ELSEVIER

Journal of Chromatography A, 694 (1995) 399–406

JOURNAL OF CHROMATOGRAPHY A

Directly coupled sample treatment–high-performance liquid chromatography for on-line automatic determination of liposoluble vitamins in milk

M.M. Delgado-Zamarreño*, A. Sanchez-Perez, M.C. Gomez-Perez, J. Hernandez-Mendez

Department of Analytical Chemistry, Nutrition and Food Science, Faculty of Chemistry, University of Salamanca, 37008 Salamanca, Spain

First received 20 July 1994; revised manuscript received 8 November 1994; accepted 15 November 1994

Abstract

We developed an on-line system for the determination of liposoluble vitamins A, D_3 and E in milk, both liquid and in powder form, using an automated sample treatment system coupled to chromatographic determination. For this, C_{18} cartridges were used because of the strong capacity of this material for the extraction and preconcentration of such vitamins, its ease in handling and the possibility of coupling it with automatic analysis systems.

Alkaline hydrolysis of the samples was performed in an on-line system comprising two confluent channels through which the sample solution and alcoholic sodium hydroxide plus ascorbic acid flowed for a given period of time. A third channel merged with the other two to neutralize the solution before it arrived at the C_{18} cartridge. The latter, inserted into a loop with a six-port injection valve, retained the soluble vitamins. The vitamins were eluted with a stream of methanol and the eluate was automatically injected into the chromatographic system. Variables affecting the on-line system were optimized: sample size, flow-rate, preconcentration, washing and elution times, etc. The recoveries for powdered and liquid milk for the three vitamins assayed ranged between 80 and 105% ($n = 10$). Additionally, a day-to-day precision (10 days) of the method of 4.5% was obtained for the different vitamins.

1. Introduction

The determination of microconstituents of samples includes a sample preparation step that usually is laborious and time-consuming. The aim of sample preparation is to extract the analytes from the matrix to be analyzed and to concentrate them, if needed, to obtain a measurable response in a chromatographic or any other type of system. Whereas the actual analytical procedure may only take a few minutes, the sample preparation step may take several hours.

Currently, chromatographers are searching for better sample preparation techniques to improve the speed and precision of analysis. An additional goal is to obtain more cost-effective, easy to use, and mostly automated methods.

The interest aroused by solid–liquid extraction

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(94)01142-7

(solid-phase extraction, SPE) has increased not only because it affords results similar to liquid–liquid extraction but also because it requires smaller amounts of solvent, is faster and gives cleaner solutions because there is an inherent filtration. Another important aspect is the possibility of using cartridges in which SPE is used in continuous set-ups.

For the determination of liposoluble vitamins in milk, sample treatment is slow and tedious. It consists of alkaline hydrolysis of the sample followed by extraction of the vitamins from the non-saponifiable matter, evaporation of the solvent and injection into the chromatographic system. Although attempts to minimize the treatment by direct extraction of the vitamins from the sample [1–3] have been described, some authors had to perform fractionation steps and clean-up of the extracts prior to quantification [4–7]. However, it was observed previously [8] that the classic treatment should be used for milk samples because the results obtained after direct extraction are too low.

There are numerous reports on the determination of different liposoluble vitamins in milk using HPLC in normal-phase [9–15] and reversed-phase [16–20] modes, usually with UV or fluorimetric [15] and amperometric detection for vitamins A and E [20]. A method has also been proposed for the simultaneous determination of vitamins A, D_3 and E in milk samples without the need of any clean-up step for the detection of vitamin D_3 [8].

The aim of the present work was to design an on-line sample treatment system with a chromatographic system for the simultaneous determination of liposoluble vitamins in liquid and powdered milk samples. No automated method for the determination of liposoluble vitamins in this kind of sample has been found in the literature consulted.

2. Experimental

2.1. Apparatus

A Spectra-Physics SP 8800 ternary pump equipped with a Spectra System AS1000 automatic injector with a 100-μl loop was used. The detectors were a Spectra-Physics SP8450 UV detector and electrochemical detector EG and PAR Model 400, with a glassy carbon working electrode. Peak areas were measured by a Spectra-Physics SP 4290 integrator.

The on-line system consisted of commercially available Sep-Pak Plus C_{18} cartridges (Millipore) placed in the sample loop of a Rheodyne Model 5020 six-port injection valve; a Gilson 231-401 microprocessor; a Gilson Minipuls-3 peristaltic pump with Isoversinic pump tubes; PTFE tubing (0.50 mm I.D.) for the rest of the channels, including the knotted reactor of 5 m of length.

