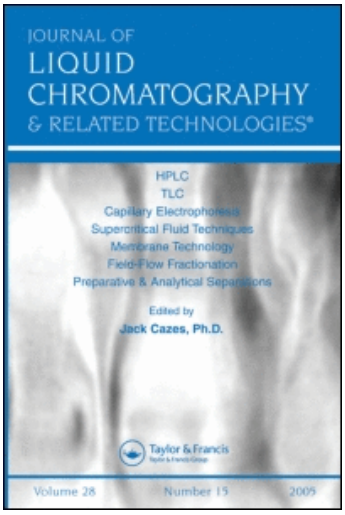

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LACTEAL MATRICES. A SINGLE GUIDE FOR EXTRACTION AND QUANTIFICATION OF FAT-SOLUBLE VITAMINS

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LACTEAL MATRICES. A SINGLE GUIDE FOR EXTRACTION AND QUANTIFICATION OF FAT-SOLUBLE VITAMINS

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ABSTRACT

The present study examines different lacteal matrices (varying in fat content) in order to develop a protocol for extraction and quantification of fat-soluble vitamins. The samples (whole milk, both liquid and powdered; milk with reduced fat content; chocolate milk, and soy-modified milk) were evaluated using various protocols. Organic phase extraction alone succeeded in isolating no more than 20 to 40% of esterified and non-esterified forms of vitamins A and E. A combination of overnight saponification isolated more than 100% of the vitamin content indicated by manufacturers' labels.

All vitamin compounds were separated by reversed-phase HPLC and detected at 265 nm, or alternatively, at the maximum λ of each individual form. The best mobile phase consisted of methanol : water 99 : 1 (v/v) used in all the samples. The following experimental factors were optimized: saponification-KOH concentration, aqueous and alcoholic concentrations, duration

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and pyrogalol concentration; extraction of individual non-polar solvents, and solvent combinations (hexane, diethylether, and light petroleum). In order to confirm the effectiveness of these methods, matrices free of fats and vitamins (zero matrices) were spiked with known quantities of all-trans-retinol, vitamins D2, D3, and α-tocopherol.

Other matrices were also spiked. Recoveries were followed by saponification with KOH methanol (1.9 N) at 30, 21, 42, and 28%. These improved to 104, 42, 85, and 96%, respectively, using KOH methanol (3.8 N). Similar conditions used diethyl-ether : light petroleum 20 : 80 (v/v) as an extraction solvent. The time required for exhaustive saponification was 3 h, since the pyrogallol content was increased from 0.1 to 1% w/v. The new extraction protocols show advantages relative to established methods for vitamins A, D, and E. This protocol could also be used for other food matrices.

INTRODUCTION

Lacteal matrices consist of solid and fluid milk-born samples in food, the solid ones being chocolate milk, infant formulas, and whole milk powder, the liquid ones being whole milk, chocolate milk, and reduced fat milk. The fat content of these foods may vary from 26% to 1% (or below) in reduced-fat products. There are several analytical techniques to evaluate fat-soluble vitamins. They involve colorimetric reactions (1,2), gas–liquid chromatography, (1–4) and recently, high performance liquid chromatography (HPLC), mainly reversed-phase (1,2,5–10) through conventional columns octadecylsilane (C_{18}), differing in end-capping level, length, internal diameter, particle size, and manufacturer (5,8–14). Recently, other techniques have been mentioned involving polymeric phase octylsilane C_8 compared to C_{18} through different mobile phases (15). Meanwhile, narrow bore columns of 2.1 mm internal diameter (i.d.), with 3.2 μm particle size have demonstrated greater efficiency than conventional columns, such as 4.8 mm (i.d.) with particle sizes of 5 or 10 μm (15–17).

The most suitable analytical techniques with respect to sensitivity and linearity combine fluorescence detection with normal phase (18–22) or alternatively, reversed phase (15–19), mainly for vitamins A and E (non-esterified and esterified forms), while vitamin D does not fluoresce (8,13,14,16,17). Simultaneous analyses are usually conducted using UV detection when there is a preponderance of vitamins A and E in the presence of vitamin D (5–7,9,10,15–18).

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The main advantage of the new column materials (small particles and narrow columns) is that they permit analyses of various chemical formulations, which assume that fat-soluble vitamins function in the presence of isomers and other compounds of minor interest. Through this technique, it is possible to estimate real vitamin value in different matrices (foods and feeds) using proper bioequivalent values that enable the estimation of the effects of processing and storage on vitamin-containing compounds (20,22–24).

The treatment of samples is laborious and expensive, mainly because of the high fat content and related compounds (cholesterol, steroids, and precursors) that are intrinsically crosslinked. The purification of esterified vitamins A and E through HPLC can be achieved by liquid–liquid extraction, mainly when fat content is below 3% (7,17), since cleanup columns have been used in conjunction (15,25–27). Moreover, a cleanup column becomes necessary when supercritical fluid is recommended for analysis of vitamins A and E following a saponification assay (25). Alternatively, lipase treatment (6) combined with *n*-pentane extraction also produces similar effects. In a recent experiment, no response was registered in several immobilized enzymes with respect to alpha-tocopheryl acetate, indicating that a single procedure for vitamins A and E quantification is problematic (28). HPLC-grade hexane is also suitable for extracting fat-soluble vitamins from parenteral nutritional products (17).

Various saponification conditions are proposed: aqueous medium (5,29,30), aqueous-ethanol (9,11–13,19), ethanol (10), or alternatively, methanol alone (8). Some conditions require the use of a hot plate (5,10,19), others are processed overnight (8,9,11–13). The non-saponificable extracts can be extracted by various solvents, whilst HPLC-grade hexane alone is recommended by the official protocol for vitamins A and E (30) after hot aqueous saponification (KOH-67% aqueous) under a stream of nitrogen. Extraction of vitamin D is excessively detrimental to analytical techniques (24,30), it being suggested that a combination of the two columns (8,11–14), i.e., one clean-up, one analytical, following hot (5,10) or overnight saponification (8,9,11–14) be utilized. Extractions with solvents can utilize light petroleum (8) or certain combinations (10–12,20) of solvents. Vitamins K and D are extremely difficult to extract (6,8,16,17,23,24). A saponification step is mandatory for the previous assay to introduce lacteal matrices into HPLC, since it has also been used in conjunction with the supercritical fluid technique (25).

Identification and detection are very difficult because of the great number of compounds related to fat-soluble vitamins and others that absorb in the UV/visible region at various wavelengths (λ). Therefore, multichannel detection is always required, through λ programming (5,6,9,10,22), or alternatively, selection of a unique λ (26,27,31,32). In fact, fortified matrices containing vitamins A and D in the ratio 300 : 1 (w/w) and vitamins E and D 3000 : 1 (w/w) pose great

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difficulties in the application of simultaneous methods of extraction and detection.

