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High-performance liquid chromatography with electrochemical detection for the simultaneous determination of vitamin A, D_3 and E in milk

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ABSTRACT

A high-performance liquid chromatographic electrochemical detection for the rapid and simultaneous determination of the vitamin A, D_3 and E is described The separation is carried out by using a C_{18} reversed-phase column and 0 1 *M* $LiClO_4$ in methanol–water (99 1, v/v) as the mobile phase The compounds are eluted with good resolution in the above order within about 15 min and are determined by amperometric detection with a glassy carbon electrode at +1050 mV (*vs* Ag/AgCl) The method gave reproducible results and the detection limits were of the order of 0 07, 4 and 0 2 ng of vitamin A, D_3 and E, respectively The method was successfully applied to the determination of vitamin A, D_3 and E in liquid cow milk and milk powder samples After saponification, fat-soluble vitamins were extracted with hexane and a methanolic solution of the dried extract was injected directly into the chromatographic system, avoiding the clean-up step that is necessary for vitamin D_3 when electrochemical detection is not used Good recoveries were obtained

INTRODUCTION

In recent years, much research has been devoted to developing sensitive, selective, rapid and reliable methods for the determination of fat-soluble vitamins in foods [1] because a deficiency of these vitamins causes serious nutritional diseases The absence from the diet of significant levels of vitamin A, D_3 and E leads to certain eye diseases, rickets and fertility disorders, respectively The determination of vitamin A, D_3 and E in milk is of major importance as dairy foods play a vital role in nutrition and these vitamins have additional roles such as in protecting against cancer, avoiding dental caries and osteomalacia and avoiding neuropathological and neuromuscular disorders, respectively

In addition to the determination of naturally occurring vitamin A, D_3 and E in milk and milk-based foods, there is also a need for the determination of these substances in processed low-fat and skimmed milk products and dairy foods fortified with these fat-soluble vitamins There are numerous reports relating to the determination of lipid-soluble vitamins in milk, although methods for the simultaneous determination of these three vitamins are lacking In recent years there have been increasing reports on the determination of fat-soluble vitamins using high-performance liquid chromatography (HPLC) in the normal-phase [2–11] and reversed-phase [12–14] modes with UV or fluorimetric detection HPLC with electrochemical detection (HPLC–

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ED) appears to be promising for fat-soluble vitamin determination Electrochemical methods have been published for the determination of vitamin A, D_3 and E [15–22] in different samples, pharmaceuticals (A and D_3) and biological samples

In this paper, a rapid method is proposed for the simultaneous determination of vitamin A, D_3 and E using reversed-phase HPLC and amperometric detection with a glassy carbon electrode The method was applied to the determination of these vitamins in milk samples In this procedure the clean-up step, after extraction of vitamins from the unsaponificable phase, which is usually used in the determination of vitamin D_3 in milk, butter and other samples, was avoided

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of an SP 8800 ternary pump (Spectra-Physics, San Jose, CA, USA) equipped with a Rheodyne (Berkeley, CA, USA) valve with an injection loop of 10 μl The detectors were a Spectra-Physics SP8450 UV detector and an EG & G PAR (Princeton, NJ, USA) Model 400 electrochemical detector Peak areas were measured with an SP 4290 integrator (Spectra-Physics)

The chromatographic columns used were an RP-18 precolumn (15 × 3 2 mm I D , 7-μm film thickness (Brownlee Labs , Santa Clara, CA, USA) and an OD-224 RP-18 column (220 × 4 6 mm I D , 5-μm film thickness) (Brownlee Labs)

A Buchi (Flawil, Switzerland) RE 121 rotavapor with a Buchi 461 water-bath was used

Reagents

All-*trans*-retinol, vitamin A was obtained from Sigma Química (Madrid, Spain), cholecalciferol, vitamin D_3 from Fluka Química (Madrid, Spain), α-tocopherol, vitamin E from Aldrich Química (Madrid, Spain), $LiClO_4$ (analytical-reagent grade) from Panreac (Barcelona, Spain) and methanol (LC grade) from Carlo Erba (Milan, Italy) Water was purified in a ElgaStat water-purification system (Elga, High Wycombe, UK)

The mobile phase was a 0 1 *M* solution $LiClO_4$ as supporting electrolyte in methanol–water (99 1, v/v) Alcoholic potassium hydroxide solution was prepared by mixing 50 ml of ethanol and 15 ml of 60% KOH solution The extractants used were hexane and hexane–chloroform–ethanol (6 3 5 0 5)

