421), milk powder, oat powder	14
	AACC 86.06 (LC)	*All-rac*-α-tocopherol	External calibration	Nonfat milk powder, corn cereal, margarine, butter, multigrain cereal, infant formula, cottage cheese, NIST SRM 1846, canned tuna in oil, cheese sauce, chicken gravy, whole egg powder	33
D	AOAC **992.26**	Vitamin D_3 + estimated previtamin D_3	No internal standard; factor of 1/0.86 used to correct for previtamin D formation during analysis	RTF milk-based infant formula	36
	AOAC **995.05**	Vitamin D_3	Vitamin D_2 internal standard used to correct for previtamin D formation during saponification/extraction	Infant formula (liquid and powdered), enteral products	37
	AOAC **2002.05**	Vitamin D_3	Vitamin D_2 internal standard used to correct for previtamin D formation during saponification/extraction	Fortified milk, gruel, cooking oil, margarine, infant formula powder, fish oil, liquid infant formula	38
	EN 12821	Vitamin D_3 + estimated previtamin D_3	Vitamin D_2 internal standard used to correct for previtamin D formation; in addition a factor of 1.05 is used to compensate for previtamin occurring in product	Margarine, milk powder (CRM 421), porridge, milk powder	15
	ISO 14892:2002, IDF 177:2002	Vitamin D_3 or D_2	Vitamin D_2 internal standard used to correct for previtamin D formation during saponification/extraction	Skimmed milk powder	19
K	AOAC **999.15**	*cis* and *trans* vitamin K_1	External standard	Milk, infant formula	39
	AOAC **992.27**	*trans* vitamin K_1	External standard	Liquid milk	40
	EN 14148:2003	*cis* and *trans* vitamin K_1	External standard	Infant formula and other foods	16

Table 4. Relative activity of tocopherols and tocotrienols

Forms	Relative activity, %
Vitamin E (natural)	
α-Tocopherol	100
β-Tocopherol	50
γ-Tocopherol	10
δ-Tocopherol	3
α-Tocotrienol	50
β-Tocotrienol	5
γ-Tocotrienol	Not known
δ-Tocotrienol	Not known

are used, several extractions with hexane are necessary to achieve a full recovery of K_1.

Direct Extraction

This approach avoids saponification by direct extraction with organic solvents. The AOAC method (31) for determining liquid milk supplemented with vitamin A palmitate has been approved as First Action. This method involves addition of ethanol containing retinyl acetate internal standard to 2 mL milk, followed by hexane and water with mixing on a Vortex mixer. After centrifugation, the hexane phase is retained for LC analysis. Numerous publications (54–61) describe similar techniques for other FSVs, but they have not yet been fully validated and approved as official procedures.

The use of a matrix solid-phase dispersion procedure avoids saponification in milk- and soya-based infant formula (55, 56). In this technique, 500 mg of reconstituted product was mixed with Bondesil C_{18}, and then packed into a reservoir under pressure. Vitamins A and E were eluted with hexane containing 0.5% isopropanol, followed by methylene chloride. The combined solvents were evaporated and redissolved in hexane before LC analysis. An interlaboratory performance test was made using spiked blank products. Recoveries between 92 and 104% were obtained.

A rapid procedure for controlling nutritional label content of β-carotene by direct injection of the drink onto an LC-UV system was reported (62).

An official general procedure for carotenoids is not available. However, a useful multimethod was reported (63) for the determination of norbixin, bixin, capsanthin, β-apo-8′-carotenal, β-apo-8′-carotenoic acid ethyl ester, β-carotene, and lycopene in processed foods. Accelerated solvent extraction (ASE) was compared with a manual extraction using a solvent mixture methanol–ethyl acetate–light petroleum (1 + 1 + 1). Echinonene was added as an internal standard. Recoveries for each analytes were 91–97% for ASE, with the exception of norbixin (67%), compared to 89–103% for the manual procedure.