Chromatographic columns: precolumn RP18 15×3.2 mm, 7 μm (Brownlee Labs.) and column OD-224 RP18 220×4.6 mm, 5 μm (Brownlee Labs.).

2.2. Reagents

All-*trans*-retinol (vitamin A) was from Sigma, cholecalciferol (vitamin D_3) from Fluka, α-tocopherol (vitamin E) from Aldrich, Ascorbic acid and NaOH (analytical-reagent grade) from Panreac and methanol (LC grade) from Carlo Erba. Water was purified in a Elga-Stat water-purification system.

The alcoholic sodium hydroxide solution consisted of 50 ml ethanol and 15 ml of 60% aqueous NaOH solution and 5 ml of 10% ascorbic acid.

The washing solution was water–methanol (60:40). The elution solvent was methanol. The mobile phase was a solution of acetic acid–sodium acetate 2.5 mM in methanol–water (99:1, v/v).

3. Results and discussion

Determination of liposoluble vitamins in milk samples usually includes saponification of the fat material, extraction of the vitamins from the non-saponifiable material and clean-up of the extracts before injection into the chromatographic system. In the present work, a flow system coupled to the chromatographic system is proposed which performs these steps automatically.

The set-up is shown schematically in Fig. 1. Alkaline hydrolysis of the samples is performed in the two-channel system through which the sample and alcoholic sodium hydroxide with ascorbic acid (to avoid oxidation of the vitamins) are passed for a given time (preconcentration time). Hydrolysis occurs in the reactor. A third channel merges with the former ones to neutralize the solutions before the C_{18} cartridge is reached. This cartridge, inserted into the loop of an injection valve, retains the liposoluble vitamins. Later, the washing solution is passed through the cartridge followed by a stream of methanol, which elutes the vitamins. The eluate is automatically injected into the chromatographic system. The two injection valves together with the integrator are controlled by a microprocessor, thus automating the process.

Fig. 1A shows the whole process in detail; initially, the two valves are set at the load position such that the hydrolyzed and neutralized sample reaches the C_{18} cartridge where the vitamins are retained, while the washing solution flows through the second valve.

After a suitable preconcentration time, the first valve is switched to the injection position, the second valve remaining unchanged (Fig. 1B). In this way the cartridge is washed with the water–methanol mixture. When the washing time has been completed, methanol is introduced through the same channel to elute the vitamins from the cartridge, thereby filling the loop of the second valve (Fig. 1C). When the loop contains the greatest amount of vitamins eluted (elution time) the second valve turns automatically, injecting the 100 μl directly into the chromatographic column where the vitamins are separated for later detection. Fig. 2 shows a diagram of the whole set-up; electrochemical and UV (280 nm) detectors were used.

The results obtained spectrophotometrically were similar to those obtained using electrochemical detection except that in some cases certain problems arose regarding spectrophotometric quantification of vitamins A and E, which were not detected. In the UV detection a 280 nm wavelength was chosen as a compromise wavelength for the detection of vitamins A, D_3 and E. UV detection is less sensitive than amperometric detection [8]. The present work only reports the data obtained by electrochemical detection.