Samples were commercial powdered milk and commercial liquid cow milk

Procedure

The mobile phase was degassed with helium and pumped at a flow-rate of 1 0 ml/min Standards of vitamins or sample extracts dissolved in methanol were injected through a Rheodyne valve The glassy carbon elctrode was pretreated daily by cathodic polarization (−600 mV for 10 min) followed by anodic polarization (+1200 mV for 30 min), both in

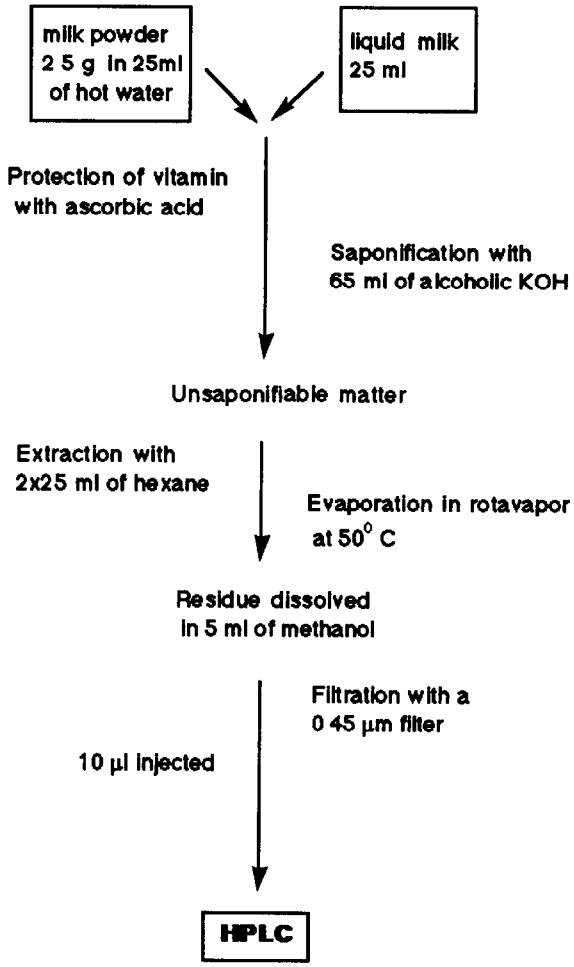


Fig 1 Procedure for the determination of vitamin A, D_3 and E in powdered and liquid milk samples

a flowing stream of mobile phase The applied potential for detection was +1050 mV For the simultaneous UV detection of three vitamins a compromise wavelength of 280 nm was chosen The procedure for the determination of the vitamins in milk samples is detailed in Fig 1

RESULTS AND DISCUSSION

Optimization of the method

The influence of the applied potential, mobile phase composition, flow-rate and concentration of the supporting electrolyte was studied In order to determine the optimum applied potential for the detection of the three vitamins, their hydrodynamic voltammograms were obtained by injecting fixed amounts of standard solutions of vitamins and varying the applied potential in 50-mV steps (Fig 2) Higher voltages produce higher signals, especially with vitamin D_3, but also an increase in background signal The working potential chosen was +1050 mV

The amount of water in the mobile phase was varied from 0 to 6% For all the water/methanol ratios tried good separations between the three vitamins were obtained, but at 3% water the retention times increased considerably (up to 22 min for vitamin E) Therefore, methanol–water (99 1, v/v) was chosen

From the study of the effect of flow-rate a value of 1 0 ml/min was chosen The chromatograms showed good resolution and acceptable retention times for the three vitamins (4, 12 and 14 min for A, D_3 and E, respectively) (Fig 3)

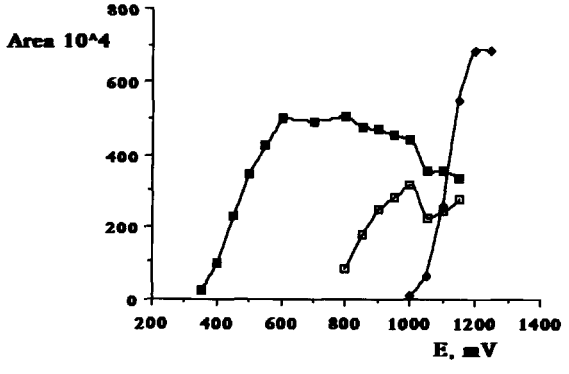


Fig 2 Hydrodynamic voltammograms Mobile phase, methanol–water (99 1, v/v) containing 0 1 *M* $LiClO_4$, flow-rate, 1 0 ml/min, Amounts injected, (□) vitamin A 2 5, (◆) vitamin D_3 19 3 and (■) vitamin E 10 8 ng