Supercritical Fluid Extraction

SFE of FSVs with supercritical CO_2 has been described in numerous publications (6, 9, 10, 64) and shows some promise. Turner et al. (65) reported an online SFE/enzymatic (lipase) hydrolysis to extract vitamins A and E from various foods. Recoveries of vitamin A were between 79 and 119% and for vitamin E, between 85 and 152%. The authors claim that their method is more automated than off-line saponification or conventional saponification techniques. However, a potential drawback is the small test portion of 500 mg which may give somewhat lower precision for heterogeneous food matrixes. The procedure was collaboratively tested (66). The following products were analyzed: milk powder, infant formula, pork and minced meat, and high- and low-fat liver paste. Intralaboratory relative repeatability was around 11%, whereas interlaboratory variation averaged 23% during the validation, and approximately 40% during the intercomparison. Ruggedness tests performed at different steps of the project showed that different types and models of equipment did not give large differences in recoveries. Hence, it was concluded (66) that the interlaboratory variations obtained were due largely to differing levels of experience in vitamin analysis between the participants.

Chromatographic Techniques

Several chromatographic techniques have been used for analysis of FSVs, including LC, GC, and capillary electrophoresis (CE). However, the most widely used is LC, and the methods discussed below refer mainly to this technique.

Table 5. Spectrophotometric methods for FSV determination

Method No.	Method title	First Action	Final Action	Ref.
AOAC **960.45**	Vitamin A in margarine	1960	Surplus 1980	89
AOAC **974.29**	Vitamin A in mixed feed, premixes, and human and pet foods (Codex adopted–AOAC method)	1974	Yes	90
AOAC **971.30**	α-Tocopherol and α-tocopheryl acetate in foods and feeds	1971	1972	91
AOAC **938.64**	Carotenoids in macaroni products	1938	Yes	92
AOAC **941.15**	Carotene in fresh plant materials and silages	1941	Yes	93
AOAC **970.64**	Carotenes and xanthophylls in dried plant materials and mixed feeds	1970	1974	94
ISO 12080-1	Dried skimmed milk—determination of vitamin A content—Part 1: colorimetric method	—	2000	17
AACC 86-02	Vitamin A—Carr Price method (milk powders)	—	1999	95

Table 6. Summary of reference materials available for fat-soluble vitamin analyses

Product	Reference	Vitamin A	Viamin D	Vitamin D	β-Carotene	α-Carotene	Lutein	Zeaxanthin	Cryptoxanthin	α-Tocopherol (vitamin E)	γ-Tocopherol	δ-Tocopherol
Margarine	BCR 122		x				x	x	x	x		
Coconut oil	NIST 1563-2									x		
Spinach	NIST 2385				x		x					
Baby food composite	NIST 2383	x	[a]		x	x	x	x	x	x	x	x
Milk powder	BCR 421		x							x		
Whole milk powder	NIST 8435	[a]	[a]									
Infant formula (milk-based)	NIST 1846		x	x							x	x
Baking chocolate	NIST 2384									x	x	x
Peanut butter	NIST 2387									x	x	x

[a] Value only indicative; not certified.

For separation of vitamins A and E by LC, the official methods (11, 12, 14, 18, 21, 22) recommend either reversed-phase (C_{18}) or straight-phase chromatography. Reversed-phase separations (C_{18}) are preferred for vitamins D (11, 15, 19) and K_1 (16, 39). However, C_{30} is useful for separating *cis-* and *trans-*forms of vitamin K_1 (67).

The European (CEN) method (13) for β-carotene recommends reversed-phase chromatography using a C_{18} column. There are no AOAC or ISO procedures for carotene analysis by LC. Thus, there is a major gap for a general purpose LC procedure for separating the full range of carotenoids, including lycopene and lutein, which are of considerable current interest in nutrition.

In the scientific literature, carotenoids are usually separated by reversed-phase chromatography on C_8 or C_{18} columns (9). It has been observed that carotenoids are susceptible to oxidation and may undergo on-column degradation; however, addition of triethylamine and ammonium acetate to the mobile phase can help to reduce these losses. A stable column temperature is also important to obtain reproducible separations. The use of C_{30} columns is advantageous for separating *cis-trans* isomers of carotenoids (68). Fávaro et al. (69) determined isomers of carotenoids in enteral feeding products. An example of a typical LC separation of carotenoids in a healthcare product, using a C_{30} column, is shown in Figure 1.

