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Review

Extraction of fat-soluble vitamins

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Abstract

An overview of the different extraction procedures of fat-soluble vitamins from human fluids, foods and pharmaceutical preparations is presented. Methods using organic solvent extraction (both liquid–liquid and solid–liquid extraction), supercritical fluid extraction and solid-phase extraction for the different types of both vitamins and matrices are discussed.

Keywords: Reviews; Extraction methods; Vitamins

Contents

1. Introduction

During the first third of the twentieth century, a major focus on research in physiological chemistry was the identification of vitamins, compounds that are essential to the health of humans and other vertebrates but cannot be synthesized by these animals and must be therefore be obtained from the diet. Early nutritional studies identified two general classes of such compounds based on their solubility in nonpolar organic solvents (fat-soluble vitamins) and those that could be extracted from foods with aqueous solvents (water-soluble vitamins). Eventually the fat-soluble group was resolved into four vitamin groups (namely, A, D, E, and K), all of which are isoprenoid compounds synthesized by the condensation of multiple isoprenoid compounds [1].

Vitamin A, or retinol, in its various forms functions as a hormone and it is also an essential component of the visual cycle. Deficiency of vitamin A leads to a variety of symptoms in humans, including dryness of the skin, eyes, and mucous membranes; retarded development and growth; and night blindness, an early symptom commonly used in diagnosing vitamin A deficiency [1,2]. Vitamin D consists of two different compounds, vitamin D_2 and D_3, both absorbed from the diet. Vitamin D_3 is also

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biologically synthesized in the skin from 7-dehydrocholesterol by UV radiation; then it is hydroxylated in the liver to 25-OH-D_3, which is also metabolized in the kidney to other dihydroxymetabolites such as 24,25-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3. Vitamin D_3 metabolites are of paramount importance in several bone diseases, such as osteoporosis and osteomalacia, in oncology and dermatology due to their actions on cell differentiation and proliferation [3,4]. Vitamin E is the collective name for a group of closely related lipids called tocopherols, which are biological antioxidants. Vitamin E deficiency in humans is very rare; the principal symptom is fragile erythrocytes. Vitamin K is an essential nutrient for animals and humans because it is required for functioning of the blood clotting cascade. Vitamin K deficiency is very uncommon in humans. Vitamin K_1 (phylloquinone) is found in green plant leaves; a related form, vitamin K_2 (menaquinone), is formed by bacteria residing in the vertebrate intestine [1,2].

The determination of these vitamins is necessary because of their importance for organic functions. An extraction step prior to determination of vitamins is normally required as sample pretreatment. There are three general types of samples where vitamins have to be determined namely: human fluids, food and pharmaceutical preparations. The importance of the determination of vitamins in human fluids is due to the relationship between vitamin deficiency states and certain pathologies. The quantitation of vitamins and their metabolites in human fluids has been considered one of the most difficult goals in clinical chemistry. The low concentration of circulating metabolites, the presence of a number of metabolites which exhibit similar chemical behavior to one another, the large amount of other related neutral lipids and the high instability of their chemical structures in the presence of UV light and heat, are the main reasons which justify this assertion [5,6]. For these reasons, a liquid–liquid extraction prior to a preconcentration step by solid-phase extraction is usually required. The determination of vitamins in food, which normally are solid or semi-solid samples, is very important in the evaluation of several biochemical and nutritional disorders. The analytical methods for the determination of vitamins in foods generally involve solid–liquid extraction as a previous step, followed by preconcentration. However, in some cases, a recent technique called matrix solid-phase dispersion (MSPD) is being used, thus avoiding the drawbacks involved in conventional solid–liquid extraction [7]. Finally, specification of vitamin contents in pharmaceuticals, makes these matrices of analytical interest.

The different procedures for removal of fat-soluble vitamins from their matrices using organic solvent extraction, (either liquid–liquid and solid–liquid extraction), supercritical fluid extraction (as an alternative to conventional extraction methods) and, finally, solid-phase extraction (both as a preconcentration and clean-up procedure) are discussed and compared in this paper.

