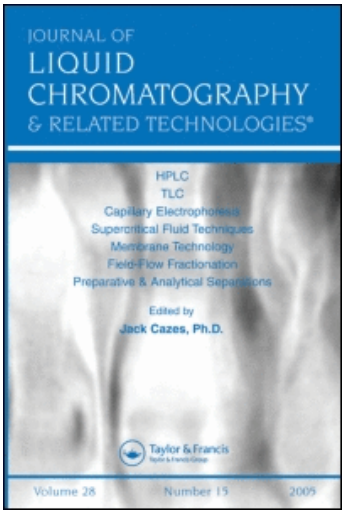

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Determination of Fat Soluble Vitamins by Reversed-Phase HPLC Coupled with UV Detection: A Guide to the Explanation of Intrinsic Variability

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Determination of Fat Soluble Vitamins by Reversed-Phase HPLC Coupled with UV Detection: A Guide to the Explanation of Intrinsic Variability

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ABSTRACT

Fat-soluble vitamins and their precursors are linked to other lipid compounds of cellular membranes of foods and feeds by physical and chemical forces. Different methods have been developed for the isolation and simultaneous determination of the main fat-soluble vitamins: retinoids, tocopherols, olefins, menadiones, and menaquinones. HPLC determinations are reported as being a suitable technique for estimating all compounds, or their derivatives, that contribute to ascertaining the vitamin value of foods. Particular advantages are outlined for single and simultaneous determinations using the HPLC technique, whilst suitable variability is intrinsic to the particular protocol. This article describes a new protocol and points to some factors that directly affect extractability and variability. Reversed phase

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ODS-2-HPLC-UV determination was used in order to develop the best procedure for the extraction of fat-soluble vitamins in lacteal matrices. Programmable λ was used for estimate A (trans-retinol) and E (α-tocopherol). Only mixtures of solvents: from 5% to 40% v/v diethylether light petroleum proved to be sufficiently better for A and E esterified alcoholic forms than the other agents evaluated: Na-EDTA, Na-EDTA-Tween, and Na-EDTA-NaOH. An alkaline force of above 20% in alcoholic medium was more destructive when associated with hot plate saponification, than with overnight saponification, to extract alcoholic forms of A and E vitamins. The time required for complete saponification was reduced from 12 h to 3 or 6 h, which achieved recovery rates of 85–106% when associated with incremental pyrogallol concentration (0.1⇒1.0% w/v). The ideal size of pool, in which the best extraction was performed, was found to be 2 L. Minimum variability was achieved by analyzing hard samples (raw milk, infant formula, blend-in cocoa), new pyrogallol and other methodological factors. Further and complete validation was expected when similar lacteal matrices were put into narrow and capillary columns.

INTRODUCTION

Fat-soluble vitamins include different classes of chemical compounds: retinoids, olefins, D_2, D_3 and its precursors, tocopherols, menadiones, and menaquinones that show distinct bioactivity according to The International Union of Pure and Applied Chemistry. These compounds are linked to other compounds of cellular membranes of foods and feeds by physical forces (H edge, Van der Waals, hydrophobic and electrostatic attraction), chemical forces or covalent linkage.[1–4] These forces may affect, above all, bioavailability.

Recently, different methods have been developed for isolated or simultaneous determination of fat-soluble vitamins. In general, biological assays are developed for allocating specific biochemical and nutritional functions.[1–3] Biochemical and histological assays are common, whilst there are no microbiological assays[1–3] involving fat-soluble vitamins. Foods and feeds that contribute a high vitamin value need precise and accurate determinations of different diet compounds that should be considered in order to estimate the total vitamin value, which includes isomers and main compounds related to fat-soluble vitamins.[1,2,4]

The spectrophotometric assay is insufficient as a single method for estimating distinct compounds, which involve interferences and isomers that contribute erroneously to the vitamin value.[1,2,4] HPLC-UV determinations coupled with λ max or λ fixed are an appropriate technique for estimating a single compound's isomers and its derivatives that contribute to determining, accurately, the vitamin value. Currently, good analytical approaches are described for retinoids and

tocopherols, mainly through the normal phase.[5,6] Meanwhile, reversed-phase HPLC has recently became an interesting alternative to conventional[7–11] and microcolumn UV detection of compounds, including D vitamin precursors,[12–13] or alternatively, through micellar medium fluorescence,[15,16] supercritical CO_2 coupled with UV detection for alcoholic forms.[15–18]

Similarly, a single liquid–liquid extraction using solvent is rarely reported, but an exception is obtained when coupled with solid phase extraction, for example, cleanup steps or preparative columns, involving natural compounds associated with lipid membranes.[19] Therefore, few studies have shown good separations without additional analytical columns, mainly for D and K vitamins that are roughly associated with other compounds of lipid membranes.[4,10,12,13,19] Some factors involved in this linkage are associated with a great number of methods described in the current literature.[1,7–19]

Saponification in alkaline medium is a good mechanism for removing interfering compounds, polysaccharide linkages, and disaggregate micellar forms, and therefore, contributes to an exhaustive liberation of fat-soluble vitamins compounds.[6,7] While D and K[6–9] vitamins are most sensitive to alkaline medium and temperature, A and E vitamins are also sensitive to O_2 and light, impaired extractability, and sensibility.[1,3,4,7]

It is noteworthy, that liquid–liquid extraction has shown itself to be an interesting technique, when coupled with a solid phase for D vitamins in the treatment of pharmaceuticals and parenteral formulas.[20–22]

Recent work has shown rough interaction of extractabilities with some factors involved in a single protocol: KOH concentration,[6,7] extraction solvents,[7] pyrogallol concentration,[7] λ detection[7] on lacteal matrices containing from 0 to 26% fat content, varying from 3 to 87% water content. Therefore, some factors involved in extraction performance were maximized and optimized using methanol medium.[7] The official method using aqueous KOH-N_2 influx discourages developing countries from rigorously policing real vitamin value in some foods.[6,7,23] Recently, a similar procedure and experimental design for saponification through aqueous KOH has been recommended equally for milk and fish.[6] Meanwhile, KOH methanol has been suggested for milk and chocolate fluid and solid formula.[7]

The particular advantages of the HPLC microcolumn have shown it to be suitable for detecting D vitamin precursors in the presence of a great number of other compounds, since it increases capacity factor and efficiency.[12,13,24] The stationary phase most used is reversed phase ($C_{18} > C_8$) for fat-soluble vitamin determination[12,13] associated with a gradient profile for complete elution.