For vitamin D, an adequate separation for quantification is not possible on a single column; therefore, a preliminary semipreparative column is used to isolate a fraction containing vitamins D_2 [internal standard (IS)] and D_3. This step can be automated by use of a fraction collector. Reversed-phase (C_{18}) columns are required to separate vitamins D_2 and D_3. Tocopherols and tocotrienols are usually separated by either normal phase or reversed-phase (C_{18} columns; 10); however, use of a C_{30} column gives an excellent separation of all 8 compounds.

Calibration Procedures

The calibration standards for FSVs must be checked for purity by spectrophotometric procedures and a correction applied. This is usually detailed in the official analytical procedure. A summary of the calibration procedures is shown in Table 3 (12, 14–19, 21, 22, 28–31, 33, 34, 36–40). Official procedures for vitamins A, E, and K_1 generally use external calibration. For vitamin A, the 2 major isomers, *all-trans* retinol and 13-*cis* retinol, are determined to give the total vitamin A content. Some differences exist between the various procedures (Table 3) for calculation of 13-*cis* retinol; however, its concentration is fairly low and any differences should not markedly affect the overall result.

The use of an IS may be advantageous and some examples are described. 3,4-Didehydroretinyl acetate was reported as an IS for vitamin A analysis in breast milk by LC (70). The authors report that it can be added to milk before saponification and is carried through the analysis as dehydroretinol (vitamin A_2). However, it is not readily available commercially. β-Cryptoxanthin was included as an

Table 7. Summary of some methods for vitamin supplements, premixes, and concentrates

Method No.	Method title	First Action	Final Action	Field of application	Analytical technique	Ref.
AOAC **2005.07**	β-Carotene in supplements and raw materials	2005	No	Raw materials and supplements from 0–200 mg/g except for beadlet materials and tablet materials made from beadlets	Liquid chromatography	98
AOAC **974.29**	Vitamin A in mixed feeds, premixes, and human and pet foods—colorimetric method (Codex adopted–AOAC method)	1974	No	Dry-mixed feeds and premixes; liquid feed supplements and premixes; not applicable to high potency vitamin A concentrates used for feed, premix, and food manufacture	Colorimetry	90
AOAC **948.26**	α-Tocopheryl acetate (supplemental) in foods and feeds	1948	1980		Colorimetry	99
AOAC **971.30**	α-Tocopherol and α-tocopheryl acetate in foods and feeds	1971	1972	Premixes, feed concentrates	Colorimetry	100
AOAC **988.14**	Tocopherol isomers in mixed tocopherols concentrate—food chemicals) Codex, USP, AOAC method)	1988	No	Mixed tocopherols concentrates	Gas chromatography	101
AOAC **989.09**	α-Tocopheryl acetate in supplemental vitamin E concentrates	1989	No	Oil and powder concentrates containing 400–1000 IU vitamin E/g	Gas chromatography	102
AOAC **975.43**	Identification of *RRR*- or *all-rac*-alpha-tocopherol in drugs and food or feed supplements	1975	1980		Polarimetry	103
AOAC **980.26**	Vitamin D in multivitamin preparations	1980	1980	Sum of previtamin D and vitamin D in multivitamin preparations containing ≥200 IU/vitamin D/g	Liquid chromatography	104
AOAC **975.42**	Vitamin D in vitamin preparations	1975	1977	Oily solutions, powders, capsules, tablets, aqueous dispersions and vitamin D resin—vitamin D concentrates—vitamin D in multivitamin preparations	Colorimetry	105
AOAC **985.27**	Vitamin D in vitamin AD concentrates	1985	No	Sum of previtamin D and vitamin D in oil, powders, and aqueous dispersion of A,D concentrates containing ≥5000 IU vitamin D/g	Liquid chromatography	106
AOAC **979.24**	Vitamin D in vitamin preparations	1979	1980	Sum of previtamin D and vitamin D in oils containing $\geq 10^5$ IU vitamin D_3/g; resins $\geq 20 \times 10^6$ IU vitamin D_3/g; and powders and aqueous dispersions $\geq 25 \times 10^3$ IU vitamin D_3/g	Liquid chromatography	107
AOAC **974.30**	Menadione sodium bisulfite (water-soluble vitamin K_3) in feed premixes	1974	1975	Feed premixes	Gas chromatography	108
AACC 86.01A	Vitamin A—ultraviolet absorption method	1972	1999	Dry vitamin mixes, beadlets, oils, and emulsions $>1 \times 10^4$ IU/g	Spectrophotometry	96
USP 29	Oil- and water-soluble vitamins with minerals tablets	2006	—	Tablets—vitamins A (retonol or esters of retinol), D_2, D_3, E (α-tocopherol, α-tocopheryl acetate, α-tocopheryl acid succinate), K_1, and β-carotene	LC-UV and spectrophotometry	97