2. Organic solvent extraction

Liquid–liquid extraction is a very common tool for the extraction of vitamins in human fluids, supported on the large variety of pure or mixed solvents with broad solubility and selectivity ranges. Also, the equipment required for conducting this separation step is simple and, in many cases, the phases are compatible with the mobile phases used in the subsequent chromatographic step [8]. Usually, vitamins D and E are individually extracted from human blood, serum and urine. A large number of organic solvents such as acetonitrile [9], diethyl ether [10] and mixtures as methanol–toluene [11], $CHCl_3$–light petroleum [12], tetrahydrofuran (THF)–diethyl ether [13] for the extraction of vitamin D, and heptane [14], xylene [15], diethyl ether [16] and hexane [17] for the extraction of vitamin E have been used.

Vitamin A is usually extracted simultaneously with vitamin E using hexane [18], $CHCl_3$ [19] and ethyl acetate–butanol mixture [20]. Cloud-point extraction has been applied to the simultaneous extraction of these vitamins from human serum and whole blood [21]. Samples (~50 μl) were mixed with 10 μl of retinyl acetate solution, 0.4 ml of 16% Genapol X-080 [a poly(ethylene oxide)/tridecanol condensate] and 0.125 g of NaCl. The mixture was vortex-stirred until the salt was dissolved, kept in a water bath at 50°C for 10 min and centrifuged. The aqueous phase was decanted, leaving the surfactant-rich phase adhering to the tube wall. Hydrophobic

proteins and most of the surfactant were precipitated with acetonitrile and the solution was filtered and prepared for analysis by reversed-phase high-performance liquid chromatography (HPLC).

In some cases, sample treatment before liquid–liquid extraction is mandatory. For example, urine samples can be hydrolyzed by incubation with β-glucuronidasa in 0.1 *M* sodium acetate buffer of pH 4.5 for 4 h at 37°C for the determination of vitamin E [16]. Proteins from serum and plasma are precipitated before vitamins extraction by adding ethanol [10,15,22], acetonitrile [20] and an inorganic salt such as sodium sulfate [11].

The determination of fat-soluble vitamins from foods and pharmaceuticals, which are solid or semi-solid samples, involves a previous leaching step. Most times this leaching step consists of a simple manual extraction with organic solvents. As in human fluids, vitamins D and E have been extracted individually from food and pharmaceuticals using different solvents such as light petroleum [23], CH_2Cl_2 [24], diethyl ether [25–27], hexane [28–30] or dimethylformamide–dimethyl or sulfoxide–$CHCl_3$ mixtures [31]. Vitamin E has also been leached with ethanol [32] or hexane [33] from feedstuff using the conventional Soxhlet procedure, which has been recommended as the official method. A mixture of propan-2-ol–hexane has been used for the extraction of vitamin K from various food matrices [34,35]. Vitamin A have been extracted from this type of samples using hexane [36], diethyl ether [37] and $CHCl_3$–acetone [38], among others.

The simultaneous extraction of vitamins from foods is very common. Thus, Li et al. used a combined extraction method for the sequential extraction of lipid-soluble vitamins A, α-E, D_3 and K_3 from feed samples [39], in which the sample was shaken with $CHCl_3$, 25% NH_4OH, Na_2SO_4–diatomaceous earth (20:3) for 30 min, then filtered and diluted for extraction of vitamin K_3 (menadione). Alternatively, 5 g sample was heated with ethanolic 0.5% ascorbic acid and 50% KOH, followed by extraction of the other vitamins (A, E and D_3) with diethyl ether. The extracts were evaporated and the residue was dissolved in methanol for HPLC analysis. A simpler one-step extraction procedure prior to HPLC analysis for the simultaneous determination of fat-soluble vitamins A, D and E and pro-vitamin D_2 in animal feeds was proposed by Qian and Sheng [40]. The sample was brought into contact with 4 ml acetone–$CHCl_3$ (3:7), vortex-mixed for 1 min and extracted, then centrifuged at 4000 rpm for 5 min. A portion of the organic extract was evaporated to dryness in a stream of N_2, the residue dissolved in 0.3 ml *n*-butanol and injected into the HPLC column. Chen et al. have used microwave irradiation in order to accelerate the extraction of vitamins A, D and E from pre-mixed feeds. Using methanol as extractant and applying microwave at 100 W for 5 min, they achieved efficiencies in the 90–110% range [41].