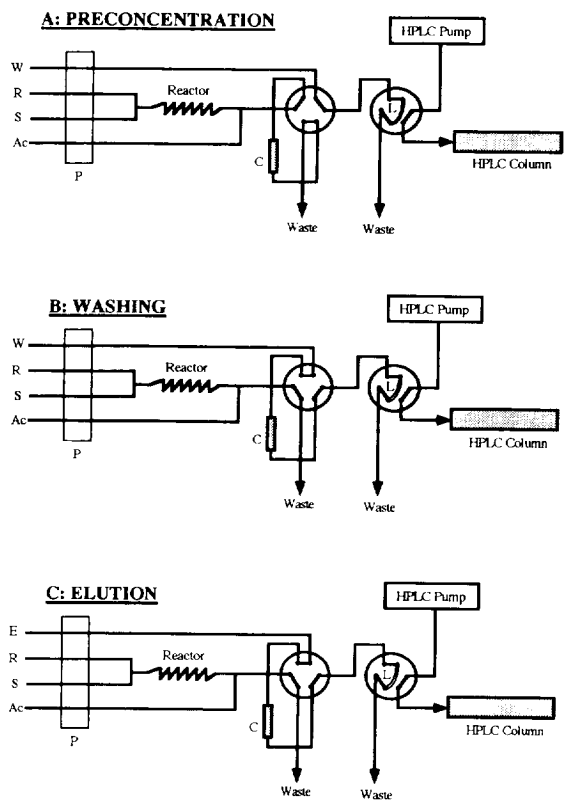

Fig. 1. Schematic diagram of the on-line hydrolysis and elution with HPLC system. P = Peristaltic pump; R = hydrolysis reagent; S = sample; Ac = acetic acid; W = washing; E = elution; C = C_{18} cartridge; L = 100-μl loop.

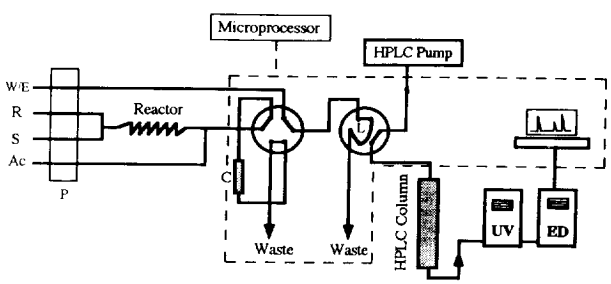

Fig. 2. Diagram of coupled on-line sample treatment–HPLC for determination of liposoluble vitamins. P = Peristaltic pump; R = hydrolysis reagent; S = sample; Ac = acetic acid; W = washing; E = elution; C = C_{18} cartridge; L = 100-μl loop. HPLC conditions: mobile phase: methanol–water (99:1), 0.0025 *M* HAcO–NaAcO; flow-rate: 1.0 ml/min; UV detection at 280 nm; electrochemical detection at + 1300 mV.

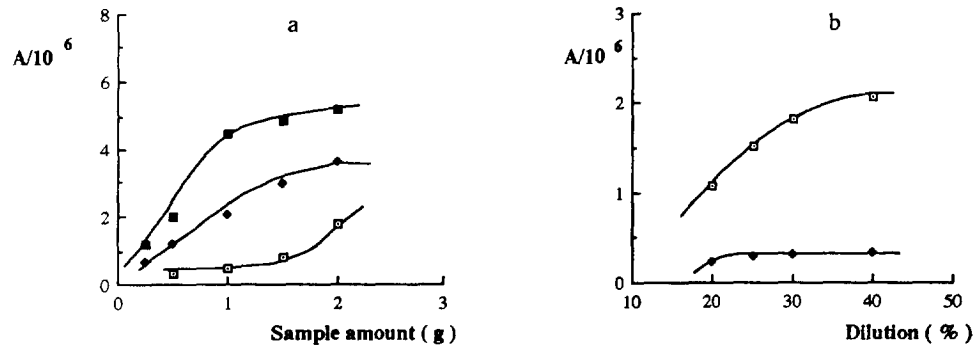

Fig. 3. Influence of sample amount. (a) Milk powder: □ = Vitamin A; ◆ = vitamin D_3; ■ = vitamin E. (b) Liquid milk; □ = Vitamin A; ◆ = vitamin E.

3.1. Optimization of variables

To automate the process, it is necessary to optimize some of the variables affecting the different steps of the system. These are:

Amount of sample: In the case of powdered milk, solutions containing 0.5–2 g were prepared in 25 ml of water, while liquid milk samples were diluted with water from 10 to 50%. The results obtained are shown in Fig. 3. For the working conditions employed, samples of powdered milk of 2 g and liquid milk diluted at 30% were chosen. The fat content of the milk powder is lower than the fat content of the liquid milk. Accordingly, larger amounts of milk powder produce a higher signal for the three vitamins. However, problems of C_{18} cartridge obstruction arose when the amount of the samples was large. In the case of liquid milk two effects are present: a larger dilution should give a smaller signal; however, alkaline hydrolysis is more complete for a larger dilution and the signal is higher, probably because vitamin-fat bonds are broken without difficulty; furthermore, no obstruction of the C_{18} cartridges is produced.