This article has pointed out and optimized some chromatographic conditions for the separation and detection of free (alcoholic or non-alcoholic) and esterified forms of fat-soluble vitamins. It has also presented a single and suitable protocol for the extraction and quantification of fat-soluble vitamins in various lacteal matrices.

EXPERIMENTAL

Apparatus and Conditions

The experiments were carried out using a HPLC system equipped with a solvent delivery system (Waters); auto sampler injector (Waters 717); multi-wavelength spectrophotometer UV-Vis (Waters 490), and a Maxima workstation with two channel options, in which λ max or other λ can be selected, in order to obtain quantification conditions. Programmable λ were obtained through a HP 1050, dual λ chromatographic system, in which some conditions of detection were adjusted to vitamins A and E. In this case, a Rheodyne injector (20 μL) was always used.

The conventional analytical column employed was a SPHERISORB ODS-2 (250 × 4.6 mm i.d.; 5 μm), acquired from Sigma Aldrich (Z226068). A ODS (from Supelco) guard column was used to protect the analytical column. The mobile phase was pumped at a flow rate varying from 1.2 to 1.4 $mL \cdot min^{-1}$ in the isocratic mode to adjust for the best k' (capacity factor) and α' (separation factor) for the chemical compounds described. Before use, the mobile phase was vacuum-filtered through a 0.45 μm nylon filter (FHLP01300) and degassed by ultrasonic bath over the required period. The chromatographic experiments were carried out at room temperature ($20 \pm 1^{\circ}C$).

Reagents and Samples

Methanol, HPLC grade, was employed as supplied by the manufacturer (Mallinckrodt, USA). Ultra-pure water was obtained through a Millipore Milli-Q Plus 18.2 MΩ system (Milford, MA, USA). Analytical grade trans-retinol (70% pure), colecalcipherol (99% pure), ergocalcipherol (99% pure), α-tocopherol (95% pure), and phyloquinone (99% pure) standards were supplied by Sigma Co., St Louis. Pyrogallol was purchased from Carlo Erba. Potassium hydroxide, ethanol, methanol, hexane, diethylether, light petroleum, analytical grade, were purchased from Vetec (Brazil).

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Individual standard solutions of the vitamins were prepared in ethanol, HPLC grade, to provide a concentration from 10 to 500 $\mu g \cdot mL^{-1}$ for trans-retinol; 0.3 to 12 $\mu g \cdot mL^{-1}$ for D2 and D3, 35 to 2800 $\mu g \cdot mL^{-1}$ for α-tocopherol, 0.72 and 72.50 $\mu g \cdot mL^{-1}$ for K1. These solutions were degassed using an ultrasonic bath and were stored in dark glass flasks at $-18^{\circ}C$. All samples were injected in triplicate within two days of preparation.

Individual standards, or mixture of standards (pools), were prepared by appropriate dilution of standard solution and filtered through a 0.45 μm fluorpore membrane (FHLP01300) before being injected into the chromatographic system. Similar lots (two bottles) of each of the matrices were well mixed before the sampling procedure. Sample analysis was run from 24 to 72 h after preparation of the extract.

The lacteal matrices investigated are described as S (solid matrix) and F (fluid matrix):

[S1] Zero solid matrix without fat-soluble vitamins and 0.5% max fat content;
[S2] Chocolate powder with fat-soluble vitamins and 3.5% max fat content;
[S3] Modified milk with fat-soluble vitamins and 12% max fat content;
[S4] Milk powder with fat-soluble vitamins and 26% max fat content;
[F1] Zero fluid matrix without fat-soluble vitamins and 0.5% max fat content;
[F2] Defatted fluid matrix with fat-soluble vitamins and 0.5% max fat content;
[F3] Integral fluid matrix with fat soluble vitamins and 3% min fat content.

Chromatographic Conditions

Prior spectrophotometric assays were conducted to obtain the ratio of max λ or unique λ for each vitamin, mainly A (325 nm : 265 nm) and E (292 nm : 265 nm), at known concentrations, in order to obtain vitamin quantification and indirectly evaluate peak purity. Previous experiments were carried out to obtain the linearity range under certain detection conditions. Different polarities in the mobile phase were used to improve separations for D2 and D3, mainly analyzing k' and α, equally applied to esterified forms of vitamins A and E. A typical chromatogram was obtained for the standard mixture that was evaluated directly by liquid–liquid extraction and saponification, which were designed for various methods of extraction. The last quantitative and recovery assays were conducted using a simple versus extracted curve at 10, 20, 40, 80, and 160 $\mu g \cdot mL^{-1}$ (trans-retinol)and 50, 100, 200, 400, and 800 $\mu g \cdot mL^{-1}$ (α-tocopherol). Vitamins D and K were not quantified through this procedure (λ

program) because of the high ratios of vitamins A to D and E to D. Previous conditions of quantification were necessary in order to evaluate different sample treatments on solid and fluid matrices.

Sample Preparation

Saponification assays were conducted with KOH-methanol, ethanol, or aqueous ethanol at various concentrations (from 1.0 to 7.2 N) and different extraction solvents (diethylether and light petroleum, hexane or light petroleum alone). Overnight saponification was compared to hot plate saponification and the best combination for solvent extraction was determined. The non-saponificable extracts were concentrated in a rotary evaporator and re-dissolved in spectroscopic grade ethanol before being injected. The extracts were maintained at $-18^{\circ}C$ for no more than 24 h to 72 h after being filtered through a 0.45 μm fluorophore membrane (FHLP01300).

Calibration and Recovery Assays

A linear range was obtained at various standard concentrations. Precision and accuracy assays were carried out on standards and samples (matrices) in order to recommend a suggested protocol. A precision assay was performed on milk powder with a 26% fat content and fluid milk (3.5% fat content), whilst accuracy was determined through standard recoveries at two levels of spiking, using a zero solid matrix (S1) and zero fluid matrix (F1), both fat-free and vitamin-free. The recovery rate was also obtained through other matrices. Finally, extractable standards (trans-retinol and α-tocopherol) were used to confirm the suggested protocol.