Distinct methods have shown minimum variability, whilst some protocols are particularly advantageous for quantitative goals. The aim of this article is to describe more fully a new protocol, and any intrinsic factors, that directly affect extraction and variability, by way of a single protocol.

EXPERIMENTAL

Apparatus and Conditions

The experiments were carried out using a HPLC system equipped with a solvent delivery system (Waters). Programmable λ were obtained through a HP 1050, dual λ chromatographic system, in which some conditions of detection were adjusted to vitamins A (325 nm) and E (292 nm). Other channels were adjusted to 265 nm. A Rheodyne injector (20 μL) was always used.

The conventional analytical column employed was a Spherisorb ODS-2, 250 × 4.6 mm i.d., ϕ 5 μm, acquired from Sigma Aldrich (serial number: Z226068). Alternatively, a Waters Spherisorb ODS-2 150 × 3.2 mm i.d.; 5 μm conventional column (serial number: 99070806) was also used. An 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 mL min^{-1} (250 × 4.6 mm i.d.; 5 μm) to 0.4 mL min^{-1} (150 × 3.2 mm i.d.; ϕ 5 μm) 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 ± 1°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). 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. Analytical grade potassium hydroxide, ethanol, methanol, hexane, diethylether, and light petroleum were always purchased from Vetec (Brazil).

Individual standard solutions of the vitamins were prepared in HPLC grade ethanol, to provide a concentration of 10–500 μg mL^{-1} for trans-retinol; 0.3–12 μg mL^{-1} for D_2 and D_3, 35–2800 μg mL^{-1} for α-tocopherol, 0.72 and 72.50 μg mL^{-1} for K_1 (Spherisorb ODS-2, 250 × 4.6 mm i.d.; ϕ 5 μm). The ratio of A to E vitamins obtained in a great number of samples was 1 : 10 w/w. A similar ratio was, therefore, maintained for standards carried out on the Spherisorb ODS-2 150 × 3.2 mm i.d.; 5 μm (from 7.50 : 75 μg mL^{-1} to 360 : 3600 μg mL^{-1}), respectively, of A vitamin : E vitamin. After preparation, these solutions were sufficiently degassed by ultrasonic bath and stored in dark glass flasks at −18°C. All standard solutions were injected in triplicate within two days of preparation.

Individual standards or mixtures 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 liquid or solid matrices were mixed well before the sampling procedure. Sample analysis was carried out from 24 to 72 hours after preparation.

The lacteal matrices investigated are described as S (solid matrix) and F (fluid matrix), encoded in Table 1.

Chromatographic Conditions

Prior spectrophotometric assays were conducted to obtain the ratio of max λ or single 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.[7] Previous experiments

Table 1. Distinct composition of lacteal matrices investigated.

Different types of matrices	A (UI $100\,g^{-1}$)	D_3 (UI $100\,g^{-1}$)	E (mcg $100\,g^{-1}$)	Fat content (%)
Cocoa formula (S1)	5,000	400	—	3
Modified soya-based milk (S2)	1,500	322	6,600	26
Powdered full-fat milk (S3)	1,500	400	6,800	26
Modified milk UHT (F1)	150	1.5	1,500	1.6
Full-fat milk UHT (F2)	60	1.8	2,000	3.0
Skimmed milk UHT (F3)	450	3.0	—	0.5
Full-fat milk UHT (F4)	75	—	1,050	3.5
Chocolate milk UHT1 (F5)	90	1.6	300	2.4
Chocolate milk UHT2 (F6)	190	0.9	3,500	2.5

Note: S, solid matrices; F, fluid matrices.

had been carried out to obtain the linearity range under certain detection conditions in order to establish better performance involving liquid–liquid extraction (polar, non-polar), or coupled with sufficient saponification before the analytical columns. A typical chromatogram was obtained for the standard mixture, through which liquid–liquid extraction alone or liquid–liquid extraction plus saponification were directly evaluated, in order to optimize some factors of this new single protocol. The last quantitative and recovery assays were conducted using a simple vs. extracted curve at 10, 20, 40, 80, and 160 $\mu g\,mL^{-1}$ (trans-retinol) and 100, 200, 400, 800, and 1600 $\mu g\,mL^{-1}$ (α-tocopherol), while 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 the solid and fluid matrices described in this paper.

Sample Preparation and Experimental Plan

A great number of treatments with similar goals for liquid–liquid extraction (polar or non polar) were evaluated and are set out in Table 2. Saponification assays were conducted through KOH–methanol, ethanol, or aqueous-ethanol and aqueous-methanol at various concentrations (from 1.0 to 3.8 N), and the different extraction procedures were evaluated and compared. Overnight saponification was compared with hot plate saponification in a different medium when the best combination for extraction solvent was determined. The time required for similar extraction obtained comparatively through overnight saponification (minimum 12 h), was evaluated as an alternative to hot-plate saponification. Pyrogallol concentrations were increased (from 0.1 to 1% w/v) in order to reduce the variability obtained for some samples, full-fat milk fluid and powder. The unsaponificable extracts were finally concentrated in a rotary evaporator and re-dissolved in spectroscopic grade ethanol before being injected for chromatography. The extracts were maintained at $-18^{\circ}C$ for 24 to 72 h after being filtered through a 0.45 μm fluorpore membrane (FHLP01300).

Precision and Recovery Assays

A linear range ($R^2 \geq 99.95$) was obtained at various standard concentrations. The new and narrow column performed comparatively at the most sensitive conditions, $Y = 531.0417 + 561.1002X$, $R^2 = 99.95$ for trans-retinol and $Y = -38.9167 + 14.5018X$, $R^2 = 99.99$ for α-tocopherol quantification. Other condi-

Table 2. Alternative experiments for liquid–liquid extraction from lacteal matrices.