Concentration of acetic acid: The strongly alkaline hydrolysis reagent should be neutralized before it arrives at the cartridge or it will destroy the C_{18} packing. In this way, possible corrosion of the stainless-steel tubes of the injection valves of the system is also avoided. To achieve this, a third channel was introduced through which a solution of acetic acid was passed. Experiments were performed with concentrations of acetic acid ranging from 1 to 3 *M* in order to reach pH values close to 7. As can be seen in Fig. 4, for concentrations of acetic acid ranging from 2.4 to 2.8 *M* the pH of the resulting solution was around 6. Acetic acid of 2.5 *M* was chosen as the working concentration.

Flow-rate: Flow-rate affects the hydrolysis of the fat material since a contact time between the sample and the hydrolysis reagent is required; it also affects the retention of the vitamins in the C_{18} cartridge. Different flow-rates in the 0.5–2.0 ml/min range were tried. As can be seen in Fig. 5, the best results were obtained for flow-rates of 1.25 ml/min, and hence, this was used as the optimum value.

Washing mixture and washing time: The mixture employed for washing was methanol–water.

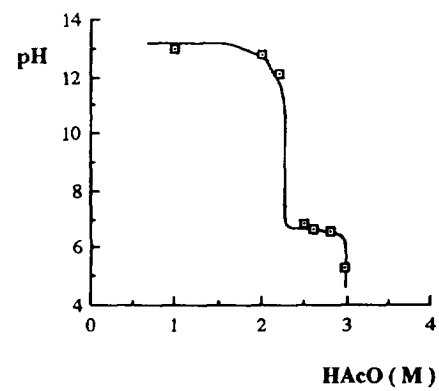

Fig. 4. Influence of acetic acid concentration on neutralization of the hydrolyzate.

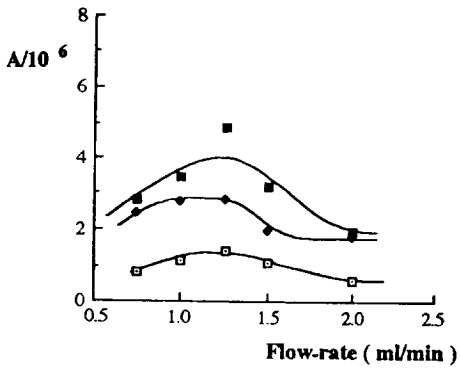

Fig. 5. Influence of flow-rate. □ = Vitamin A; ◆ = vitamin D_3; ■ = vitamin E.

The composition of this mixture and the washing time are important variables to be controlled since if a mixture whose composition in methanol is too high is used, the vitamins may elute in the washing step. Mixtures of water–methanol ranging between 50:50 and 0:100 and washing times between 2 and 5 min were tried (Fig. 6). The best results were obtained with a water–methanol (60:40) washing mixture and a washing time of 4.0 min.

Preconcentration time: Extraction of the vitamins from the non-saponifiable material of the hydrolyzate is performed by the SPE cartridge inserted in the injection loop, at the same time performing a preconcentration of the analytes. It is therefore important to control the time during which the hydrolyzate passes through the cartridge, the preconcentration time, since the amount of vitamins extracted will be proportional to this time. Preconcentration times ranging from 1.0 to 6.0 min were investigated, as can be seen in Fig. 7. For times above 5 min, the analytical signal remained almost constant. Moreover, times above 6 min led to obstructions in the cartridge. Accordingly, a time of 5.0 min was chosen as the most suitable preconcentration time.

Elution time: The vitamins are eluted with methanol and are transported by the flow system to the loop of the injection valve of the chromatograph automatically. It is therefore important to control the time of elution since on one hand the liposoluble vitamins must be eluted and, on the other, the richest fraction should fill the 100 μl of the loop to be injected. Elution of the vitamins is a function of their polarity; first vitamin A is eluted and then D_3 and E, which are less polar. So, optimal elution of all three vitamins had to be determined. As can be seen in Fig. 8, a maximum in the signal for vitamins D_3 and E occurred at around 4.2 min while the maximum for vitamin A appeared before this time. However, other substances retained by the cartridge also eluted before vitamin A, potentially overlapping partly with this vitamin (Fig. 9). The optimum elution time chosen was 4.0 min.