RESULTS AND DISCUSSION

Chromatographic Conditions

This study evaluated combinations of mobile phases (binary and ternary systems), in order to determine the best combinations. The best performance was methanol : water (99 : 1 to 95 : 5) which lead to its recommendation as being sufficient for separating vitamins D2 and D3 using a conventional column. Conversely, monohydroxylated D2 or D3 can be selectively isolated with phosphate buffers in methanol, when the identification of bone disease is necessary (32). Other mobile phases are presented in Table 1. Various

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combinations permit similar results. Non-esterified and esterified forms of vitamins A and E were eluted with methanol alone. This mobile phase was recommended (17) when vitamins D and K were included in special analyses of nutritional parenterals. Binary and ternary systems or other polar solvents, such

Table 1. Selectivity Index from Different Mobile Phases

Mobile Phase Composition	k_1 trans-Retinol	k_2 Retinol Acetate	α k_2/k_1	k_3 D2	k_4 D3	α k_4/k_3
Methanol : water						
99 : 1	0.80	1.28	1.60	2.52	2.73	1.08
98 : 2	0.87	1.43	1.64	3.07	3.32	1.08
95 : 5	1.37	2.54	1.85	5.56	6.00	1.08
Methanol : acetonitrile						
95 : 5	0.71	1.14	1.63	Nd	Nd	
90 : 10	Nd	Nd		2.46	2.66	1.08
80 : 20	1.01	1.71	1.69	2.67	2.88	1.08
70 : 30	Nd	Nd		3.10	3.35	1.08
60 : 40	Nd	Nd		3.64	3.94	1.08
50 : 50	Nd	Nd		3.77	4.05	1.07
40 : 60	Nd	Nd		4.02	4.34	1.08
30 : 70	Nd	Nd		5.04	5.82	1.15
Methanol : acetonitrile : water						
95 : 2 : 3	Nd	Nd		2.93	3.23	1.10
90 : 5 : 5	Nd	Nd		4.69	5.13	1.09
90 : 8 : 2	Nd	Nd		2.60	2.96	1.13
90 : 3 : 7	Nd	Nd		9.09	9.72	1.07
80 : 15 : 5	Nd	Nd		9.18	9.86	1.07
70 : 25 : 5	Nd	Nd		8.61	9.22	1.07
Methanol : water : ethylacetate						
97 : 2 : 1	Nd	Nd		4.38	4.59	1.04
88 : 2 : 10	Nd	Nd		2.77	2.98	1.07
Methanol : water : ethanol						
97 : 2 : 1	Nd	Nd		4.45	4.83	1.08
95 : 2 : 3	Nd	Nd		3.41	3.71	1.08
Methanol : water : isopropanol						
97 : 2 : 1	Nd	Nd		3.46	3.71	1.08

Nd = not determined.
k taken in relation to solvent deflection point and α was measured from k_2/k_1 and k_4/k_3, respectively.

as water and ethanol did not show satisfactory k' and α. In some cases, interferences were removed. Isocratic elution is better than gradient, but simultaneous separation can be performed using methanol:acetonitrile:water systems (9,10,16,22).

The combination methanol : acetonitrile (95 : 5 to 80 : 20 v/v) was probably similar to methanol : water (95 : 5) when k' and α were compared (Table 1). Other mobile phases evaluated increased k' without improving performance (α). Conversely, ternary and binary systems separated cis- and trans-isomers of vitamin A, mainly using aqueous and ethanol polar solvents (25,31). Other isomers and interfering compounds were handled under different conditions of sample preparation without sufficient identification. Up to now, fewer chromatograms of different samples have been discernable. Therefore, an organic polar mode offers a good explanation of this separation (25,27,32,34–36).

In order to detect and quantify fat-soluble vitamins, various curves were performed at λ max for A, D, E, and K; other detection systems were also tried, such as 290, 280, and 265 nm. A linear response was observed in these λ ($R^2 \geq 0.95$), but at 265 nm it was fixed for optimizing certain extraction conditions in both solid and fluid matrices. For comparison, a similar quantification was carried out using programmable λ for vitamins A (325 nm) and E (292 nm).

UV detection is recommended when adjusted to the max λ (best filter) of each vitamin compound, enabling the quantification of various compounds in the same run. Interfering compounds may, therefore, result in low recovery rates. Obviously, other factors inherent to the methodology can offer appropriate conditions. On the other hand, vitamin D absorbs at 265 nm but vitamin A absorbs at 325 nm and vitamin E at 292 nm. Shifting λ may result in baseline drift and, consequently, unsatisfactory chromatograms.

A spectrophotometric assay was also carried out in order to obtain a factor capable of predicting the purity parameter, since a diode array detector was not available. The relationship obtained from trans-retinol ($\sim 7.25\,\mu g \cdot mL^{-1}$) gave 1.333 units of absorption (UA) at 325 nm and 0.161 UA at 265 nm; the 325 nm : 265 nm absorption ratio being used to compare and standardize the effects of the matrices (ideally above 8.2). A similar procedure was designed for α-tocopherol ($\sim 132\,\mu g \cdot mL^{-1}$), whose 292 nm : 265 nm absorption ratio was ~ 5.75. These parameters were taken as a reference to measure the peak of each vitamin (A and E, respectively). Table 2 summarizes these results from fluid matrices.

The quantitative curves for trans-retinol and α-tocopherol and other vitamins were thus determined. The individual procedure can estimate samples and adjust parameters to extraction conditions (λ programmable for vitamins A and E or λ fixed at 265 nm for all fat-soluble vitamins), whilst the extractable

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Table 2. Ratio Absorbance λ Max of Each Vitamin and Other λ

	N	trans-Retinol 325:265 nm	α-Tocopherol 292:265 nm
Standards through spectrophotometric conditions	07	8.27 ± 0.08* [8.18–8.42]	5.72 ± 0.06* [5.62–5.78]
Standards through HPLC conditions	16	9.66 ± 0.66* [9.24–11.43]	7.86 ± 0.57* [6.36–8.62]
Fluid samples evaluated through HPLC conditions	13	7.70 ± 0.45* [7.30–8.90]	6.28 ± 0.69* [4.95–7.92]

*Mean ± standard deviation.
[ ] Minimum and maximum.
N = number of repetition.

procedure was reliable only at three points for vitamins A and E. It is probable that the internal standard will correct this problem.

An automatic method for simultaneous determination of vitamins A, D, and E (280 nm) in milk powder or fluid (27,31,32,34) has recently been developed. Through simultaneous determination of retinoids, tocopherols, and carotenoids, elution can be monitored through two channels: max λ or unique λ (280 nm). Visible detection at 436 nm enabled quantification of seven pro- and non-vitamin carotenoids in milks and similar matrices (33,34). λ programs for retinoids and tocopherols are most suitable when vitamin D is present (5,6,9,10,21,26). Chromophore fluorescence (fluorophore) is a sensitive and practical method for retinoids and tocopherols, but vitamin D does not fluoresce, therefore, only UV detection has shown itself to be suitable (21,26). A quantification procedure was developed in order to define better extraction methods, such as power extraction by alkaline alcoholic medium and other treatments through fixed λ and programmable λ (Table 3).