Extraction solvent	Exposure time (min)	Antioxidant
Non-polar solvents		AA/BHT
Hexane	30, 60 and 720	AA/BHT
Hexane : diethylether	30, 60 and 720	AA/BHT
Light petroleum	30, 60 and 720	AA/BHT
Light petroleum : diethylether	30, 60 and 720	AA/BHT
Diethylether	30, 60 and 720	AA/BHT
Non-polar solvents after polar dissolution		
Na-EDTA	30 and 60	AA/PG
Na-EDTA-NaOH	30 and 60	AA/PG
Na-EDTA-Tween 20	30 and 60	AA/PG
Na-EDTA-Tween 80	30 and 60	AA/PG
Ethanol	30 and 60	AA/PG
Non-polar solvents before polar dissolution	30 and 60	AA/PG
Lipase treatment	30 and 60	AA/PG

Note: All combinations of treatments were performed with AA, ascorbic acid (0.1–1.0% w/v); BHT, butylhydroxytoluene (0.1–1.0% w/v); PG, pyrogallol (0.1–1.0% w/v).

tions were described in a later article.[7] Precision and accuracy assays were carried out on standards and samples (matrices) in order to recommend a new protocol. A precision assay was performed on milk powder with a 26% fat content and fluid milk with 3.5% fat content, whilst accuracy was determined through standard recoveries at two levels of spiking, using a full-fat solid matrix (S3) and fluid matrix (F2). The recovery rate was also obtained through other matrices.

RESULTS AND DISCUSSION

Direct Extraction

Recently, a single technique for simultaneous determination, in which esterified and non-esterified forms of A and E vitamins are included has required an additional step, such as cleanup columns,[19,20] enriched bed,[12,13] capillary columns,[12,13,16] and micellar medium,[14,15] suitably validated for each particular case.

Therefore, lacteal matrices containing from 0.1 to 26% fat content and from 3 to 87% water content require a new approach to this possibility. Solely liquid–liquid extraction for introducing samples, such as food and feed into the

chromatographic system, has been shown to be insufficient. However, this has recently been demonstrated on fluid lacteal matrices with 3% fat content coupled with an enrichment technique.[12,13] Full-fat milk and butter were also analyzed using this approach since coupled with alcoholic saponification.[12,13]

Previous experiments were carried out in order to fix the best combination of solvents, see Table 2—Experimental. Comparative assays were conducted to establish some ways of disaggregating micellar forms, burnt polysaccharides, and proteins. Promising agents were found: Na-EDTA, Na-EDTA-NaOH; Na-EDTA-Tween.

In this study, profuse emulsions were always formed when samples contained above 3% fat. One explanation is the small size of samples with respect to the quantity of solvent, another is the minimal interaction force of this solvent.[24] This hypothesis can be ascertained only through capillary columns. The best combination of solvents found was diethylether : light petroleum, which yielded an extraction power of 20–40% of the stated value for the main compounds. This low extractability was probably a result of not having used fluided bed, enrichment column or cleanup column,[12,13] saponification,[6,7] or supercritical CO_2.[15–18] The minimum emulsion was obtained by using diethylether : light petroleum (from 5 to 40% v/v).

Lacteal matrices containing above 3%, rendered dense oil, which obligatorily required an additional cleanup step through extraction → saponification → extraction. A washing step managed to break up this emulsion. Along these lines, some agents: NaCl and KCl–methanol, ethanol, or acetona medium were investigated and were found to differ greatly in efficiency.

Only mixtures of solvents: 5%–40% v/v diethylether : light petroleum were better for A and E esterified alcoholic forms detected in solid and fluid matrices. On the other hand, the association of Tween 20 and Tween 60 coupled with Na-EDTA proved insufficient for breaking down aggregates and micellar forms. Although digitonin is a good clarifying agent (from 0.5 to 1.5% w/v) in methanol medium at $-33^{\circ}C$, the A and E peaks disappeared after redissolution.[1,2,25] Its efficiency is probably coupled with D and K vitamin extraction, because of rough associations of these vitamins with cholesterol and other steroids.[10,12,13,19] However, other methodological steps can interfere in the action of digitonin.

Figure 1 shows a typical chromatogram resulting from the exclusive use of 10–20% diethylether in light petroleum in some matrices as a single step of extraction. It, therefore, constitutes a rough indication of chemical linkage of lipids and cholesterol to some fat-soluble vitamins, mainly esterified forms of A and E vitamins. In this case, esterified vitamins are limited when intended for use as an internal standard in order to prove the best quantification system. In addition, reference material with zero fat-soluble vitamins and zero fat content to improve precision and accuracy studies for different lacteal matrices is almost impossible to obtain.

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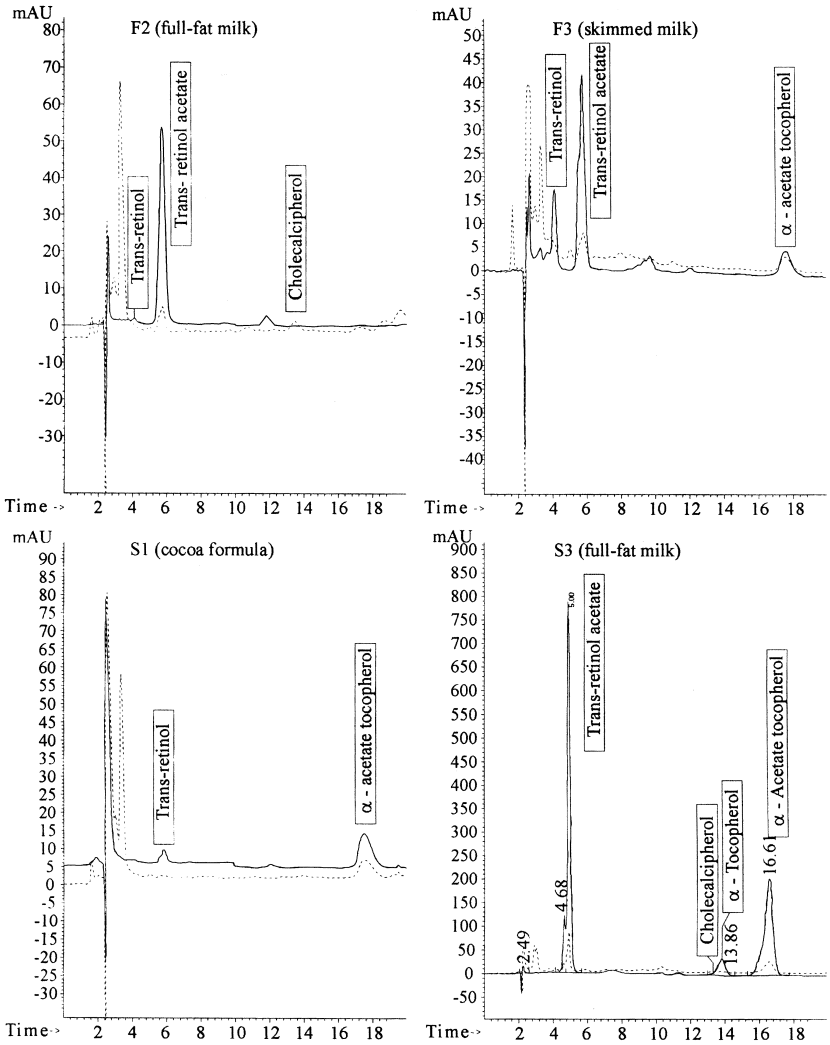

Figure 1. Extraction performance using non-polar solvents without saponification in fluid and solid lacteal matrices. *Key*: ___, λ programmed (325 nm until 8.0 min, from 8.01, 292 nm); ...., λ fixed at 265 nm. Column: Spherisorb ODS-2, 250 × 4.6 mm, methanol : water (99 : 1), flow-rate: 1.0 mL min^{-1}.