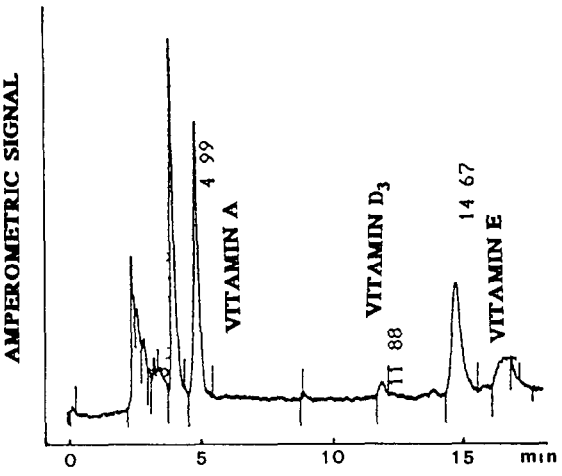


Fig 3 Chromatogram obtained after application of the proposed method to a sample of liquid cow milk

The influence of $LiClO_4$ concentration in the mobile phase was studied in the range 0 02–0 18 *M* The peak areas increased with increasing electrolyte concentration, reaching a constant value for concentrations >0 05 *M* Hence an $LiClO_4$ concentration of 0 1 *M* was chosen for the proposed procedure

Analytical characteristics of the HPLC–ED method

The relationships between chromatographic peak area and vitamin concentration injected were linear in the ranges 4 62 10^{-8}–2 31 10^{-6} *M* vitamin A, 2 06 10^{-6}–3 24 10^{-5} *M* vitamin D_3 and 4 48 10^{-8}–1 55 10^{-6} *M* vitamin E The equations relating peak areas to concentration are shown in Table I

The limits of detection (signal-to-noise ratio = 3) obtained were 3 7 10^{-8}, 1 1 10^{-6} and 4 4 10^{-8} *M* (0 07, 4 3 and 0 19 ng injected) for vitamin A, D_3 and E, respectively, relative standard deviations of 4 2, 3 1 and 5 7% were obtained when amounts of 0 66, 25 and 1 15 ng of vitamin A, D_3 and E were injected (n = 10)

Table II shows the calibration fittings for UV detection at 280 nm The detection limits obtained were 6 48 10^{-8}, 2 1 10^{-6} and 7 7 10^{-7} *M* (0 12, 10

TABLE I

LINEARITY BETWEEN VITAMIN CONCENTRATIONS AND CHROMATOGRAPHIC PEAK AREAS USING THE HPLC–ED METHOD

Calibration fitting $A = a + bx$

Vitamin	Concentration range (M)	a 10^{-3}	b 10^{-10}	r ($n = 10$)
A	(0 46–23) 10^{-7}	150 ± 70	359 ± 7	0 998
D_3	(2 1–32) 10^{-6}	−80 4 ± 39	15 6 ± 0 2	0 999
E	(0 45–16) 10^{-7}	3 9 ± 0 1	446 ± 13	0 997

TABLE II

LINEARITY BETWEEN VITAMIN CONCENTRATIONS AND CHROMATOGRAPHIC PEAK AREAS USING THE HPLC–UV METHOD

Calibration fitting $A = a + bx$

Vitamin	Concentration range (M)	a 10^{-3}	b 10^{-10}	r
A	(0 46–23) 10^{-7}	0 73 ± 0 49	2 41 ± 0 05	0 997 ($n = 10$)
D_3	(2 1–32) 10^{-6}	−1 07 ± 2 0	1 61 ± 0 01	0 999 ($n = 7$)
E	(8 9–18) 10^{-7}	5 8 ± 1 4	1 20 ± 0 16	0 991 ($n = 5$)

and 3 3 ng injected) for vitamin A, D_3 and E, respectively, the relative standard deviation being 1% for the three vitamins As shown by the detection limits and slope values, electrochemical detection provides higher sensitivity for the determination of these vitamins

Analytical applications

The determination of fat-soluble vitamins usually includes saponification, extraction and clean-up of the samples before injection into the HPLC system In order to avoid the saponification step, direct extraction of the vitamins from the milk powder,