Extraction Conditions

Only organic solvents (polar plus non-polar or non-polar directly) have shown insufficient and non-exhaustive protocols for extraction of fat-soluble vitamins, principally when the matrix has a fat content of 3% or higher. Therefore, exhaustive extraction in solid and fluid matrices, such as new milk products which incorporate dry serum milk, soy milk (high in steroids and vitamin D precursors), corn oil (high in steroid vitamins and non-vitamin compounds), and other ingredients was not possible. Vitamin formulas and

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Table 3. Adjustable Parameters Linear Regression to Quantify Some Fat Soluble Vitamins

Analytical Standards $\lambda = 265$ nm	Response Range ($\mu g \cdot mL^{-1}$)	A (Y Intercept)	B (Angular Constant)	R^2
All-trans-Retinol	0.72–580	0.3471	0.08108	99.96
D2-ergocalcipherol	0.69–11.04	−0.0242	0.3490	99.88
D3-colecalcipherol	0.81–12.98	−0.01142	0.3778	99.97
α-Tocopherol	1.32–6600	0.3052	0.00858	99.88
K1 (phyloquinona)	0.72–72.50	−12.24	9.1422	99.68
λ Programmable: Punctual curve				
All-trans-Retinol	10.75–166.00	−653.25	176.09	99.60
α-Tocopherol	50–807	−43.09	0.1011	99.88
λ Programmable: Extractable curve				
All trans-Retinol	10.75–166.00	−8.25	99.52	99.06
α-Tocopherol	50–807	−7.49	3.39	99.25

All points were injected from three to five repetitions (at least were performed two assay).

fortified products can include particles and substances in order to encapsulate the vitamins (through gelatin and cyclodextrins) because of their sensibility to some processing agents, such as temperature and oxygen (23,26). Meanwhile, this practice of direct extraction is recommended for some fat-soluble vitamins (principally esterified forms), present in pharmaceutical capsules (15) and enteral and parenteral formulas (17).

Probably, if there had been accuracy in isolating fat in these matrices, there would also be an effective way for extracting fat-soluble vitamins without saponification, principally when interfering compounds, such as neutral and acid lipids (35), cholesterol, and steroids (8,11,12,25,31,35) are minimal. On the other hand, fat-soluble vitamins can be sensitive to any saponification condition, official protocols, or non-official methods suggesting N_2 influx and pyrogalol, in order to control lipid oxidation products (25,30,31,36–39). In our study, it was necessary to adjust the pyrogallol content to improve extraction, without using N_2, because of its high cost.

KOH Concentration in Fat-Soluble Vitamin Extraction

KOH-aqueous (67% w/v) is an official method (30) for isolated vitamin A and E, but KOH-ethanol-water (11,12,18,19) through hot plate saponification is equally recommended for different matrices. From 30 to 60 minutes of exhaustive saponification are usually recommended when samples have a fat content of

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above 3%. Care should be taken when recommending hot saponification for extracting fat-soluble vitamins because of isomeric compounds, principally those obtained from vitamin A (trans-retinol), D2, and D3 (1,2,13,14,26,31).

On the other hand, although overnight KOH methanol (35% w/v) is only recommended for extraction of vitamin D2 and D3 (8), it appeared as a problem when applied in this study; all matrices described in this paper showing additional peaks, probably vitamins A and E (evaluated through the k' obtained). Other studies of overnight saponification have been described but poorly defined, since no differences in matrix chromatograms, mainly using KOH-ethanol (10), or alternatively KOH-ethanol-aqueous (11,12,18,19), were shown.

Previous studies have enabled the adjustment of these parameters: hot saponification has shown itself to be unsatisfactory in some matrices; KOH-ethanol or KOH-ethanol-water has proved ineffective in extracting vitamins D and E when combined with extractions using different solvents. While KOH-aqueous is recommended by official methods for vitamins A and E (31,37–39), its use is questionable because dipolar moments and redox potential on the aqueous oil surface are maximized, this probably being the principal cause for the dependence of saponification conditions on N_2. Figs. 1 (solid matrix) and 2 (fluid matrix) compare different concentrations (KOH–methanol) and can optimize the extraction of vitamins A, D, and E from solid and fluid matrices, respectively. These experiments suggest a higher sensibility of A (trans-retinol) and E (α-tocopherol) vitamins to alkali conditions (above KOH 20%), both peaks being reduced to around half the maximum extractability when submitted to KOH 40% ($\sim$7.1 N). On the other hand, when these matrices were submitted to KOH 5% ($\sim$1.0 N), non-exhaustive extraction was obtained.

Better and more suitable extraction solvents were obtained after varying diethylether from 0% to 40% in light petroleum, probable steroid extraction being obtained in this study, whilst other chromatographic conditions, such as mass-capillary-HPLC would confirm this hypothesis (36–39). Therefore, from 20 and 10% diethylether (lowest peroxides than 1 : 1 v/v) was recommended, and showed sufficient combination for matrices in which minimal quantities of these interfering compounds are found, such as milk powder and fluid milk (high recovery). Conversely, infant formulas and chocolate milk were sometimes poorly quantified. In contrast to the official method, only pure chromatographic grade hexane (30), or alternatively, diethylether light petroleum (1 : 1) are usually recommended for simultaneous determination (10). Our results are in agreement with solvent extraction combined with KOH-ethanol-aqueous saponification for vitamin D (11,12).

A fluorimetric detector, when attached to a reversed phase system, is a good practice for these compounds in infant formulas, chocolate milk, or modified milk (high steroids) on which simultaneous extraction can be performed. Inadequate extraction of vitamin D associated with low resolution of C_{18} using conventional

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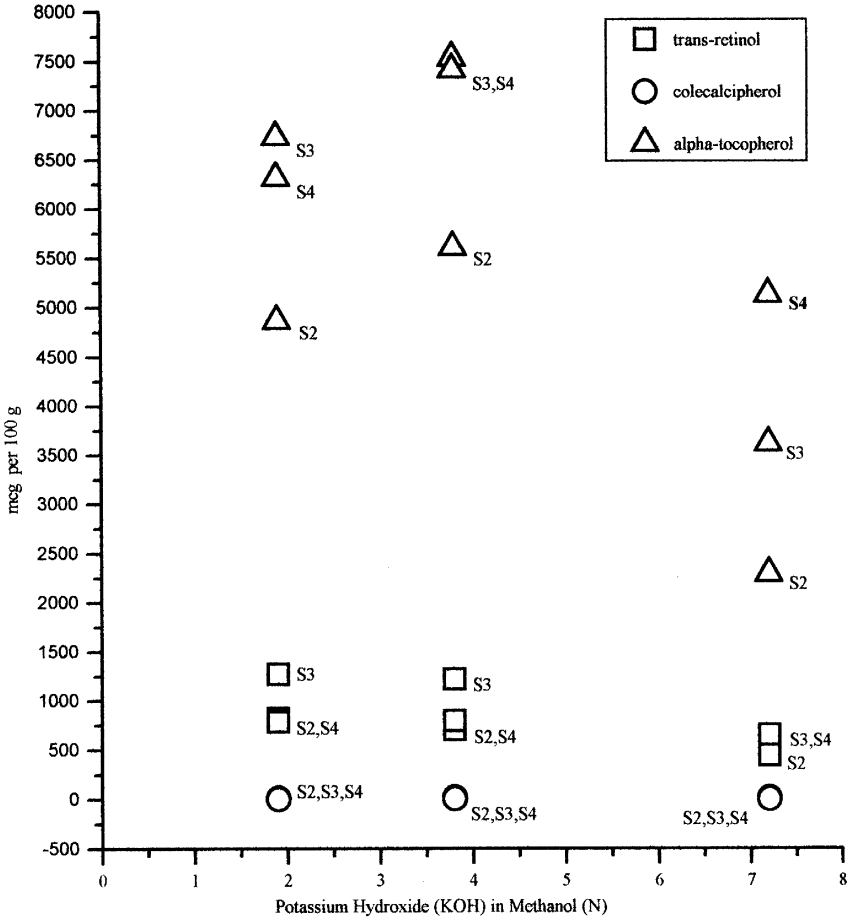