Until now, sustainable theories of liquid–liquid extraction have been so poorly discussed that they are considered insufficient, probably because of the low accessibility of most solvents, among other problems. Furthermore, the inadequate removal of interfering compounds from natural and modified products greatly inhibits micellar disaggregation in enriched foods and feeds.

Saponification Media

An official method for the extraction of A and E vitamins recommends aqueous KOH, in which oxireduction potential is elevated, the use of N_2 influx during saponification associated with pyrogallol solution[23] being recommended. Therefore, rough interactions can be adjusted in this case. Some researchers use only pyrogallol associated with alcoholic aqueous[6,7] medium and alcohol, because of its polar and non-polar characteristic and high recovery rates.[1,2]

Aqueous medium using the official method, can extract only trans-retinol (A vitamin) or α-tocopherol (E vitamin) in isolation.[23] Meanwhile, ethanol-aqueous medium can extract A and E vitamins, and methanol medium can sufficiently extract both vitamins associated with other tocopherols and D vitamins.[6,7,9,11–13] Therefore, through simultaneous determination by HPLC, it was possible to verify KOH concentration[6,7] always below that indicated by the official protocol (67% KOH-aqueous). This suggests a similar effectiveness of alcoholic[7] medium, below 40%, mainly because of differences in their solubility: $\leq 0.4\ \text{g KOH mL}^{-1}$ (methanol/ethanol medium) and $\cong 1.0\ \text{g KOH mL}^{-1}$ in H_2O medium.[6]

In a recent study,[7] KOH 10% (1.9 N) and KOH 20% (3.8 N) showed similar extraction performance, although KOH 20% registered a high recovery rate, mainly using a fat-free matrix (zero fat-soluble vitamins). Fluid matrices and solid matrices were used to optimize extraction solvents whose recovery rates were always above 70% for alcoholic forms of A and E vitamins. It is probable, that through capillary column[12,13] and micellar medium[14] these recovery rates could be improved.

From this perspective, the conditions which guaranteed maximum survival of vitamin compounds were investigated. Hot plate saponification using ethanol medium yielded trans-retinol and α-tocopherol (Table 3), but minimal survival of α-tocopherol because of the presence of H_2O and OH^-. Intermolecular forces can partially destroy A and E vitamins in the absence of N_2. However, alkaline forces above 20% in alcoholic medium were more destructive when associated with hot plate saponification (Table 3) for vitamins A and E than with overnight saponification (Table 4).

The extraction power of A and E vitamins through KOH 10% and KOH 20% proved to be sensitive to high temperatures (above 60°C), associated with ethanol and methanol media. Interactions of extraction power with the

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Table 3. Extraction performance using hot-plate saponification in different media.

Saponification media	Matrix	Trans-retinol (UI per 100 g)	Cholecalciferol (UI per 100 g)	α-Tocopherol (μg per 100 g)	Phyloquinone (μg per 100 g)
KOH : Ethanol : Water (25% w/v)	S1	1,782 (11)	300 (13)	702 (6)	ND
	S2	1,513 (17)	ND	4,550 (9)	14 (10)
KOH : Methanol (10% w/v)	S1	1,530 (18)	ND	ND	ND
	S2	786 (15)	ND	2,260 (12)	38 (10)
KOH : Methanol (20% w/v)	S1	3,273 (9)	454 (15)	ND	ND
	S2	66 (10)	ND	ND	ND
KOH : Methanol (40% w/v)	S1	270 (44)	ND	ND	ND
	S2	40 (37)	216 (18)	1,100 (18)	ND

Note: All values expressed as mean ± (relative standard deviation); ND, not detected; S1, cocoa formula containing ≅ 3% fat content; S2, modified soya-based milk ≅ 26% fat content.

saponification medium are poorly discussed in the literature, because other non-vitamin compounds can interfere through quenching or other biochemical processes. Signal suppression can be performed through interactions, which limit HPLC assays of lacteal matrices with high fat content (up to 26%) and high water content (87%), by causing an underestimation of A and E vitamins.

On the other hand, D and K vitamins are difficult to extract, although achieved through capillary columns coupled with UV detection.[12,13] D vitamin assays after alcoholic saponification, are well performed, whilst K vitamin detection appears to be more sensitive to alkaline methanol medium, mainly when extracts were injected 24 h after their extraction.[7]

Reversed phase conventional columns appear to be less efficient than normal phase ones in obtaining optimal separations of tocopherols in the presence of trans-retinol, D_2, D_3 and K vitamins, mainly through UV detection. Whilst, D vitamins are not fluorescent, UV is considered to be the only procedure for better quantification. It is possible to improve the quantification system when HPLC-capillary or further analytical technologies are associated. Fluorescence detection is poorly resolved by reversed phase because of the particular effects of methanol and ethanol in the mobile phase.

Time Required for Exhaustive Saponification

Previous assays were carried out in order to effectively reduce the time spent on the procedure of saponification, whilst hot saponification did not prove satisfactory for the determination of either alcoholic forms of A and E vitamins. This study achieved the effective reduction to 3 h or 6 h by increasing pyrogallol concentration from 0.1% to 1% w/v in fluid[7] and solid matrices, as described in Figs. 2, 3(a), (b). Recovery rates are set out in Table 5.

Solid matrices with up to 26% fat content were considered the most difficult. This single procedure showed itself to be an acceptable approach in effecting the re-dissolution of samples until the introduction of HPLC systems. Fat content and water content were, therefore, considered good parameters for drawing conclusions about exhaustive saponification and sufficient validation. In addition, liquid lacteal matrices containing less than 3% fat can undergo a similar procedure without disadvantages. These parameters should guide a complete validation.[26–28]

Recent studies have pointed to a relationship g KOH : g total solids : g sample as indicative of a single procedure of saponification.[6,7] Meanwhile, lacteal matrices and feed containing up to 3% fat can have any fat-soluble vitamins extracted using solvents, such as dichloromethane : dimethyl-sulphoxide : chloroform[29] or, alternatively, *n*-pentane.[30] These solvents effectively rupture the polysaccharide and gelatin matrix by isolating mainly

Table 4. Extraction performance using overnight saponification in different media.