A method for the automated analysis of vitamin E in butter samples with minimum sample manipulation was proposed by Delgado-Zamarreño et al. [8]. Following dissolution of the samples in a micellar medium (methanol–Triton X-100–water), on-line sample treatment involving alkaline hydrolysis was coupled to a separation and enrichment system in a cell containing a silicone membrane; the analyte-rich phase was finally injected into a liquid chromatographic system controlling the whole system automatically. The experimental setup shown in Fig. 1 consists of two parts: one of them carries out the sample treatment and the other performs separation and detection of the vitamin.

A contribution to the present trend in routine laboratories of replacing time-consuming manual steps, which constitute bottlenecks in the overall analytical procedure, by automated counterparts which are either faster or easier to implement on a 24-h working-day basis, was performed by Gámiz-Gracia et al. [42]. A fully automated robotic method for the determination of vitamins A and E in milk-based products, involving an extraction step based on the AOAC official method of analysis was developed. The whole procedure was carried out automatically in a robotic station coupled to a liquid chromatograph using a sample volume of 5 ml.

3. Supercritical fluid extraction

Supercritical fluid extraction (SFE), particularly using CO_2 as extractant, has proved to be one of the most significant achievement in solid sample pretreatment during the last 2 decades [43]. Extraction with carbon dioxide is considered very suitable for

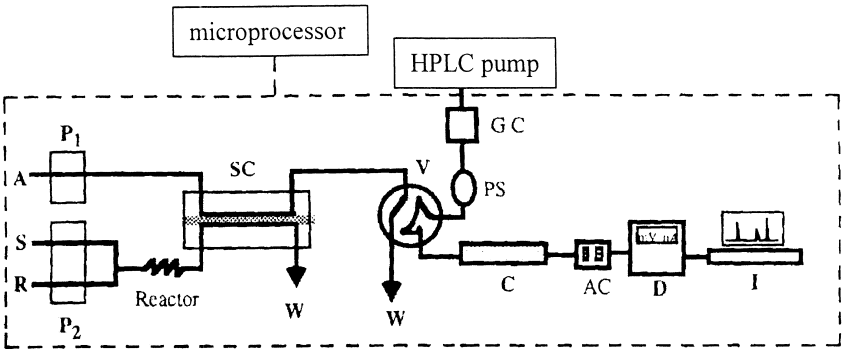

Fig. 1. Diagram of coupled on-line sample treatment–HPLC for the determination of vitamin E. A, Acceptor; S, sample; R, hydrolysis reagent; P_1, piston pump; P_2, peristaltic pump; SC, separation cell; GC, guard cell; PS, pulse suppressor; V, injection valve (20 μl loop); C, HPLC column; AC, analytical cell; D, coulometric detector; I, integrator; W, waste. From Ref. [8].

extracting fat soluble substances from different matrices as supercritical CO_2 has a high dissolving power for non-polar compounds. This technique has been applied for the removal of vitamins from matrices such as food [44–46] and serum [47], but principally for pretreatment in pharmaceutical analysis of samples such as tablets [48,49], creams [50], powdered infant formulas [51] and ointments [52].

The extraction of vitamin E from pharmaceutical formulations has been performed by Salvador et al. [48]. Ground, homogenized tablets or dietetic product (powdered milk) were subjected to SFE in polymer cartridges for 30 min at 400 atm and 60°C using supercritical CO_2 (1 atm=101 325 Pa). Efficiencies within 96.4–104.5% were obtained.