Figure 1. Extraction power related to KOH concentration in solid matrix. All points were measured in triplicate for each matrix.

columns (250 × 4.6 mm, 5 μm) can jeopardize this technique. Better results and a complete validation procedure can be obtained using a capillary column adjusted to the quantification of vitamin D in the presence of β,δ,γ-tocopherols and other steroids (11,21,25,29,37–39). Official methods for vitamin D are very expensive and laborious, cleanup columns and proper analytical columns being recommended (13–15,24). This subject is appropriate to a similar mobile phase (only methanol) used in a narrow-bore column (100 mm × i.d. 2.1 mm, ϕ 3.0 μm), with which a suitable range of 2.5 min and similar k were obtained

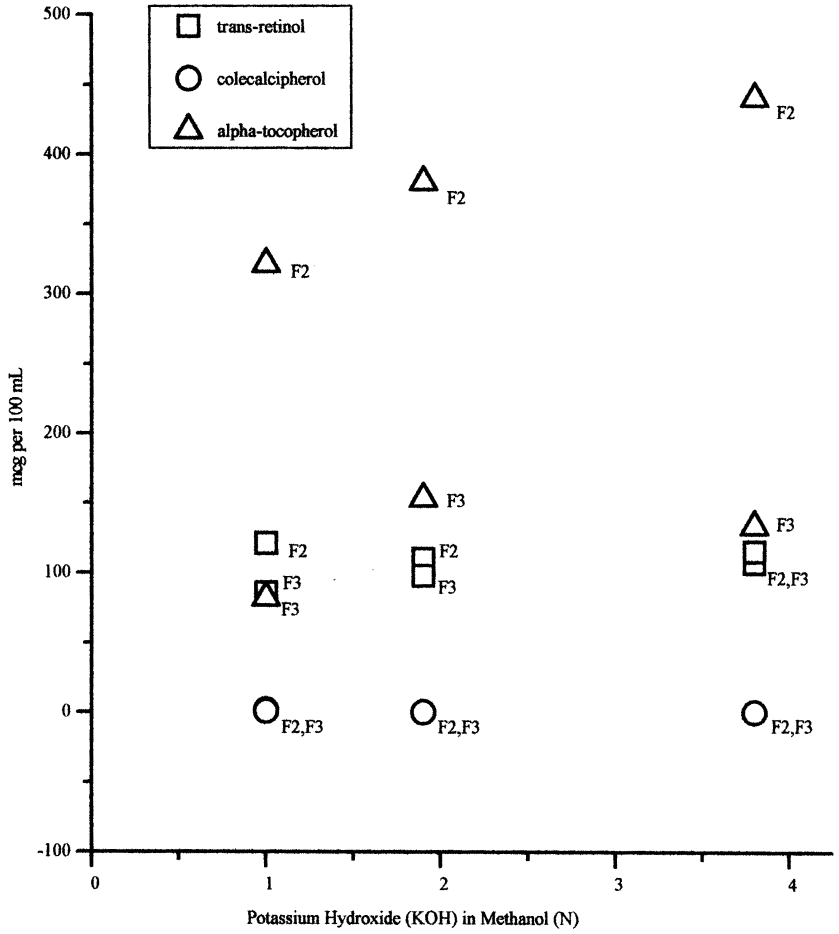

Figure 2. Extraction power related to KOH concentration in fluid matrix. All points were measured in triplicate for each matrix.

when vitamin D was quantified in the presence of trans-retinol and α-tocopherol in parenteral formulas (17).

Intrinsic crosslinkage related to fluid or solid matrices may be distinguished between proper saponification, 10% (1.9 N) and 20% (3.8 N) KOH-methanol, respectively. Solid matrices may show interferences in compounds when other λ are input instead of max λ because of a higher weight : weight ratio for fortified foods, mainly vitamins D : E or A : D. Phospholipids, triglycerides, and other neutral and acid lipids are intrinsically related to the effects of the matrices (35).

In order to obtain partial validation, parameters were spiked with known quantities of vitamin A, D2, D3, and E, particularly in the zero matrix (free of vitamins and almost fat-free). The results shown in Table 4 are indicative of adequate conditions for the removal of micellar forms, such as aggregate-polysaccharides, gelatin, or cyclodextrin used in combination with

Table 4. Recovery Rate (%) in Solid and Fluid Lacteal Matrices

	All-trans-Retinol	Ergocalci-pherol	Colecalci-pherol	α-Tocopherol
Quantify by λ Fixed at 265 nm				
KOH-methanol (1.9 N)				
Level 1	28 (11) a	28 (10) a	44 (22) a	24 (8) a
Level 2	31 (0.3) a	14 (3) a	41 (9) a	32 (11) a
KOH-methanol (3.8 N)				
Level 1	113 (4.8) b	40 (10) a	87 (1.4) b	91 (8) b
Level 2	95 (10) b	45 (19) a	83 (4.3) b	95 (4.2) b
Quantified Through λ Programmed				
KOH-methanol (1.9 N)				
F1	67 (4) b^1			77 (5) b^1
	64 (5) b^2			76 (8) b^2
F2	75 (5.5) b^1			97 (2) b^1
	69 (6.0) b^2			92 (3) b^2
F3	58 (6) b^1			89 (3) b^1
	65 (6) b^2			97 (2) b^2
KOH-methanol (3.8 N)				
F1	28 (4) a^1			49 (5) b^1
	59 (6.5) b^2			81 (3) b^2
F2	55 (4) b^1			77 (6) b^1
	60 (4) b^2			91 (2) b^2
F3	73 (4) b^1			97 (9) b^1
	68 (4) b^1			100.2 (3) b^2

*Assay was conducted in triplicate, means ± relative standard deviation ( ).