Saponification media	Matrix	Trans-retinol (UI per 100 g)	Cholecalciferol (UI per 100 g)	α-Tocopherol (μg per 100 g)	Phyloquinone (μg per 100 g)
KOH : methanol : water (40% w/v)	S1	1,755 (11)	365 (11)	1,000 (12)	ND
	S2	705 (11)	360 (14)	1,380 (16)	ND
KOH : methanol (10% w/v)	S1	1,975 (11)	430 (16)	ND	ND
	S2	1,120 (17)	301 (20)	3,700 (8)	26 (15)
KOH : methanol (20% w/v)	S1	3,060 (6)	422 (11)	ND	ND
	S2	1,185 (10)	215 (13)	3,500 (11)	16 (18)
KOH : methanol (40% w/v)	S1	2,070 (11)	365 (12)	ND	ND
	S2	782 (12)	382 (9)	1,380 (14)	ND

Note: All values expressed as mean ± (relative standard deviation); ND, not detected; S1, cocoa formula containing ≅ 3% fat content; S2, modified soya-based milk ≅ 26% fat content.

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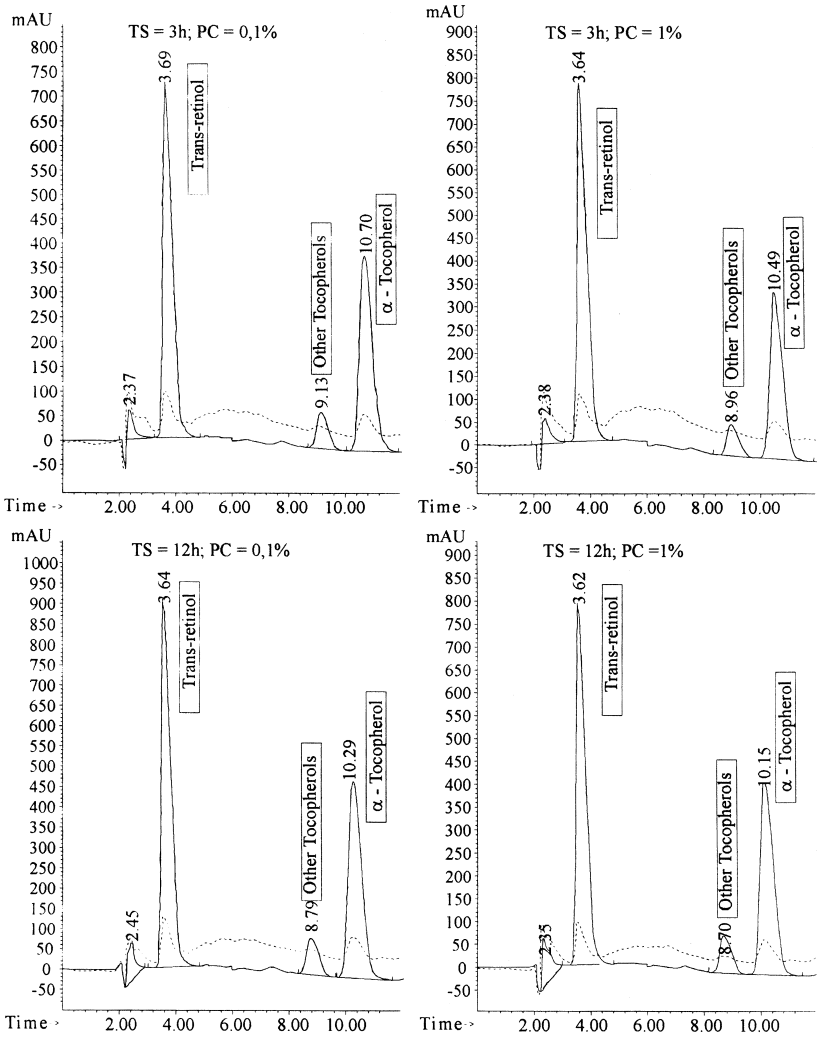

Figure 2. Time required for exhaustive saponification (TS) vs. pyrogallol content (PC) in solid matrix (S3). *Key*: ___, λ programmed (325 nm until 8.0 min, from 8.01, 292 nm);. . ., λ fixed at 265 nm. Column: Spherisorb ODS-2, 150 × 3.2 mm, methanol : water (99 : 1), flow-rate: 0.4 mL min^{-1}.

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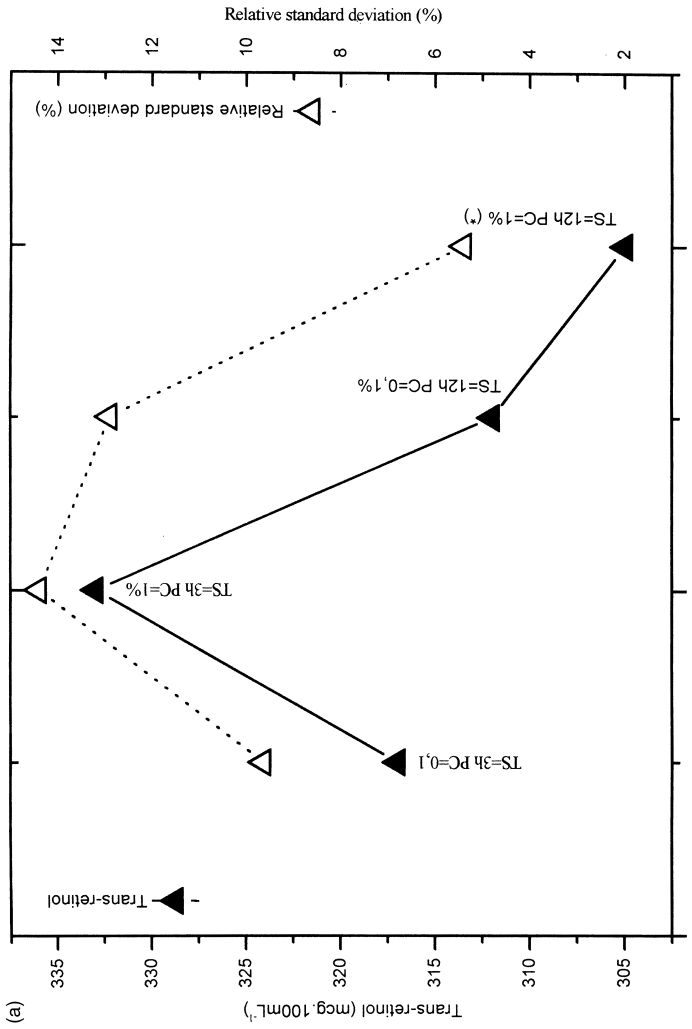