Optimization of chromatographic variables: In a previous work [8] a systematic study of the variables affecting the chromatographic system was described. The mobile phase was water–

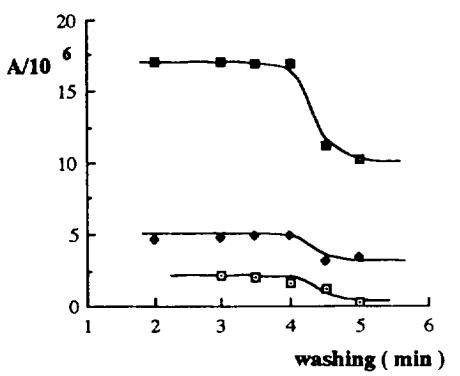

Fig. 6. Influence of washing time. □ = Vitamin A; ◆ = vitamin D_3; ■ = vitamin E.

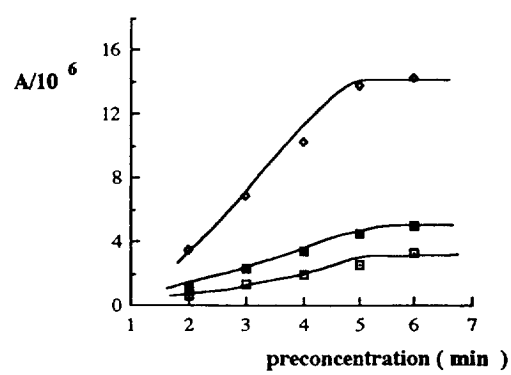

Fig. 7. Preconcentration time. □ = Vitamin A; ◇ = vitamin D_3; ■ = vitamin E.

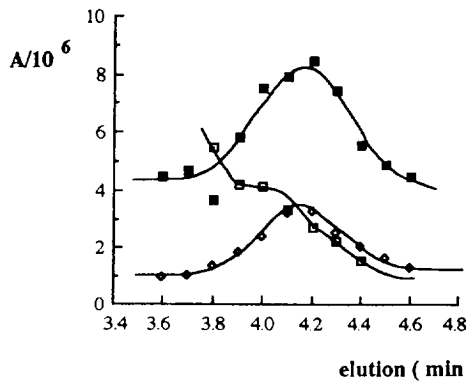

Fig. 8. Elution time. □ = Vitamin A; ◇ = vitamin D_3; ■ = vitamin E.

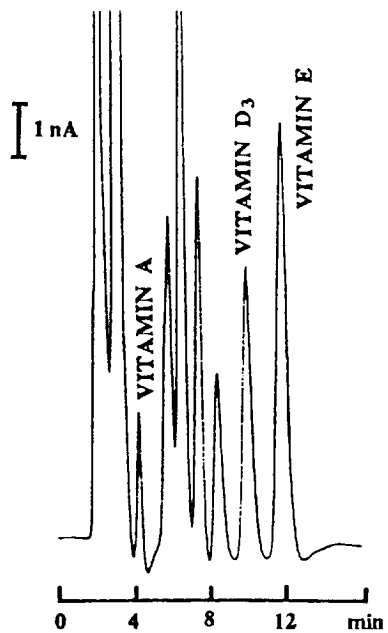

Fig. 9. Chromatogram obtained after application of the proposed method to a sample of milk powder.

methanol (1:99) with 2.5 m*M* acetic acid–sodium acetate, at a flow rate of 1.0 ml/min.

3.2. Analytical application

With the proposed system we obtained the corresponding calibration curves for samples containing the three vitamins at amounts similar to those contained in the milk samples. The calibration curves are shown in Table 1.

The detection limits (signal-to-noise ratio 3) obtained under the proposed working conditions were the following: $3.49 \cdot 10^{-8}$, $1.77 \cdot 10^{-6}$ and $3.11 \cdot 10^{-7}$ *M* (0.10, 6.8 and 1.34 ng injected) for vitamins A, D_3 and E, respectively.

The precisions of the method obtained with 10

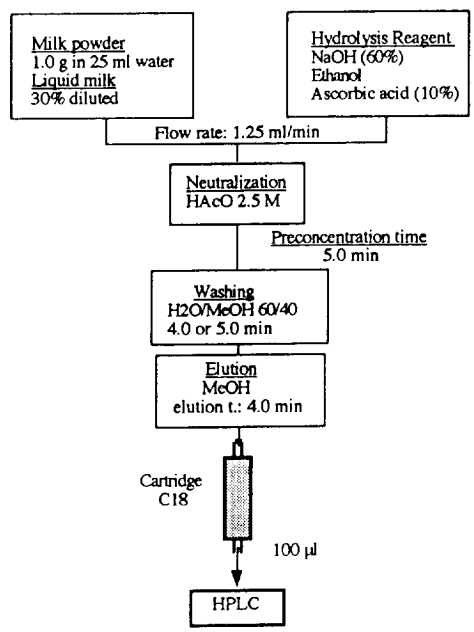

Fig. 10. Scheme of proposed procedure.