λ fixed at 265 nm

Fixed extraction solvents = 20% diethylether in light petroleum.

Similar distinctive weren't statistical different Tukey ($P \leq 0.01$).

Level 1 = added 100, 1.80, 1.44 and 700 $\mu g \cdot mL^{-1}$ for trans-retinol, D2, D3 and α-tocopherol.

Level 2 = added 250, 3.60, 2.88 and 1400 $\mu g \cdot mL^{-1}$ for trans-retinol, D2, D3 and α-tocopherol.

λ Programmed

[1] Extraction solvent (5% diethylether in light petroleum p.a.).

[2] Extraction solvent (20% diethylether in light petroleum p.a.).

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non-polar compounds (target analyte) for enrichment of lacteal matrices. On the other hand, when KOH 5% ($\sim$1 N) was used, it was possible to detect acetate-trans-retinol, suggesting low efficiency saponification in lipid fractions and related compounds.

Matrices varying in fat content were also evaluated through recovery rates. In fact, some interaction of these matrices was obtained, recoveries below 80% being registered particularly for vitamins A and E, the most important compounds that may quench partially towards lipid oxidation products. Meanwhile, vitamins D3 may be spiked around 200%, it being suggested that chocolate powder (S2), modified (S3), and soy milk, may detect other compounds, such as steroids and other tocopherols (mainly β,δ,γ,ζ-toc) through fixed or programmed λ.

Comparative experiments were carried out using fluid matrices in KOH (1.9 versus 3.8 N), associated with the best extraction solvents, since max λ was used. Table 4 summarizes the principal effects on the zero matrix. The most sensitive (neither sufficiently extractable nor recovered) was D2, below 40% in both imposed conditions. Some entrapments were obtained using KOH 1.9 N, probably resulting in lower recoveries. In contrast, D3 recoveries were around 90%, the value expected for accuracy studies. This is supposedly a good factor that imposed N_2 influx on extraction of vitamin D, whilst from 89 to 113% of alcoholic forms of vitamins A and E were recovered. Better recovery rates may be obtained using a capillary column and, therefore, a complete validation procedure, including tests for ruggedness, reproducibility, and others (24,34,36–39).

The practical way for the quantification of vitamins A and E is to use unique λ (265 nm) instead of wavelength program when the ratios of vitamins E : D and A : D were sufficiently large. In fact, it must be pointed out that in the case of D3 and other compounds (below 1 μg), the recovery rate was critical but acceptable. Through recovery rates, it was possible to fix the ratio between solid and fluid sample and saponification solution. Therefore, when fluid matrices are put through this protocol, they should rigorously obey the ratio X : 2X in w/v or v/v, whilst solid matrices should be adjusted to X : 10X (because low dissolution takes place below 1 : 9). Extraction solvent volume was fixed from saponification solution (always obeying the ratio 1 : 4) in two phases of liquid–liquid partition for exhaustive extraction. Recovery rates from fluid matrices were fixed using a calibration curve based on two levels: 40 and 200 $\mu g \cdot mL^{-1}$ of trans-retinol and α-tocopherol, or alternatively 80 and 400 $\mu g \cdot mL^{-1}$, respectively.

Pyrogallol Effect on the Quantification of Vitamins A and E

The effect of butylhydroxytoluene (BHT) on fat-soluble extraction was recently demonstrated through chromatography grade hexane (17) using parenteral formulas, whilst pyrogallol is the antioxidant recommended by official

methods (30), usually added simultaneously to the saponification step. The effect was magnified on vitamin contents of 20 to 40% when chromatograms are compared and trans-retinol, D2, D3, α-tocopherol, and phylomenadianon are quantified in the presence of BHT (17). In this study, similar effects were observed with quantified trans-retinol and α-tocopherol, since vitamin D quantification problems were the last to be pointed out and vitamin K was unable to be quantified because of some destructive effects appearing, as suggested in the direct extraction of parenteral formulas (17).

Initial interest was directed towards the adjustment of pyrogallol content in relation to the solvent extraction (w/v) or saponification solution (w/v) step. In general, concentrations of around 0.1% w/v were sufficient when overnight saponification (from 12 to 18 h) was recommended, except with fat-free fluid matrices. On the other hand, matrices with a high lipid content, such as whole milk, may demonstrate a lower extraction rate (from 20 to 80%) in the absence of this antioxidant. This fact suggests a rough interaction of pyrogallol with the fat content of matrices in the presence of other lipids compounds, well documented in biological and food samples (40,41).

The best performance of the method when the pyrogallol content was increased to 1% w/v was also investigated. Therefore, it was possible to reduce the required time for exhaustive saponification. Overnight saponification was too long because of the oxidation products. From the initial step, lipid oxidation products were maximized with 3 h saponifications, which therefore, required high levels of pyrogallol. Fig. 3 summarizes these effects.

Precision and accuracy tests (Table 5) were carried out on these matrices, with low and high levels of fat content, their day-to-day variability demonstrating good practice, since other effects, mainly on vitamins A and E extraction, were minimized.

Sampling Technique for Fluid Matrices

No published results have yet indicated the best way of sample sizing. Similar batches were pooled to get 1000 mL or 2000 mL and were evaluated according to whether they had been pre-heated to 40°C (at least 30 min) or not. When a pool of 2000 mL was heated, there was a minimum intrinsic variability of below 5% that is in agreement with vitamin content determined by HPLC. A high variability (around 30%) was observed when the sample size was below 2000 mL and the fluid matrix was not pre-heated. Therefore, a similar procedure for sampling (two bottles of 454 g) was followed in precision and accuracy studies performed on solid and fluid matrices (Table 5). This procedure is advantageous when compared to official methods for vitamins A and E, which are deficient in