Figure 3. (a) Time required for exhaustive saponification (TS) vs. pyrogallol content (PC) on A vitamin extraction performance. (b) Time required for exhaustive saponification (TS) vs. pyrogallol content (PC) on E vitamin extraction performance. *Key:* Only this treatment showed significant differences ($p < 0.01$).
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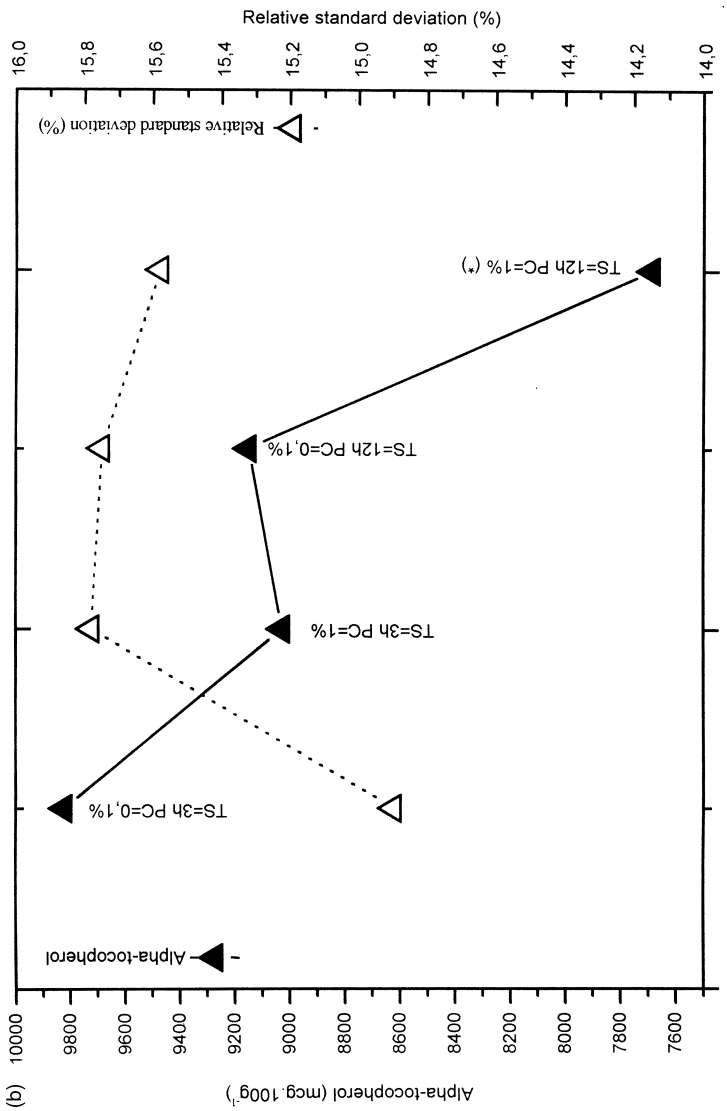

Figure 3. Continued.

Table 5. Recovery rates (%) of vitamins A and E in full-fat solid (S3) and liquid matrix (F2).

Time required for saponification	Trans-retinol	α-Tocopherol
3 h	[68–92] (15)	[92–98] (6)
6 h	[67–91] (8)	[67–99] (15)
12 h	[72–95] (8)	[92–99] (4)

Note: Assay was conducted in triplicate and expressed as [minimal and maximal value] and (relative standard deviation); level of spiking (40 μg of trans-retinol and 200 μg α-tocopherol).

vitamin A and other fat-soluble vitamins, when coupled with lipase treatment. Other sources of enzymes have recently been unsuccessfully investigated for improved α-tocopherol quantification through the single procedure.[17,18]

Antioxidant Effect on Extraction Performance

Butylhydroxytoluene is only soluble in non-polar solvents. Ascorbic acid is soluble in polar solvents and pyrogallol is soluble in both, polar and non-polar solvents. Therefore, pyrogallol is mostly recommended for experiments using liquid–liquid extraction or, alternatively, when this extraction procedure is combined with any previous saponification, as described in this paper.

Assays carried out in the absence of pyrogallol can produce low extraction and high variability, whilst our experiments were conducted to prove the maximum survival rate for A and E vitamins, mainly in overnight saponification using from 0.1% to 1% w/v pyrogallol concentration [Figs. 3(a), (b)]. There was a statistical tendency to reduce extraction performance (significant $p < 0.01$) when 1% of pyrogallol concentration was combined with 12 h saponification. Time required for maximum saponification and pyrogallol concentration are shown in Figs. 2, 3(a), (b). This fact is probably explained by the presence of oxidation lipid products interacting with E vitamins, quenching in the absence or insufficiency of pyrogallol.[7] On the other hand, products of lipid oxidation can be minimal in solid matrices [Figs. 3(a), (b)].

This is too difficult to measure because there are no reference materials for fluid matrices. Products of lipid oxidation depend mainly on processing agents, such as temperature, acid medium, fat content, and type of sample. It is, therefore, highly improbable that a comparative guide for this evaluation

will appear. The biochemical mechanism for this hypothesis is the quenching process involving oxy, peroxy-lipid reparation with E vitamins.

Natural and Modified Products

Reference material is only available for solid matrices, such as infant formula (SRM1846). Material for soya milk powder, skimmed milk powder, full-fat milk powder, and chocolate is more difficult to obtain in this grade. In contrast, fluid matrices achieved high variability and sensibility to pyrogallol concentration, showing them to be more unstable than other solid matrices.

Particular interest has recently been directed towards enriched foods containing from 30 to 60% of the daily requirements of each vitamin. However, in some cases it is possible to find less than this level of enrichment. Fluid matrices, being more unstable, were investigated because they showed less than 3.5% fat content and high enrichment levels (Table 6).

Other tocopherols (β,γ,δ) are usually found in products, which are derived from cocoa, grains, oils, and soya associated with cows milk products.

