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Column liquid chromatography determination of vitamins A and E in powdered milk and local flour: a validation procedure

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Abstract

A high-performance liquid chromatography method was developed for the simultaneous routine determination of vitamins A and E in powdered milk and flour made from local plants and purchased from open markets in the Ivory Coast. The method involves saponification followed by extraction with a mixture of organic solvents. The vitamins were resolved by reversed-phase HPLC and detection at a single wavelength. The main tests of method validation were applied to the procedure. The results show the reliability of the analytical method for the intended application.

Keywords: Validation; Vitamins; Food analysis

1. Introduction

Due to the nutritional importance of vitamins A and E in the health and growth of children in developing countries, research has been performed to develop a simple and sensitive method for their routine determination in foodstuffs in Ivory Coast [1]. Several methods for the separate determination of vitamin A [2–8] and vitamin E [3,4,9–12] are described in the literature, as well as methods for their simultaneous determination [3,4,13–16]. High-performance liquid chromatographic methods are claimed to have greater specificity than colorometric and fluorimetric procedures [5,10]. Sample treatment for HPLC analysis can be performed by different procedures, such as direct extraction by organic solvents, saponification followed by extraction with organic solvent or enzymatic hydrolysis [8,14,11]. Normal and reverse-phase HPLC separation are equally used [4], normal-phase separation usually being a complicated procedure in routine determination practice such as in our case. Although the chosen method with saponification and extraction as sample treatment followed by reverse phase chromatography and UV detection is not new [4,13,14], it was found to give a satisfactory separation of the vitamins particularly vitamin E isomers in less than 15 min at a single wavelength. This particularity in routine determination, seems important since α-tocopherol is the tocol with the highest vitamin E activity in food of animal origin and vitamin E activity derives also from the other isomers in food of plant origin [11]. The linearity, specificity, precision, accuracy and sensitivity of the method should

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be sufficiently acceptable to permit quantification of these vitamins in powdered milk and flour [17].

2. Experimental

2.1. Materials

Extracts were evaporated in a Büchi rotary evaporator (Flavil, Switzerland). The chromatographic system used for validation consisted of a 5010 Hewlett Packard (Waldbronn, Germany) pump attached to a Model 7010 Rheodyne injection valve fitted with a 20 μl loop, a Waters (Saint Quentin en Yvelines, France) Model 486 tunable absorbance detector, a Gilson (Villiers le Bel, France) Model 234 autoinjector, a Gilson Model 101 column oven and a Pye Unicam (Cambridge, UK) Model PU 4880 integrator. Conical-shaped disposable plastic vials of 700 μl volumes were used. Peak homogeneity and selectivity were assessed with a Waters Model 510 pump fitted with a 20 μl injection loop coupled to a Waters Model 991 diode array detector (DAD), a Waters model 5200 printer and a Powermate SX plus Nec data handling system. Separation was carried out on a Lichrosorb (Merck, Darmstadt, Germany) RP 18 cartridge (125 mm×4.5 mm I.D.) packed with 5 μm particles.

Absolute ethanol, diethylether, petroleum ether, methanol and sodium hydroxide (analytical reagent grade), acetonitrile (HPLC grade) and Milli-Q water were used. Vitamin A (retinol 'all trans' ref. R 7632) and vitamin E isomers (α-tocopherol ref. T 3251; γ-tocopherol ref. T 1782 and δ-tocopherol ref. T 2028) were purchased from Sigma (Saint-Quentin Fallavier, France).

Solutions of sodium hydroxide (50% w/v in water); hydroquinone (20% m/v in ethanol); separate stock solutions of retinol and tocopherols (1 g/l in ethanol) may be stored for up to 15 days in the dark at 4°C; two mixed working standard solution of 2.5 mg/l of retinol and 5 mg/l of each tocopherol isomer; 5 mg/l of retinol and 20 mg/l of each tocopherol isomer in ethanol, prepared daily by dilution of the stock solution are to be used within 6 h of preparation at room temperature.