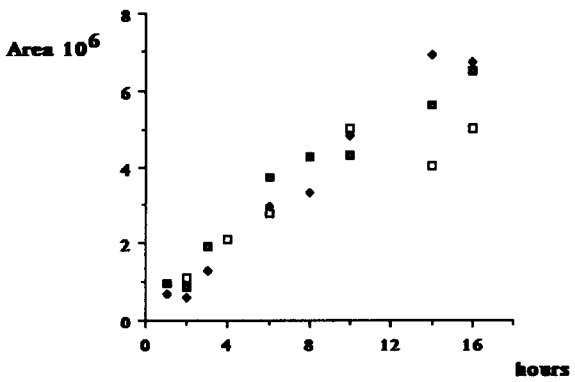
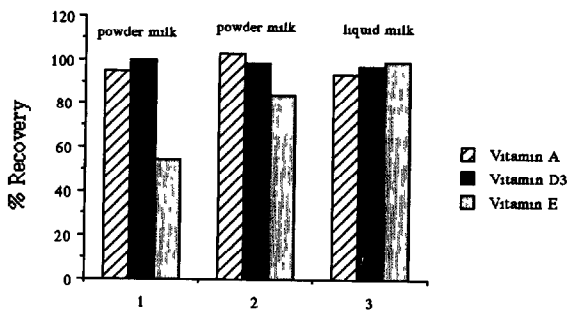


Fig 4 Influence of extraction time in the extraction of vitamins from milk powder with hexane–chloroform–ethanol (6 0 3 4 0 5) □ = Vitamin A, ◆ = vitamin D_3, ■ = vitamin E



1 without hydrolysis, 2 and 3 with hydrolysis

Fig 5 Recoveries of the proposed method for samples of milk (1) without and (2 and 3) with hydrolysis

TABLE III

DETERMINATION OF VITAMIN A, D_3 AND E IN DIFFERENT MILK SAMPLES

Results from three replicate analyses

Milk sample	Powdered milk					
	A (μg per 100 g)		D_3 (μg per 100 g)		E (mg per 100 g)	
	Found	Quoted by supplier	Found	Quoted by supplier	Found	Quoted by supplier
1	362 ± 0 5	450	10 3 ± 0 4	7 5	2 90 ± 0 1	2 7
2	441 ± 2	450	10 8 ± 3 6	10	2 65 ± 1 4	2 7
3	553 ± 3	450	3 9 ± 1 5	3	4 94 ± 1 3	4 4
	Liquid milk (found)					
	A (μg per 100 ml)		D_3 (ng per 100 ml)		E (μg per 100 ml)	
1	18 7 ± 0 4		296 ± 4		331 ± 1	
2	45 5 ± 0 4		388 ± 1		285 + 2	
3	36 5 ± 1 7		383 ± 3		185 ± 1	
4	35 3 ± 1 3		311 ± 1		67 ± 2	
5	66 1 ± 1 4		224 ± 2		50 ± 1	

using the procedures included in different reports [23], was tried The samples (2 5 g of powder milk, three replicates) were extracted with 25 ml of hexane–chloroform–ethanol (6 0 3 5 0 5), with constant stirring in the dark From the study of the extraction time a value of 10 hours is proposed (Fig 4) After centrifugation, the organic phase was removed in a rotavapor at 50°C, the remaining residue being dissolved in 5 ml of methanol After filtration an aliquot of this solution was injected into the chromatographic system Data for the recoveries were obtained by spiking the samples of milk with standard solutions of vitamins in amounts of the same order as contained in the samples ($1\ 10^{-6}$–$6\ 10^{-6}$ *M*) Although good recoveries were obtained for vitamin A and D_3, they were not for vitamin E (Fig 5), moreover, the amounts found in all the samples were lower than those specified by the manufacturer Therefore, we conclude that milk powder samples must be saponified prior to the determination of vitamins

The samples (2 5 g of powdered milk or 25 ml of liquid milk) were saponified with an alcoholic solution of potassium hydroxide After extraction of the vitamins with hexane and evaporation of the solvent, the residue was dissolved in methanol and injected into the chromatograph after filtration (0 45-μm filter) without any clean-up step (Fig 3) By applying this procedure the recoveries (Fig 5) were in the ranges 84–103% (milk powder) and 93–99% (liquid cow milk) The amounts found for each vitamin in the different samples are summarized in Table III The day-to-day precision was obtained by replicate analyses (n = 5) of standard solutions, the values found being 6 6, 5 21 and 6 68% for vitamin A, D_3 and E, respectively

The vitamin contents obtained in the milk powder were compared with those stated by the manufacturer but it was not possible to do so for the liquid milk because this value is not available for the commercial products In all instances good reproducibility in the analysis and acceptable relative standard deviations were found

CONCLUSION

The proposed method allows the simultaneous determination of vitamin A, D_3 and E in milk,

avoiding the clean-up steps usually necessary in vitamin D_3 determination No interferences were found with either detector (UV or electrochemical) and no washing step between samples is necessary Moreover, the high sensitivity of the electrochemical detection allows the determination of vitamin D_3 in unenriched liquid milk, which is not possible using UV detection without a preconcentration step Both the precision and the accuracy of the method make it suitable for routine milk (liquid and powder) analysis

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