Vitamin A has been removed from liver [53] and cereal products [45] using SFE with static extraction times of 1 and 15 min, respectively, in order to favour the solubility of the vitamin. The detection limit in cereal products was at the ng ml^{-1} level.

Determination of vitamin K in serum by direct-coupled supercritical fluid extraction–supercritical fluid chromatography (SFE–SFC) was performed by Hondo et al. [47]. Phylloquinone, menaquinone and menaphthone in a mixture dried on filter-paper were extracted into supercritical CO_2 and separated on a column of Finepak SIL operated at 40°C with supercritical CO_2 modified with 0.005% of acetonitrile as mobile phase.

The two forms of vitamin D, i.e., D_2 and D_3, were extracted from pharmaceuticals using SFE for the first time by Gámiz-Gracia et al. [54]. The recovery of the extraction was enhanced by direct addition of ethyl ether to the sample contained in the extraction cell. This method was applied to two different kinds of drops, one powder and one granulated, providing recoveries close to 100% in all instances, with precisions, expressed as relative standard deviation, of 3.8 and 6.3% for vitamin D_2 and D_3, respectively, and the quantitation limit of the method was 4.1 μg for both vitamins. The sample size depended on the content of the target analytes in the pharmaceuticals.

Scalia et al. used SFE for the simultaneous extraction of vitamins A and E from two different types of pharmaceutical samples (cosmetic creams and tablet preparations). Cosmetics (0.1–0.15 g), either cream or lotion, were mixed with celite and extracted with supercritical CO_2 using a Dionex SFE-703 system. The extracted analytes were collected in 4 ml THF–methanol (4:1) at 0°C, diluted to 5 ml and analysed by HPLC. The recoveries were >91.6% [50]. Powdered tablets were extracted using the same system in the dynamic mode. Similar samples were extracted using a standard solid–liquid extraction process. The SFE method was more efficient than the standard method; recoveries were >95% [49].

A method for the analysis of the natural contents of vitamins A and E in milk powder based on SFE, a miniaturized alkaline saponification procedure and reversed-phase HPLC was proposed by Turner and Mathiasson [46]. Modifications of the sample matrix, combination of static and dynamic extraction modes and effect of changes in extraction parameters such

as temperature, flow-rate, time, collection solvent and collection temperature were optimized, obtaining recoveries of 99% and 96% for vitamins A and E, respectively. The measurements gave a with a within-day RSD of 4% for both vitamins, and between-day RSDs of 4 and 8% for vitamins A and E, respectively. Another application to the simultaneous extraction of vitamins A and E, in this case from hydrophobic ointment, was developed using an SFE–SFC assembly [52].

Vitamin precursors, such as β-carotene, have also been extracted with supercritical CO_2. One of the most recent contributions in this sense involved continuous monitoring of the extraction of this analyte using pulse thermal lens spectrometry (PTLS), thus reporting for the first time the behaviour of this technique in supercritical fluids [55]. The positive and negative features of PTLS for this monitoring as compared with other optical techniques [56] deserves a more in-depth investigation.

4. Solid-phase extraction

Solid-phase extraction (SPE) has been the most used technique for sample preparation and extract cleaning in recent years. This technique provides methods which are rapid, versatile and selective, and affords good recoveries using smaller amounts of organic solvents than other techniques such as liquid–liquid extraction. Besides the saving of time and solvents, other advantages of SPE are the decrease of contamination problems and the easy automation of the process. In addition to allowing a simplification of the sample matrix treatment, the use of SPE cartridges also enables the analytes of interest to be concentrated [57–59].

The use of SPE has proved to be the most efficient way for increasing clean-up and preconcentration for the determination of vitamins. The marriage between SPE and HPLC constitutes an effective tool for the quantitation of vitamins in clinical and food samples.