Sampling Technique

Previous studies defined the size of pool and homogenization procedure.[7] In this experiment, the pool size was increased from 2 to 4 L, considering pasteurized and raw milk as an intrinsic factor of variability. It was, therefore, possible to quantify a cutoff for the size of pool; above 2 Lbrought a lower extraction performance and higher variability. A similar occurrence was registered for both matrices [Figs. 3(a), (b), 4]. Neither the official method nor the unofficial method is able to define this question.

Quantification Technique

In these experiments, external standards were used, although an internal standard is good practice for samples that show high variability (more than 5%). Raw milk and hard samples (cocoa, grain, and soya) were more problematic. Other problems appeared because there are no reference materials available yet. At the time of writing there is only SRM 1846 for infant formula.

The validation procedure was limited to certain procedures, but those involving simultaneous quantification[6–16,24,26–28] were so divergent as to restrict further accuracy studies.

Table 6. Enrichment performance of ready-to-drink milk and chocolate beverages available on the brazilian market.

Conditions of process	Fat content	Trans-retinol (Vit. A) ($\mu g\,100\,mL^{-1}$)	α-Tocopherol (Vit. E) ($\mu g\,100\,mL^{-1}$)	Ratio value stated : quantified	
				Trans-retinol (Vit. A)	α-Tocopherol (Vit. E)
Modified milk UHT (F1)	1.6	52	2,617	0.35	1.74
Full-fat milk UHT (F2)[a]	3.0	48	3,501	0.80	1.75
Skimmed milk UHT (F3)	0.5	43	501	0.48	1.67
Full-fat milk UHT (F4)	3.5	50	2,237	0.26	0.64
Chocolate drink UHT (F5)	2.4	72	2,138	0.16	ND
Chocolate drink UHT (F6)	2.5	47	1,340	0.63	1.28

Note: ND, value not declared by labeling.
[a]Only this matrix complied with rigorously Brazilian law.

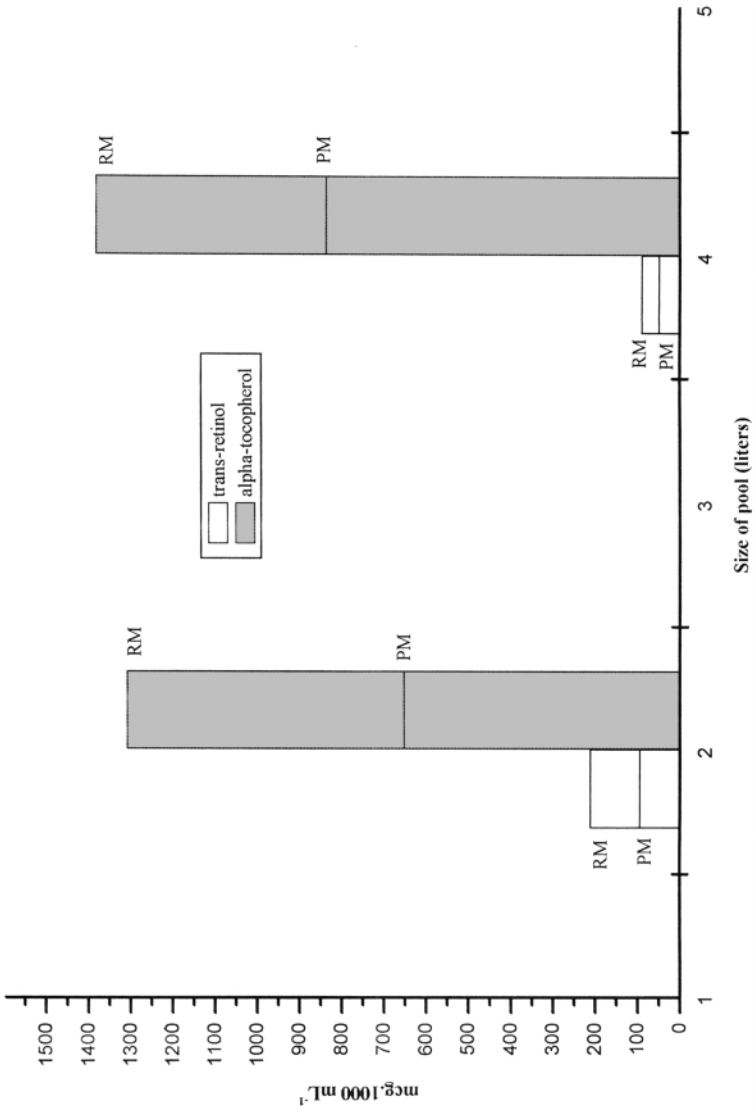

Figure 4. Influence of size of pool on extraction performance of A (trans-retinol) and E (α-tocopherol) vitamins.
Key: RM, raw milk; PM, pasteurized milk.

Table 7 shows results that describe some intrinsic factors, which may directly affect extraction performance and variability of A and E vitamins extracted by way of a single procedure, as described previously.[7] Aged matrices and out-of-date pyrogallol compromise quantitative results to some extent.

Good practice for quality control of enriched or non-enriched foods can be adjusted by using two or more extraction procedures (accuracy studies). Alternatively, UV spectrum absorption and mass spectrum are good tests for completing the validation procedure.[24,26–28]

Pool and punctual calibration curves were compared in two columns and were quite similar in quantitative response to A and E vitamins. Other vitamins: D_2, D_3, δ β and γ-tocopherols, and K_1 are possible to quantify, but only through capillary columns for sufficient separation through normal and reversed phase.

Recently, separation techniques involving automated determination[31] and liquid–liquid–solid extraction[20,32,33] have proven to be selective and practical analytical approaches for water- and fat-soluble vitamins through reversed-phase separation. Tandem column and detector[24] are very important complementary techniques, because they provide minimal variability and better vitamin value estimates.

CONCLUSIONS

Liquid–liquid extraction using organic solvents was insufficient (below 40% of value stated on label) to isolate the main fat-soluble vitamins.

Table 7. Intra-assay evaluation using powdered milk (commercial infant formula).