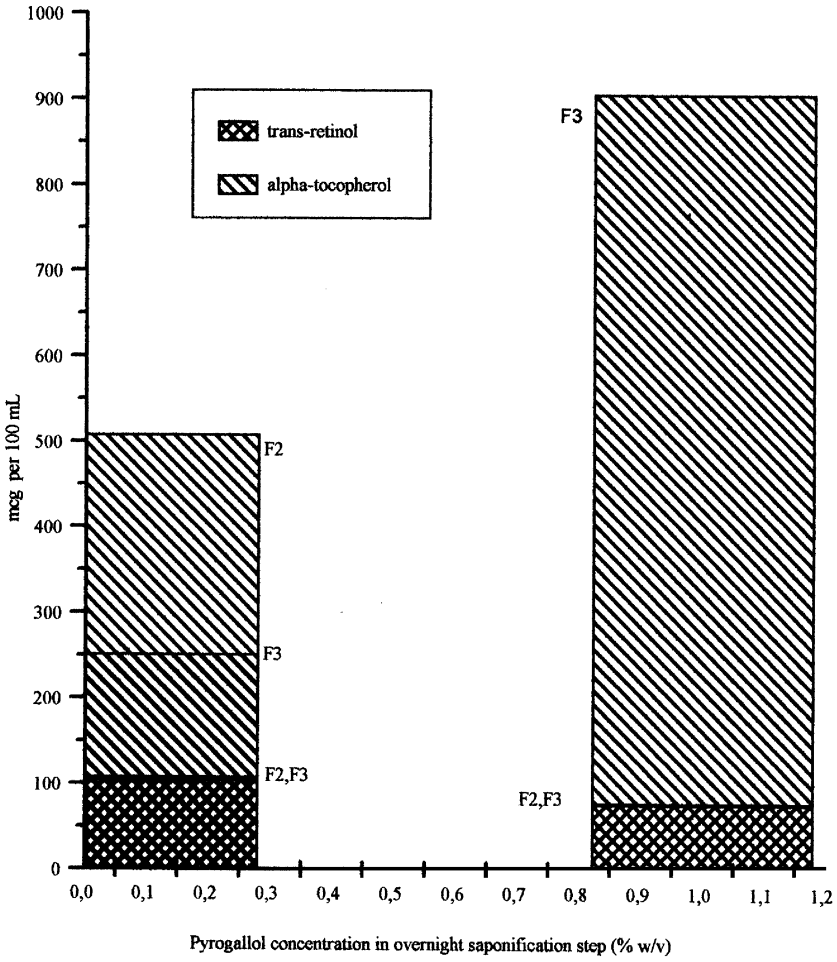

Figure 3. Influence of pyrogallol concentration on vitamin A and E extractions. All points were measured in triplicate for liquid matrices.

sample homogenization. A particular innovation was obtained when sample size and pre-heating were combined (as pointed on Figs. 4a and 4b).

Heating milk to 40°C is a mandatory step for the solubilization of fat-soluble compounds and dissolution of fat globules. It also promotes interaction with greasy matter (phospholipids, triglycerides, and steroids) and effectively releases fat-soluble vitamins in distinct matrices that vary in fat content. This

analytical approach has been pointed out as an innovation but it is believed that other methodological factors provide minimizing intermolecular forces, which improves selectivity and thus extraction power. Fig. 5 summarizes several typical chromatograms obtained from lacteal matrices, solid, and fluid samples.

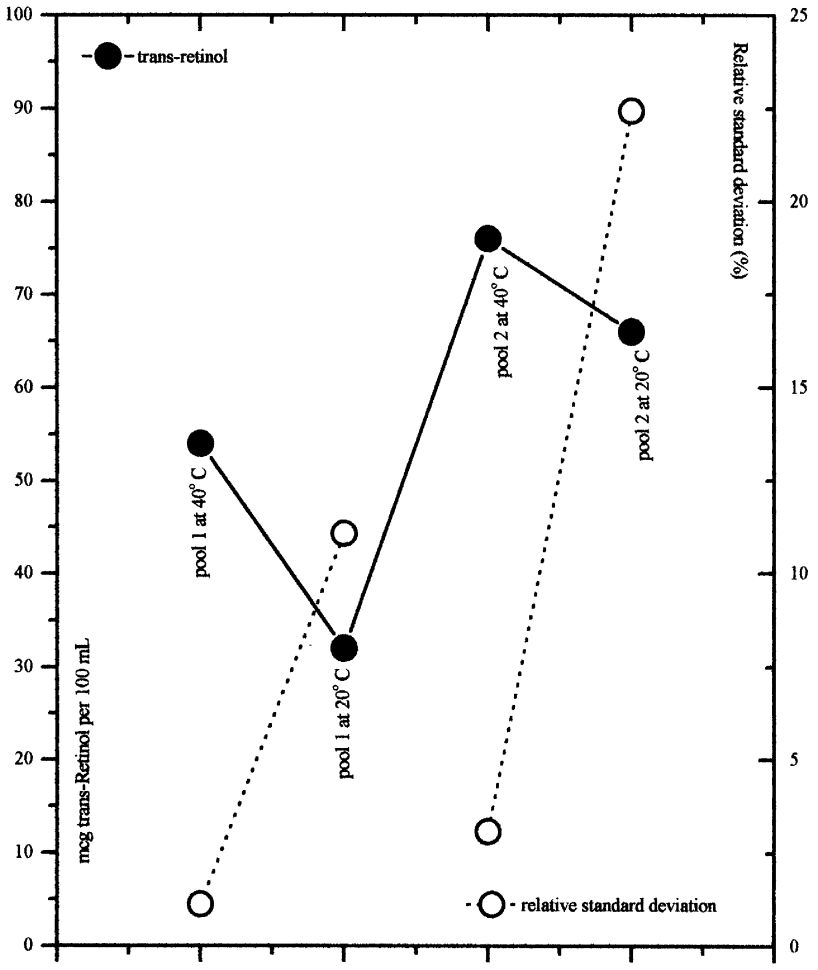

Figure 4(a). Trans-retinol extraction as function of sampling technique. All points were measured in triplicate for liquid matrix (F3).