The majority of applications using SPE in the clinical field are devoted to the determination of vitamin D and its hydroxy metabolites. Methods for removal either other fat-soluble vitamins such as vitamin K_1 or various vitamins simultaneously, have also been reported [60–86].

A simple, rapid, sensitive, highly selective and reproducible method for the determination of vitamin K_1 was developed by Kao and Heser [61]. The determination of low concentrations of vitamin K_1 (6–10.5 p*M*) in nutritional supplements with a high fat content (the concentration ratio of plant oils to vitamin K_1 was around 25 000:1) was performed by SPE and HPLC with fluorescence detection after reduction on a platinum oxide catalyst.

The separation step based on solid-phase extraction, which has been used since the earliest methods for the quantitation of vitamin D_3 and its metabolites [61–63], underwent a remarkable expansion with the commercialization of liquid–solid extraction cartridges, which virtually substituted the manual column packing. Since 1985, more than 15 papers have appeared dealing with the discontinuous clean-up/preconcentration of vitamins D and its metabolites using different types of solid sorbents. The most common sorbent used is C_{18} [64–70]; however, other sorbents such as silica [71], Florisil [72] and a combination of sorbents (C_{18} and silica) [73,74] are also used, obtaining good efficiencies most times. Nevertheless, the use of a polar sorbent such as aminopropyl silica has proved to be an advantageous alternative to nonpolar sorbents for these analytes [80]. The improvement of extraction of vitamin D_3 hydroxy metabolites with this sorbent as compared with the use of C_{18} yielded the following results: (a) lower detection and quantitation limits (5 and 30 pg ml^{-1} versus 10 and 50 pg ml^{-1}); (b) similar extension of the linear range as a consequence of saturation of the minicolumns; (c) better RSD values (below 5.2% versus 6.4%).

Different manual methods for the simultaneous extraction of other lipid-soluble vitamins have been proposed using C_{18} [75,76] or silica [77] as sorbent.

A miniaturized alternative to the extraction procedure to be used with small sample volumes (in the order of μl), is solid-phase microextraction (SPME). Various SPME fibres [such as polymethylsiloxane (PDMS), PDMS–divinylbenzene and Carbowax–templated resin] and solvents (such as aqueous acetonitrile, methanol and porpan-2-ol) have been investigated for the extraction of vitamins A, D_3 and E [78]. However, apart from this study there is not others that apply SPME for the extraction of fat-soluble vitamins. The method of in-tube SPME

coupled with HPLC developed by Wu et al. [79] using an open tubular capillary coated with polymer as the SPME device instead of the conventional SPME fiber, allows for convenient automation of the extraction process, which not only shortens the total analysis time but also provides better accuracy, precision and sensitivity relative to manual techniques. This method has not been applied for lipid-soluble vitamins extraction and would be an advantageous alternative.

A clean-up/preconcentration–LC method for the determination of vitamin D_3 hydroxymetabolites has been developed by Ortiz-Boyer et al. using the integrated system for continuous clean-up/preconcentration and HPLC separation shown in Fig. 2 [80–82]. The clean-up/preconcentration subsystem permits simultaneous clean-up of the sample and concentration of the target analytes prior to their injection into the separation subsystem. The pre-treated sample was passed through the aminopropyl–silica preconcentration minicolumn inserted in the sample loop of a low-pressure injection, and both the target analytes and interferents with similar features were retained. In order to remove the interferents, the minicolumn was washed after a 20 min preconcentration time by switching valve SV. Then, the analytes were eluted with a methanol solution by switching the high-pressure injection valve of the chromatograph. This valve had been modified by changing the conventional loop for a 100 cm length tubing of 0.25 mm I.D. The volume of methanol containing the eluted analytes was trapped by switching the HPLC injection valve 95 s after switching the low-pressure injection valve and the analytes were thus introduced into the column. The method provides enough sensitivity and precision for the determination of 24,25-$(OH)_2$-D_3 and 25-(OH)-D_3 in human plasma with excellent preconcentration fac-