Table 1
Calibration fits: area (units) = $a + bC$, where C is concentration in *M*

Vitamin	*a*	*b*	Correlation coefficient
Vitamin A	$(2.90 \pm 3.2) \cdot 10^{5}$	$(8.73 \pm 0.13) \cdot 10^{12}$	0.9997 ($n = 7$)
Vitamin D_3	$(5.62 \pm 4.96) \cdot 10^{4}$	$(4.32 \pm 0.08) \cdot 10^{11}$	0.9995 ($n = 7$)
Vitamin E	$(1.92 \pm 0.95) \cdot 10^{4}$	$(6.19 \pm 0.14) \cdot 10^{11}$	0.9992 ($n = 7$)

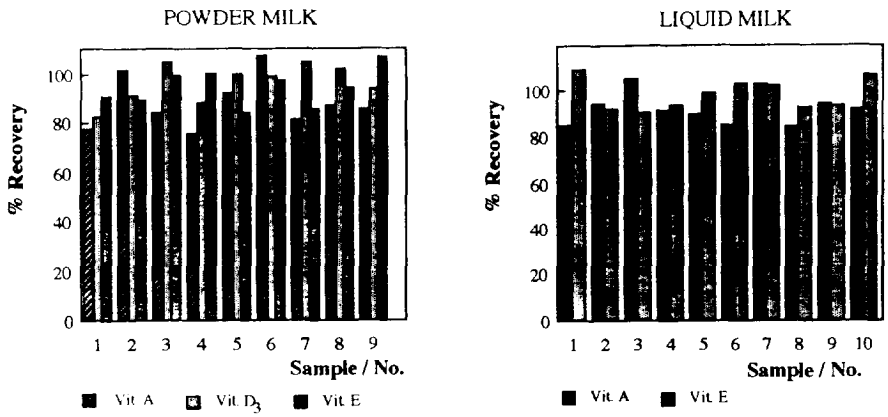

Fig. 11. Recoveries of the proposed method for samples of milk.

samples containing $1.18 \cdot 10^{-6}$ *M* of vitamin A, $3.85 \cdot 10^{-6}$ *M* vitamin D_3 and $3.10 \cdot 10^{-6}$ *M* of vitamin E were 3.8, 5.0 and 3.7%, respectively.

3.3. Analysis of milk samples

The procedure proposed for automatic determination of vitamins A, D_3 and E is shown in Fig. 10. Fig. 10 also specifies the conditions for the analysis of milk samples. The proposed method was applied to nine samples of infant formula milk and ten samples of liquid cow's milk. An example of the chromatograms obtained is shown in Fig. 9. Application of this procedure affords recoveries in the 88–105% range (Fig. 11), vitamins D_3 and E being those having the best recovery factors. The results obtained regarding the contents of the corresponding vitamins are shown in Tables 2 and 3.

The vitamin contents obtained in the powdered milk can be compared with those quoted by the manufacturer although it was not possible to do the same with the liquid milk samples because this value was not available for the commercial products.

The day-to-day precision was obtained by replicate analyses ($n = 10$) of powdered milk and liquid milk, the values found being (R.S.D., %):

Table 2
Determination of vitamins A, D_3 and E in different powdered milk samples (results from three replicate analyses)

Sample No.	Vitamin A (μg/100 g)		Vitamin D_3 (μg/100 g)		Vitamin E (mg/100 g)	
	Found	Quoted by supplier	Found	Quoted by supplier	Found	Quoted by supplier
1	394 ± 4	450	10.4 ± 0.3	10	3.0 ± 0.1	2.7
2	458 ± 3	450	13.6 ± 0.7	10	2.8 ± 0.2	2.7
3	412 ± 7	450	2.3 ± 0.9	3	4.7 ± 0.8	4.4
4	503 ± 3	550	8.7 ± 0.3	7.5	4.5 ± 1.7	5.5
5	409 ± 5	450	8.2 ± 0.2	10	3.5 ± 0.2	3.5
6	481 ± 4	465	8.5 ± 1.5	7.7	4.2 ± 0.8	5.7
7	421 ± 7	453	8.3 ± 1.4	7.5	5.3 ± 0.8	6
8	440 ± 2	456	6.1 ± 0.8	7.6	8.9 ± 0.7	8.6
9	479 ± 6	450	12.4 ± 2.3	10	4.0 ± 0.3	4