IS for determination of vitamins A and E, but was added after saponification (71). Retinyl acetate was used as an IS in the AOAC Method **2002.06** (31) using direct extraction of retinyl palmitate from fortified milk (72).

Some differences exist between nutritional labeling in Europe and the United States when defining vitamin E. The AOAC procedure determines vitamin E as DL-α-tocopherol (34) via external calibration. However, the EN procedure (14) specifies that vitamin E activity includes all 4 tocopherols. These different approaches in calculating vitamin E activity could lead to a significant bias in declared vitamin E content. The relative activities of tocopherols and tocotrienols are shown in Table 4. Tocol is a useful IS (48).

For determination of vitamin D_3, vitamin D_2 (15, 36–38) is usually used as an IS and vice versa if vitamin D_2 has to be determined. The 2 forms are not usually both present together in fortified food products. The IS corrects for transformation of vitamin D to previtamin D during saponification, since both vitamers form equivalent amounts of their respective previtamin (73). The EN method (15) also applies a factor of 1.05 to the result to correct for any existing previtamin D in the product. It should also be noted that the vitamin D added to products via a premix contains in theory about 7% previtamin D, which is not detected in the food product due to interferences around the small previtamin D peak. This value of 7% is the equilibrium value obtained after 30 days at 20°C.

The external calibration is generally used for analysis of vitamin K_1. An IS is not normally used, even though many vitamin K analogues are available.

For determination of carotenoids, numerous ISs have been reported in the literature. Typical examples are β-Apo-8′-carotenal (74) and echinenone (75). It is preferable to add the IS during the post-heating steps to avoid losses (75). *trans*-β-apo-8′ (or 10′ or 12′)-carotene oxime is more stable and can be added before saponification.

Detection

The various international methods describe conditions for the analysis of each FSV separately. For vitamin A (both *all-trans* and 13-*cis* retinols), UV detection is normally used (11, 12), but fluorescence detection may also used as an alternative (12).

For vitamin E, fluorescence detection (14) is usually preferred over UV detection particularly when using normal phase chromatography since it provides about 10 times higher sensitivity and better specificity. A comparison was made between fluorescence detection, UV, and evaporative light scattering for determination of tocopherols and tocotrienols in olive oils (76). The best results were obtained with the fluorescence detector. Fluorescence detection is recommended by ISO (20) for determining tocopherols and tocotrienols in vegetable oils and fats, but UV detection is not recommended. For routine analyses, laboratories may determine vitamins A (*all-trans* and 13-*cis* retinols) and vitamin E (α-tocopherol) simultaneously by LC, with UV and fluorescent detectors connected in series (77). Dual-amperometric detection (78) is a useful alternative detection system but has not found wide application.

For vitamin D, UV detection, in the range 254–265 nm, is used in all the official procedures (11, 15, 19, 73). However, in spite of the preliminary preparatory column cleanup, UV detection is not totally specific, and occasionally co-eluting interferences occur under the vitamin D_2 and D_3 peaks. This is especially apparent if high-fat products are incompletely saponified. An electrochemical detection system might offer greater specificity. It has been evaluated for analysis of vitamin D in fish (49), fortified milk, and infant formulas (79) with promising results but needs further evaluation on a wider range of matrixes. The evaporative light scattering detector was shown to be less sensitive than UV for determining α- and γ-tocopherols in human milk, and thus showed no advantage (80).