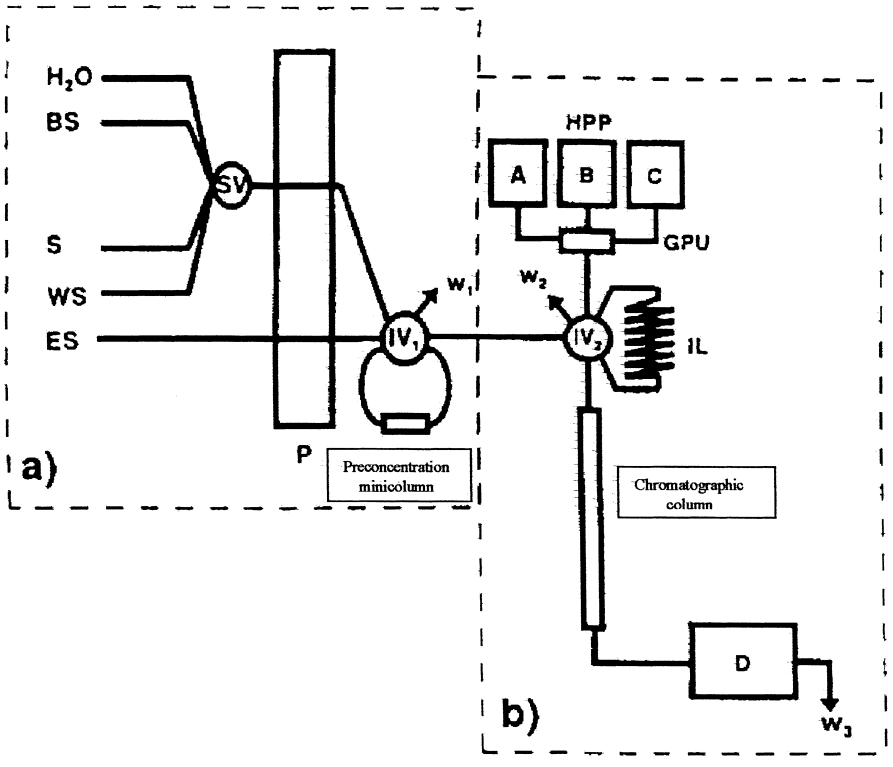

Fig. 2. Integrated continuous assembly for the determination of vitamin D_3 hydroxymetabolites. (a) Clean-up/preconcentration flow subsystem, (b) modular chromatograph for separation and UV detection. P, Peristaltic pump; SV, selecting valve; W, water; B, buffer; S, sample; WS, washing solution; E, eluent; IV_1, low-pressure injection valve; HPP, high-pressure pump; A, B, C, solvent reservoirs; GPU, gradient programmable unit; IV_2, high-pressure injection valve; IL, injection loop; D, detector; W1, W2 and W3, waste. From Ref. [80].

tors, but a sample volume within 5 and 8 ml is mandatory. However, the sensitivity is insufficient for quantitation of 1,25-$(OH)_2$-vitamin D_3 (normal values in plasma <60 pg ml^{-1}).

Improvements of the method for lowering the determination limit have been based on the integration of the two previous subsystems (namely, clean-up/preconcentration using SPE, HPLC separation) with post-column derivatization reactions. One of these reactions was based on the [4+2] Diels–Alder cycloaddition between the triene structure in seco-steroids and 4-phenyl-1,2,4-trioazoline-3,5-dione (PTAD) to form an adduct which absorbs at 337 nm [84]. The on-line derivatization increases the sensitivity about fivefold with respect to that provided by the UV method based on intrinsic absorbance of the hydroxy metabolites [82]. The new method makes possible to quantify 1,25-$(OH)_2$-D_3 at concentrations lower than sub-ng ml^{-1}. A second improved procedure was based on a fluorimetric derivatization [85]. Thus, the sensitivity was increased drastically and for the first time, quantification of 1,25-$(OH)_2$-D_3 at concentrations lower than part per trillion (that is, lower than its usual concentration in human plasma) was possible. Finally, the fluorescence of the derivatization product [86] was induced by a laser in order to enhance the sensitivity as compared with conventional fluorimetry. In this case, a limit of detection of 0.01 pg ml^{-1} was obtained for each analyte, with a linear range over four orders of magnitude and excellent regression coefficients (>0.9922 in all instances).