Table 3
Determination of vitamins A, D_3[a] and E in different liquid milk samples (results from three replicate analyses)

Sample	Vitamin A (μg/100 ml)	Vitamin E (μg/100 ml)
1	24.9 ± 0.3	67.8 ± 3.4
2	48.8 ± 0.7	93.4 ± 0.9
3	31.8 ± 0.2	170 ± 0.7
4	49.1 ± 0.3	145 ± 2.0
5	50.9 ± 0.4	141 ± 8.5
6	42.9 ± 0.7	193 ± 2.5
7	37.8 ± 3.3	118 ± 0.8
8	36.2 ± 0.8	125 ± 7.5
9	55.0 ± 0.9	165 ± 3.4
10	80.9 ± 0.3	91.4 ± 0.9

[a] Vitamin D_3 was not detected in samples of liquid cow's milk because of the low amounts present.

powdered milk: vitamin A, 3.9; vitamin D_3, 6.8; vitamin E, 5.4; liquid milk: vitamin A, 5.1; vitamin E, 1.2.

In all instances, good reproducibility of the analyses and acceptable relative standard deviations were found.

4. Conclusions

A method has been set up for the automatization of the analysis of liposoluble vitamins in milk; the method includes sample treatment and HPLC determination. In this way, sample treatment is very rapid; a sample takes some 25 min since its introduction into the system until evaluation of the corresponding chromatogram. The reproducibility of the method is very acceptable, as may be deduced from the values obtained in the day-to-day precision. The automatization of the method and its precision make it suitable for routine milk (liquid and powdered) analysis.

Acknowledgement

Financial assistance from the DGICYT (Ali 93-1164) is gratefully acknowledged.

References

[1] R.S. Mills, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 56.
[2] S.F. O'Keefe and P.A. Murphy, *J. Chromatogr.*, 445 (1988) 305–309.
[3] A. Takeuchi, T. Okano and N. Tsugama, *J. Micronutr. Anal.*, 4 (1988) 193.
[4] W.O. Landen, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 183.
[5] W.O. Landen, D.M. Hines, T.W. Hamill, J.I. Martin, E.R. Young, R.R. Eitenmiller and A.M. Soliman, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 509.
[6] M.L. Grace and R.A. Bernhard, *J. Dairy Sci.*, 67 (1984) 1646.
[7] W. Ye, L. Liu and H. Li, *Sepu*, 10 (1992) 2240.
[8] M.M. Delgado Zamarreño, A. Sanchez Perez, C. Gomez Perez and J. Hernandez Mendez, *J. Chromatogr.*, 623 (1992) 69.
[9] E.J. de Vries and B. Borsje, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 1228.
[10] D.C. Sertl and B.E. Molitor, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 177.
[11] X. LI, L. Zhang and J. Zuo, *Yingyang Xuebabo*, 5 (1983) 103; *C.A.*, 99 (1983) 103811p.
[12] H. Johnsson and H. Hessel, *Int. J. Vitam. Nutr. Res.*, 57 (1987) 357.
[13] V.K. Agarwal, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 19.
[14] C.J. Lammi-Keefe, *J. Pediatr. Gastrenterol. Nutr.*, 5 (1986) 934.
[15] W. Knifel, F. Ulbert and U. Winkler-Macheiner, *Dtsch. Lebensm-Rundsch.*, 83 (1987) 137; *C.A.*, 107 (1987) 95416c.
[16] S.L. Reynolds and H. Judd, *Analyst*, 109 (1984) 489.
[17] A.F. Wickroski and L.A. Mc Lean, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 62.
[18] J. Ballester, E. Cortes, M. Moya and M.J. Campello, *Clin. Chem.*, 33 (1987) 796.
[19] H. Johnsson, B. Halen, H. Hessel and A. Nyman, *Int. J. Vitam. Nutr. Res.*, 59 (1989) 262.
[20] C. Bauer, J. Mattusch, B. Vorger and G. Werner, *Lebensmittelchemie*, 45 (1991) 110; *Anal. Abstr.*, 55 (1993) 6H195.