A Codex-AOAC Method (**992.27**; 40) exists for *trans*-vitamin K_1 in milk and infant formulas. However, this procedure using UV detection is limited by inadequate selectivity, precision, and sensitivity (67), and has been largely superseded by AOAC Method **999.15** (39), which is equivalent to the EN method (16). This procedure determines total vitamin K_1 by means of a post-column reduction with Zn and fluorescent detection (67). A similar technique (81) can also be used to detect vitamin K_3 in pet foods, however, no official procedures are available at the moment. Vitamin K_1 can also be quantified by dual-amperometry (78).

Diode array detection (DAD) is very useful (82) for identification and quantification of carotenoids in fruit and vegetable juices.

A recent development is the use of LC/MS or LC/MS/MS as a sensitive and selective means of determining one or more FSVs in foods. Stoeggl et al. (83) compared UV, DAD-UV, fluorescence, and MS detection for tocopherols and tocotrienols in several food matrixes. A preliminary study (84) was reported for the determination of α-tocopherol in fortified food products using the selective ion monitoring (SIM) mode with deuterium labeled α-tocopherol as an IS. MS is also a very useful technique for identification and determination of carotenes (9, 10). SIM was used to determine lycopene and carotene in tomatoes, mango, and kiwi fruits (85).

LC with a single-quadrupole mass spectrometer is capable of providing good sensitivity and quantitative linear calibration over several orders of magnitude. Vitamins A, D_3, and E can be quantified with SIM detection in infant formula (48). Both *all-trans*-retinol and 13-*cis*-retinol can be quantified to provide the total vitamin A content. The use of the readily available vitamins D_2 and 5,7-dimethytocol as IS improved the quantification of vitamins D_3 and E (α-tocopherol), respectively, by LC/MS.

Vitamin K_1 was determined (86) in vegetables using UV detection at 247 nm or by particle beam MS. Another LC procedure was reported (87) for determining a range of Vitamin K derivatives. Both UV and APCI-MS detectors were used. This approach was only applied to plasma.

Future Developments in LC Procedures

A recent trend is to use LC columns (59, 88) with 3 μm particle size. This trend is likely to continue with the recent development of ultra performance liquid chromatography (UPLC) and Rapid resolution (or Fast)-LC which use even smaller particle sizes (1.7–2.5 μm) and very short chromatographic run times. Chromatographic separations of several vitamins could be reduced to a few minutes and should help to improve sample throughput and lower consumption of solvents. This potential for faster separation should be coupled to either MS or MS/MS detection for better selectivity and sensitivity for multimethods. Fluorescence detection would also offer a useful alternative or complementary technique to MS.

Spectrophotometric Methods

The various AOAC, AACC, and ISO methods using spectrophotometry are listed in Table 5 (89–95). Many of these procedures are time consuming and have largely been supplanted by more selective LC procedures. Methods for vitamin A may be subject to interference if high concentrations of β-carotene are present in the food product.

Enzyme-Linked Immunosornent Assay

Immunoassay kits for vitamins A and D in milk and milk powders have been developed commercially. First trials on infant formula show that these procedures require some further optimization to render them sufficiently robust for routine analyses.

Method Validation

Many of the earlier AOAC methods have been validated for analysis of FSVs in infant formula. Some of the more recent methods are applicable to a wider range of food products (Table 3) and have been well validated: vitamin A (72), vitamin D (73), vitamin K_1 (53). A gap exists within the AOAC procedures for a horizontal LC method for vitamin E determination.

The CEN procedures (12–16) were developed to be horizontal and applicable to total vitamin content in a wide range of food products. However, in some cases, the matrixes chosen for validation were rather limited, including only milk powders and margarine, which do not really represent the full range of fortified products. For vitamin K_1 the CEN method (16) is equivalent to AOAC Method **999.15** (39), which was validated for milk and infant formula containing fortified levels of the vitamin. The field of application of this CEN procedure was extended to other types of foodstuffs (fortified and nonfortified). This decision was based on the complementary study of Woollard et al. (67). The selectivity of the method is influenced by the choice of LC column, but the C_{30} column is advantageous since it can separate the biologically active *trans*- and inactive *cis*-vitamin K_1 isomers. The *cis*-vitamin K_1 isomer contributes up to 15% of total phylloquinone in certain foods.