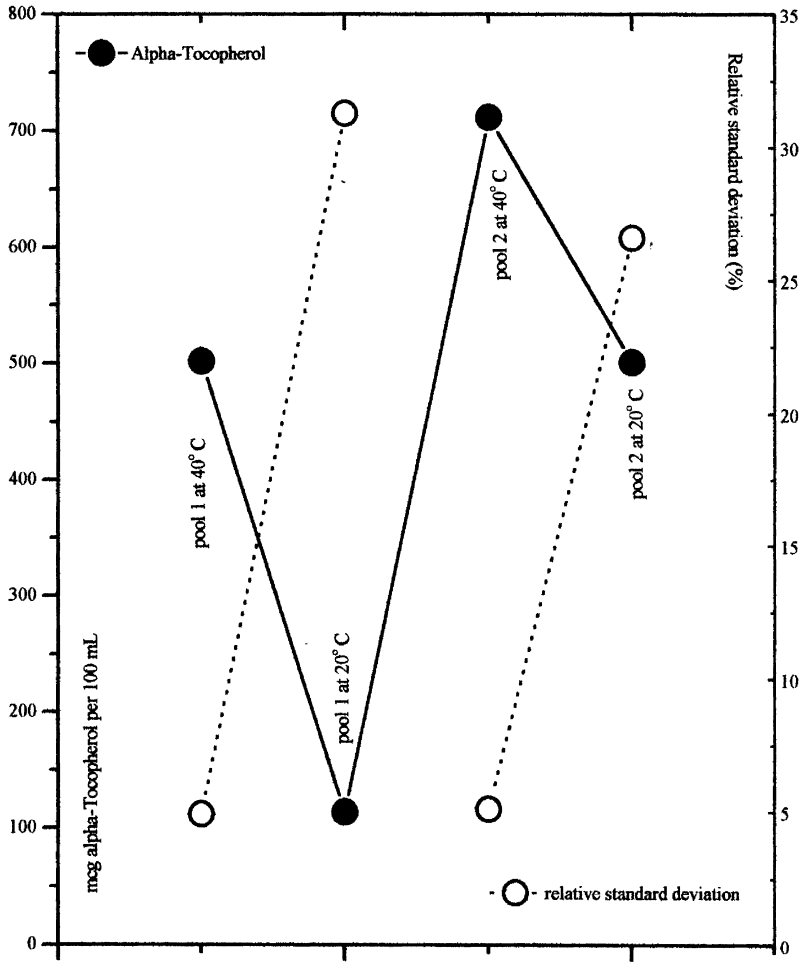

Figure 4(b). Alpha-tocopherol extraction as function of sampling technique. All points were measured in triplicate for liquid matrix (F3).

The results suggest that a fluid matrix may require low quantities of sample : g KOH ($\sim$0.24 g total solids : g KOH) while, for a solid matrix ($\sim$0.48 g total solids : g KOH), since other methodological factors were rigorously constant. This is a rough indication that fluid matrices (containing intermolecular H_2O) may facilitate accessibility in these samples. In contrast, solid matrices with dehydration effects (minimal intermolecular H_2O) may result

Table 5. Precision and Accuracy Assay

	All-trans-Retinol	Colecalcipherol	α-Tocopherol
Intra-Assay Analysis	**Quantified by λ Fixed at 265 nm**		
S2*	2362 (5.8) [2000]	536 (29) [165]	5.08 (18) [6.81]
S3*	4085 (6.4) [2400]	866 (9.3) [310]	6.84 (5.6) [5.5]
S4*	2657 (16) [1557]	360 (28) [464]	6.74 (22) [6.40]
	λ Fixed at Maximum of Each Vitamin		
F2**	400 (5.10) [500]	Nq	0.45 (12) [0.5]
F3**	320 (5.12) [300]	Nq	0.35 (5.24)
Inter-Assay Analysis	**λ Fixed at Maximum of Each Vitamin**		
S4*	2641 (5.18) [1500]	Nq	7.05 (3.28) [6.4]
F3**	274 (5.07) [300]	Nq	0.75 (4.25)

*Estimate in $IU \cdot 100\,g^{-1}$.
**Estimate in $IU \cdot 100\,mL^{-1}$.
( ) Relative standard deviation.
[ ] Value vitamin declared.
Nq Not quantified.

in low accessibility. In addition, methanol is a smaller alcohol than ethanol, the supposed effects of oxireduction potential (higher in aqueous medium) thus, being sufficiently minimized.

CONCLUSIONS

Both methanol : water or methanol : acetonitrile were selectively better as a mobile phase to quantify D3 or D2 in the presence of vitamins A and E.

Detection conditions may be adjusted to single λ (at 265 nm), or alternatively, by programming λ for each vitamin compound.

Extraction procedures were adjusted, particularly KOH concentration, extraction solvents, pyrogallol content, size of sample, and homogenization method.

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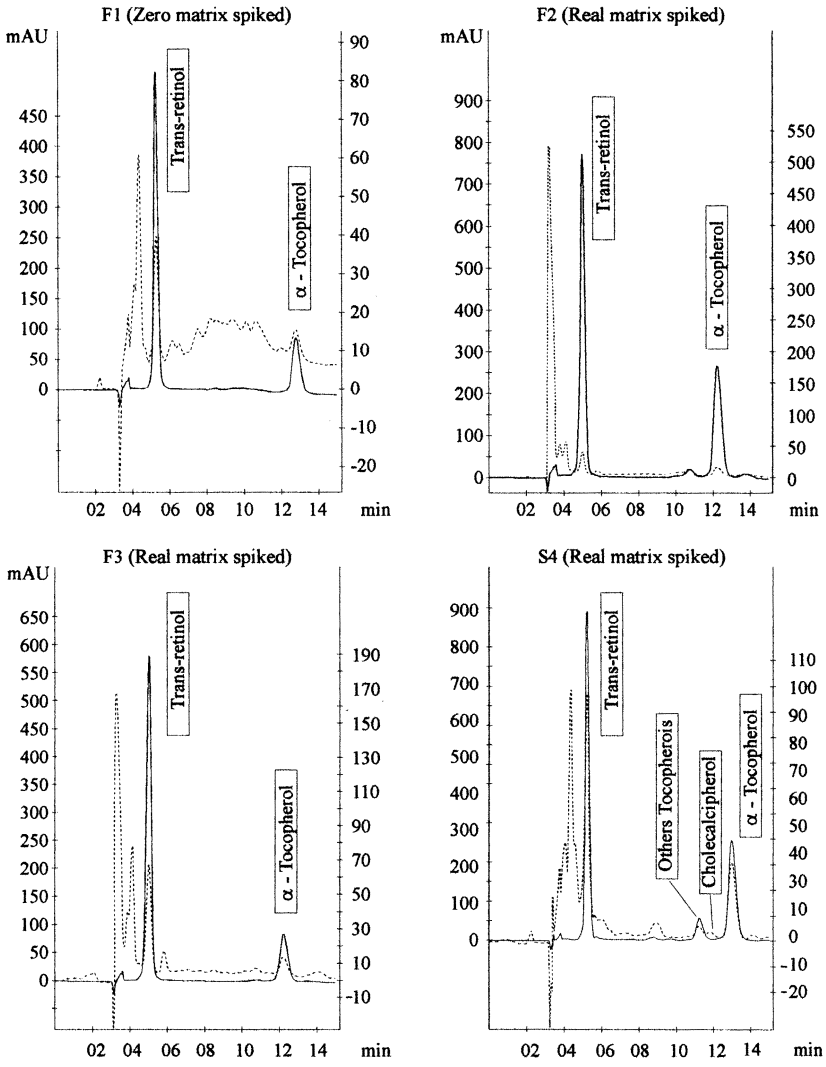

Figure 5. Typical chromatograms obtained from fluid and solid lacteal matrices. Legend: λ programmed (325 nm until 8.0 min, from 8.01, 292 nm), λ fixed at 265 nm. Column: Spherisorb ODS-2; 250 × 4.6 mm; methanol : water (99 : 1); flow rate: $1\,mL \cdot min^{-1}$.

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A single protocol for lacteal matrices of up to 3% lipids (fluid matrix), or up to 26% (solid matrix), was developed.

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