The proposed methods constitute the basis for both implementation of low-cost routine analyses of vitamin D_3 hydroxy metabolites in hospital and easy, cheap automation of the overall process.

The previous method in its basic version, that is, with measurement of the intrinsic absorbance of the analytes, has been expanded and applied to the determination of vitamins A, D_2, D_3, E, K_1, K_3 and several hydroxy metabolites of vitamin D_3 [83]. The modified procedure required the introduction of several changes in the above described clean-up/preconcentration procedure in order to both improve the retention capacity of other fat soluble vitamins in the microcolumn and facilitate the elution towards the separation subsystem. The manifold was that depicted in Fig. 2 and the procedure was quite similar to that described above, respectively. As can be seen in Fig. 3, the preconcentration step endows the method with enough sensitivity for the determination of vitamins E, D_2, D_3, 24,25-$(OH)_2$-D_3, 25-(OH)-D_3, K_1 and K_3 in human plasma, as their normal levels, even at concentration much lower than the limits at which a deficiency of these vitamins can be determined. In short, the method exhibits linear ranges between 0.005 and 100 ng ml^{-1} for vitamins D_2, D_3 and their hydroxy metabolites; between 0.1 and 100 ng ml^{-1} for vitamins A, K_1 and K_3 and between 1 and 100 ng ml^{-1} for vitamin E; with regression coefficients higher than 0.99 and using sample volumes between 5 and 8 ml.

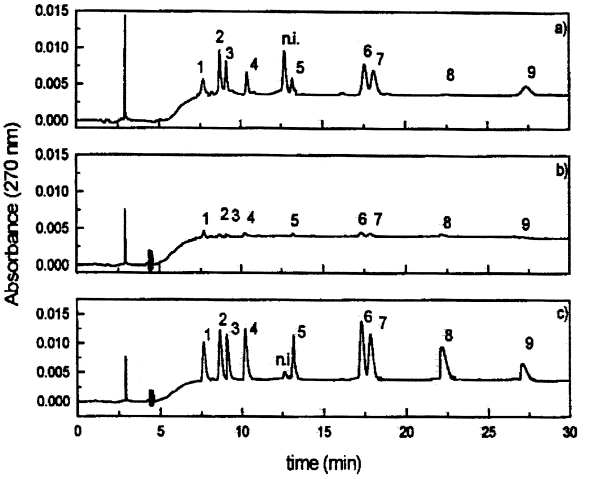

Fig. 3. Chromatograms obtained for (a) 1000 ng ml^{-1} of each analyte without preconcentration step, (b) 100 ng ml^{-1} without preconcentration step, and (c) 100 ng ml^{-1} after preconcentration step. 1, Vitamin K_3; 2, 24,25-$(OH)_2$-vitamin D_3; 3, 1,25-$(OH)_2$-vitamin D_3; 4, 25-(OH)-vitamin D_3; 5, vitamin A; 6, vitamin D_2; 7, vitamin D_3; 8, vitamin E; and 9, vitamin K_1; n.i., not identified. From Ref. [83].

5. Conclusions

Determination of fat-soluble vitamins is an important goal in analytical chemistry due to their importance in human health. An extraction step prior to chromatographic determination is required for removal, clean-up and/or preconcentration of the target analytes. Different approaches have been developed with these purposes, then applied to the

different types of sample. Nowadays, organic solvent extraction, supercritical fluid extraction and solid-phase extraction are the main pretreatment approaches used in this field. The present trends are focused at the automation of the extraction step coupled to determination in order to enhance both the operation time and sensitivity, and to minimise or avoid human manipulation.

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