The field of application and validation of ISO methods only covers skim milk products (18, 19) and animal feeds (21, 22). It is recommended that for future collaborative studies, food matrixes should be chosen that include a wider range of fortified products, such as infant formula, cereals (infant and breakfast), clinical nutrition products, sports nutrition, powdered drinks, oils and fats, and beverages as well as unfortified products like meat and cereals.

In spite of some improvements in validation of recent procedures, method validation protocols need further improvements. In general, the reported values for repeatability and reproducibility are acceptable for the products studied. However, a collaborative study involving only repeatability and reproducibility is incomplete, since it gives little information about the accuracy of the procedure and its fitness for purpose. In some cases, several parallel methods are available for a particular vitamin, but comparisons have not been made to determine if they all give equivalent values. This may be a problem for compliance monitoring.

The introduction of the Horwitz ratio (HorRat) is of great value, but the calculation of intermediate reproducibility, SD(iR), would also be useful. This is generally carried out by determining an analyte on at least 6 different days in the same laboratory by one or more analysts and then calculating the median reproducibility from the various values. An estimate of measurement uncertainty (MU), which is required by laboratories accredited by ISO 17025 or related systems, can then be calculated as MU = 2 × SD(iR).

A recent trend is to use spiked blank matrixes in collaborative tests, but this only provides information about the efficacy of the extraction and cleanup procedures. It does not take into account the uncertainties arising from the heterogeneity of the sample test portion or the forms of vitamin present in the product.

The use of a reference material is a prerequisite for checking correct application of analytical methods. A range of commercially available reference materials is shown in Table 6.

Dietary Supplements

The AOAC (11), AACC (96), and U.S. Pharmacopeia (97) have published a range of procedures for supplements, concentrates, and premixes (shown in Table 7; 90, 96–108) involving colorimetry, polarimetry, and LC. However, the majority of the AOAC methods were issued more than 20 years ago and need to be replaced by more modern procedures. The German official collection of methods (LMBG §35) lists saponification/LC methods for determining vitamins A and E in supplements (L49.00-3, L00.00.62, respectively; 109). The U.S. Pharmacopeia (97) lists recent LC procedures for vitamins A, D, and E, and several carotenoids. However, incomplete method performance data are provided.

Conventional methods for determination of vitamins A and E and carotenoids use colorimetry or fluorometry, together with thin-layer chromatography or open column chromatography with alumina or silicic acid. These assays

lack specificity, are time consuming, and are not amenable to simultaneous determination of the vitamins from a single preparation (110). LC procedures are preferable since they offer better specificity and are able to separate the *cis-trans* isomers of β-carotene and vitamin A. The *cis*-forms have lower biological activities than the *all-trans* forms.

AOAC has an ongoing mandate to develop well-validated analytical methods for dietary supplements. Recently, a procedure was established for the determination of β-carotene by LC (111), involving a single-laboratory validation. The procedure was then collaboratively studied (112) and published as AOAC Method **2005.07** (98). It is applicable to the determination of *all-trans* and total β-carotene in raw materials and supplements from 0 to 200 mg/g except for beadlet materials and tablet materials made from beadlets. For soft gels and tablets, analyzed for total β-carotene, the reproducibility relative standard deviation (RSD_R) ranged from 3 to 23% and the HorRat values ranged from 1.1 to 3.7. For *trans* β-carotene, the RSD_R ranged from 4 to 23% and the HorRat values ranged from 0.9 to 3.4. The RSD_r and HorRat values in the analysis of a beadlet raw material were substantial, and it is believed that the variability within the material itself introduced significant variation in subsampling. Further investigations are in progress to improve the methodology for beadlets. An isocratic C_{18} chromatographic procedure is generally applied, except for products with a high α-carotene content, where the *cis*-isomers of α-carotene are known to interfere with the quantitation of β-carotene. In this case, a C_{30} reversed-phase LC system is preferred. A similar procedure was reported (110) for simultaneous determination of vitamin A (including retinyl acetete and palmitate) and β-carotene in soft gel capsules and tablets.