Intrinsic variability related to development of method[a,b]	Trans-retinol ($\mu g\,100\,g^{-1}$)	α-Tocopherol ($\mu g\,100\,g^{-1}$)
S3 out-of-date with aged pyrogallol	333 (9.98)	11,507 (7.75)
S3 out-of-date with new pyrogallol	325 (10.20)	11,087 (10.80)
S3 within expiry date with new pyrogallol	381 (1.80)	11,874 (3.27)
S3 stated value	450	7,260

Note: Chromatographic conditions: Column Spherisorb ODS-2 150×3.2 mm ϕ 5 μm, mobile phase-methanol : water (99 : 1), flow-rate: 0.4 mL min^{-1}; all values expressed as mean ± (relative standard deviation); out-of-date, samples opened more than six months prior to experiment; aged pyrogallol, opened more than two years prior to experiment; new pyrogallol, opened less than six months prior to experiment.
[a]Time fixed for complete saponification (3 h).
[b]Pyrogallol concentration fixed at 1% w/v.

Methanol and ethanol saponification media without heat promised to be the best conditions for simultaneous quantification of fat-soluble vitamins.

Maximum extraction performance for vitamin A was achieved through 20% KOH in alcoholic medium with hot plate and overnight saponification. However, E vitamin survival was only achieved through overnight saponification.

The time required for saponification was adjusted to 3 h, provided it was combined with sufficient pyrogallol.

The ideal sample size for this sampling technique was considered to be 2 L for fluid samples and 2 lb for solid samples.

Natural differences in some enriched and non-enriched matrices that should be considered are mainly physical–chemical properties, such as fat and water content and other compounds found in soya- and cocoa-based food.

Intrinsic variability was affected mainly by type of samples or matrices, drugs and reagents, sampling plan, and particular composition of food and feed.

REFERENCES

1. Ball, G.F.M. *Fat Soluble Vitamin Assays in Food Analysis. A Comprehensive Review*; Elsevier Science Publishers Ltd: England, 1988.
2. Machlin, L.J. *Handbook of Vitamin: Nutritional, Biochemical and Clinical Aspects*; Marcel Dekker, Inc: New York, 1984.
3. Lambert, W.; Scott, K.J. Internat. J. Vit. Nutr. Res. **1996**, *66*, 270–297.
4. Paixão, J.A.; Stamford, T.L.M. Quím. Nova **2002**, (prelo).
5. Hewavitharana, A.K.; Brakel, A.S.V.; Harnett, M. Intern. Dairy Journal **1996**, *6*, 613–624.
6. Salo-Väänänen, P.; Ollilainen, V.; Matilla, P.; Lehikoinen, K.; Salmela-Molsa, E.; Piironen, V. Food Chem. **2000**, *71*, 535–543.
7. Paixão, J.A.; Stamford, T.L.M. J. Liq. Chromatogr. & Rel. Technol. **2002**, *25* (2), 217–239.
8. Delgado-Zamarreño, M.M.; Sanchez-Perez, A.; Gomes-Perez, M.C.; Hernandez-Mendez, J. J. Chromatogr. A **1995**, *694*, 399–406.
9. Gong, B.Y.; Ho, J.W. J. Liq. Chromatogr. & Rel. Technol. **1997**, *20* (15), 2389–2397.
10. Po, E.S.M.; Ho, J.W.; Gong, B.Y. J. Biochem. Biophys. Methods **1997**, *34*, 99–106.
11. Qian, H.; Sheng, M. J. Chromatogr. A **1998**, *825*, 127–133.
12. Blanco, D.; Fernández, M.P.; Gutiérrez, M.D. Analyst **2000**, *125*, 427–431.

13. Gomis, D.B.; Fernández, M.P.; Gutiérrez Alvarez, M.D. J. Chromatogr. A **2000**, *891*, 109–114.
14. Ramos-Lledó, P.; Vera, S.; San Andrés, M.P. Frenesius J. Anal. Chem. **2001**, *369*, 91–95.
15. Buskov, S.; Møller, P.; Sørensen, H.; Sørensen, J.C.; Sørensen, S. J. Chromatogr. A **1998**, *802*, 233–241.
16. Ibáñez, E.; Tabera, J.; Reglero, G.; Herraiz, M. J. Agric. Food Chem. **1995**, *43*, 2667–2671.
17. Turner, C.; Mathiasson, L. J. Chromatogr. A **2000**, *874*, 275–283.
18. Turner, C.; King, J.W.; Mathiasson, L. J. Agric. Food Chem. **2001**, *49*, 553–558.
19. Demopoulos, C.A.; Antonopoulou, S.; Andrikopoulos, N.K.; Kapoulas, V.M. J. Liq. Chromatogr. & Rel. Technol. **1996**, *19* (4), 521–535.
20. Wielinski, S.; Olszanowski, A. Chromatographia **1999**, *50*, 109–112.
21. Moreno, P.; Salvadó, V. J. Chromatogr. A **2000**, *870*, 207–215.
22. Papadoyannis, I.N.; Tsioni, G.K.; Samanidou, V.F. J. Liq. Chromatogr. & Rel. Techonol. **1997**, *20* (19), 3203–3231.
23. Vitamins and others nutrients. In *Official Methods of Analysis*, 19th Ed.; Deutsch, M.J., Ed.; AOAC International: Washington, DC, 1998.
24. Snyder, L.R.; Kirland, J.J.; Glajch, J.L. *Pratical HPLC Method Development*, 2nd Ed.; A Wiley Interscience Publication, John Wiley & Sons, Inc.: New York, 1997.
25. Muniz, J.F.; Wehr, T.; Wehr, H.M. J. Assoc. Off. Anal. Chem. **1982**, *65* (4), 791–797.
26. Jenke, D.R. J. Liq. Chromatogr. & Rel. Technol. **1996**, *19* (5), 719–736.
27. Jenke, D.R. J. Liq. Chromatogr. & Rel. Technol. **1996**, *19* (5), 737–757.
28. Jenke, D.R. J. Liq. Chromatogr. & Rel. Technol. **1996**, *19* (12), 1873–1891.
29. Woollard, D.C.; Woollard, G.A. New Zealand J. Dairy Sci. Technol. **1981**, *16*, 99–112.
30. Barnett, S.A.; Frick, L.W.; Baine, H.M. Anal. Chem. **1980**, *52*, 610–614.
31. Gámiz-Gracia, L.; Velasco-Arjona, A.; Luque De Castro, M.D. Analyst **1999**, *124*, 801–804.
32. Li, H.B.; Chen, F. Chromatographia **2001**, *54*, 270–273.
33. Delgado-Zamarreño, M.M.; Sanches-Perez, A.; Sanches-Rodriguez, M.; Gomes-Perez, M.C.; Hernandez-Mendez, J. Talanta **1996**, *43*, 1555–1563.

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