A multimethod was reported for determining vitamin A, vitamin A acetate, vitamin A palmitate, vitamin E, vitamin E acetate, and coenzyme Q_{10} by reversed-phase LC (C_{30}) in soft gel capsules and tablets (113). Dual wavelength monitoring at 280 and 450 nm was used. Either vitamin A palmitate or lutein could be used as IS. Another report (114) described an isocratic LC-DAD procedure for determining β-carotene and vitamins A, D, E, and K_1, K_2, and K_3 in cosmetic and pharmaceutical products.

A series of methods have been published for emulsified nutritional supplements. A procedure was developed for vitamin D_2 (115). The supplement (10 g) was dissolved in 0.2 M K_2HPO_4 solution with 1 mM $EDTA·2Na·2H_2O$, followed by a C_{18} cartridge cleanup. It was analyzed by column switching with a preparatory column of Hitachigel® 3011-0, followed by reversed-phase LC-UV. Another procedure was optimized for retinol palmitate (116). This involved use of monosodium L-glutamate to dissolve the product, followed by SPE before LC analysis. Similar procedures were also reported for vitamin K_1 and tocopherol acetate involving dissolution and SPE (117, 118).

AOAC Method **979.24** is the current reference procedure for vitamin D_3 concentrated powders (107). It involves saponification, extraction of unsaponifiable matter into hexane, evaporation of solvent under vacuum, and LC-UV analysis. An alternative procedure was reported for determining vitamin D_3 microencapsulated with starch or cellulose. This involved dissolution in 10% (v/v) aqueous dimethyl sulfoxide solution and extraction of the vitamin D_3 into isooctane (119). Similar results were obtained to those by the reference method AOAC **979.24** (107).

Vitamin Premixes

AOAC (11) has issued a few official methods for vitamin premixes which are listed in Table 7. These methods involve colorimetry, LC, or GC, but are now rather dated, and well-validated LC procedures would be welcomed by the analytical community.

Premixes used for human and pet foods often contain minerals and water-soluble vitamins, as well as FSVs. The latter are often encapsulated and in different concentrations which places high demands on the selectivity and robustness of the extraction and detection steps.

The LC-UV procedures used within the premix and food industries are usually in-house, and are not harmonized. They may yield different results due to variations in extraction procedures. Typical methods involve dissolution in 0.1 M HCl–ethanol or 0.1 N H_3PO_4–ethanol in an ultrasonic bath. The extracts are then centrifuged and re-extracted into *n*-hexane before LC analysis. If iron(III) phosphate or iron(III) fumarate are present in the premix, then 2% ammonia solution is used in place of the acidic extraction solution. If other minerals are present, then ethylene diamine tetraacetic acid (EDTA) or diethylene triamine pentaacetic acid (DPTA) can be added to complex them.

Separation of FSVs may be performed on a silica or C_{18} column with a DAD or variable UV detector. For best results, the UV detection wavelengths need to be optimized due to the very large differences in concentration between the various FSVs. For determination of vitamin K_1, the postcolumn fluorescence zinc reduction procedure developed for food products (53, 67) provides better selectivity and accuracy than UV detection.

Stary et al. (120) reported a procedure for determining pure and encapsulated vitamin A acetate, vitamin A palmitate, vitamin E acetate, vitamin D_3, and vitamin K_1 by LC. Vitamin premixes were dissolved in 0.1 N H_3PO_4–ethanol, and FSVs were then extracted into hexane. The 5 FSVs were separated on a C_{18} column with detection at 248 nm. Recoveries were in the range of 95–104%.

For vitamin D, the only official method available for multivitamin preparations is AOAC Method **980.26** (104), which is applicable to preparations containing more than 200 IU (5 μg) vitamin D/g. Vitamin D is calculated as the sum of previtamin D and vitamin D. The method includes a saponification step, a semi-preparative LC on a C_8 column followed by analytical LC, on a silica column, with the use of an IS (delta 4,6-cholestadienol), and determination of a conversion factor previtamin D/vitamin D. The use of a saponification step is essential to provide a complete extraction of vitamin D from coatings which contain medium chain triglycerides. Theoretically, the ratio of previtamin D in premixes is about 7%

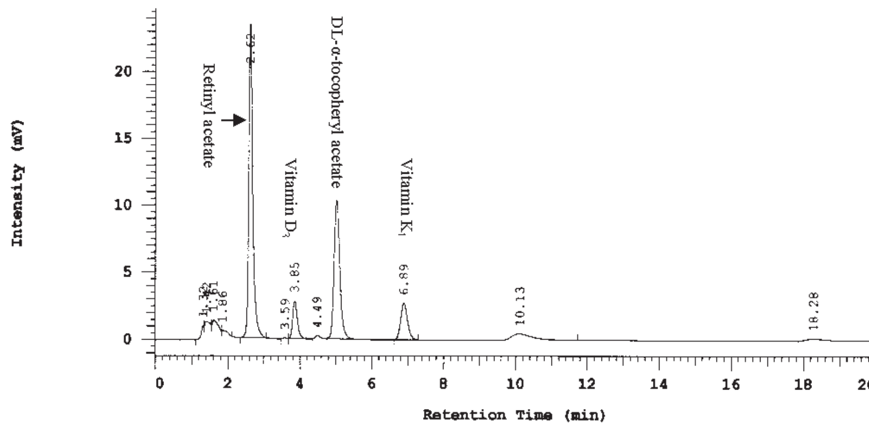

Figure 2. Separation of several fat-soluble vitamins in a vitamin premix by LC-UV.

of total vitamin D. However, different premix suppliers adopt different values. In practice, the previtamin D content often lies in the range of 1–6.5% with an average value of 4%.

Figure 2 shows separation of several FSVs in a vitamin premix by LC-UV. There are no official procedures to determine several FSVs simultaneously in vitamin premixes. A need exists for a harmonized reference procedure by LC to permit suppliers of vitamin premixes and food companies to determine that the FSV contents of vitamin and vitamin/mineral premixes are in accordance with the specifications.

Conclusions

Various international methods (AOAC, CEN, ISO) are currently available, usually based on LC, for determining vitamins A, E, D, and K in a variety of food matrixes. The repeatability and reproducibility of these procedures are generally acceptable for regulatory compliance monitoring when the required tolerance is around ±20%. However, some of these methods are not validated on a sufficiently large range of matrixes and may not be applicable to all food sectors.

For food products, and particularly for infant nutrition and baby food products, a gap exists in adequately describing the sampling and size of the test portion. The test portion needs to be described in a more standard and uniform manner, since this may have a major impact on the method precision. It is suggested that the product's recommended serving size be used as the test portion.

Because compliance monitoring requires significantly higher numbers of analyses, faster methods of analysis are needed. Multimethods need to be developed to extract vitamins A, D, E, and β-carotene simultaneously. SPE should be used to replace LLE since it is more rapid and consumes lower volumes of solvents.

Faster chromatographic separations using UPLC or Rapid resolution (Fast)-LC coupled with MS or MS/MS detection is a most promising technique for the future and merits further investigation, particularly as vitamins A, D, and E can be detected simultaneously. The single quadrupole MS detector (SIM) is of interest because it is suitable for use by analysts in a routine laboratory situation. MS/MS detection is very useful for quantification of carotenoids but may not be applicable in every laboratory due to the high cost and need for experienced chemists conversant with interpretation of MS/MS. Applications of LC/MS for vitamin K analysis in foods are lacking at the moment, but may not offer significant advantages over the current fluorometric detection procedure using a postcolumn zinc reduction.

A gap exists for an LC method capable of analyzing a wide range of carotenoids including lycopene and lutein in fortified food products. For this purpose, reversed-phase chromatography with C_{30} phase offers more efficient chromatographic separations than C_{18} or C_8 columns. MS detection offers the best selectivity, although DAD is probably the most useful for labeling compliance monitoring.

AOAC has developed a validated procedure for β-carotene in dietary supplements, but further methods are required for other FSVs and carotenoids. CEN is also seeking to develop methods in this area.

There is a lack of modern official methods for simultaneous analysis of FSVs in vitamin premixes using LC-DAD or UV detection. The current AOAC methods date from the 1980s and need updating, particularly as encapsulating materials and encapsulation techniques are changing and the analytical techniques to extract FSVs need to keep pace with these developments.

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