2.2. Method and validation procedure

Samples analysed were soy flour prepared from local grains and powdered milk purchased from a local market in Abidjan (Ivory Coast). Carry out sample handling in such a way as to protect samples from light and avoid temperature degradation. Weigh accurately approximately 1.25±0.01 g of powdered milk or soy flour in a 100 ml conical flask. Add 5 ml of a 50%(m/v) aqueous solution of sodium hydroxide. Heat in a water bath maintained at 30°C±2°C for 3 min. Add 25 ml of ethanol, 0.5 ml of a 20% (m/v) hydroquinone solution. Place in a water bath thermostated at 80°C±2°C for 30 min. Then, allow to cool under a stream of water and transfer content to a 250 ml separatory funnel. Add 25 ml of water and shake manually, add 12.5 ml of diethylether and 12.5 ml of petroleum ether, shake manually for 1 min. then allow the phases to separate. Drain the lower aqueous phase and withdraw the upper organic phase which contains the vitamins (retinol and tocopherol). Repeat the procedure. Combine organic phases and wash twice with 25 ml of water. For milk samples, filter the organic phase through a cellulose filter. Evaporate the extracts to dryness in a rotavapor at 40°C under vacuum. Dissolve the residue in 2 ml of methanol. This solution is the test solution to be injected within 5 h following preparation at room temperature.

Chromatographic method was carried out as described in the following steps. Inject the standard and test solutions in duplicate and in a bracketing sequence . Conduct the LC separation at 0.8 ml/min. Use acetonitrile as mobile phase and detect at 292 nm. Determine the content in retinol (μg/100 g); α, (β+γ), δ-tocopherol and total tocopherols (mg/100 g).

Representative chromatograms from a mixed standard solution and test solutions of milk are given in Figs. 1 and 2. Under the conditions used, retinol is eluted between 3–4 min, tocopherol isomers between 8–13 min.

Concerning solution stability studies, the response factor (RF=concentration/peak area) of a standard solutions of retinol and tocopherol prepared in duplicate (10 mg/l of retinol and 20 mg/l of each tocopherol) and kept at ambient temperature and under diffuse light were respectively compared to

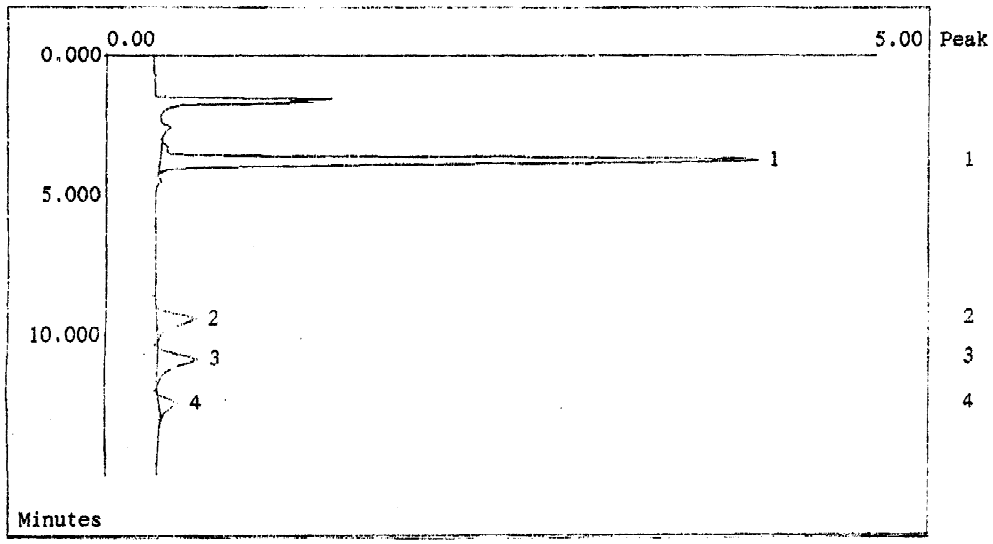

Fig. 1. Chromatogram of a mixed standard solution of 10 mg/l of retinol and 20 mg/l of tocopherol isomers. Peaks: 1=retinol; 2=δ-tocopherol; 3=$\beta+\gamma$-tocopherol; 4=α-tocopherol.

those of standard solutions freshly prepared. The RF of a standard solution of retinol and tocopherol kept at 4°C were also compared at different time intervals over a period of 15 days. The same procedure was applied to test solutions over a period of 5 h at ambient. temperature and under diffuse light. Retinol and total tocopherol content was determined by reference to a standard solution freshly diluted on each occasion.

Selectivity was evaluated by the freedom of interference of potential degradation products originated from photo-decomposition or thermodegradation of the vitamins under stress conditions. The photo-decomposition was tested by exposing a glass

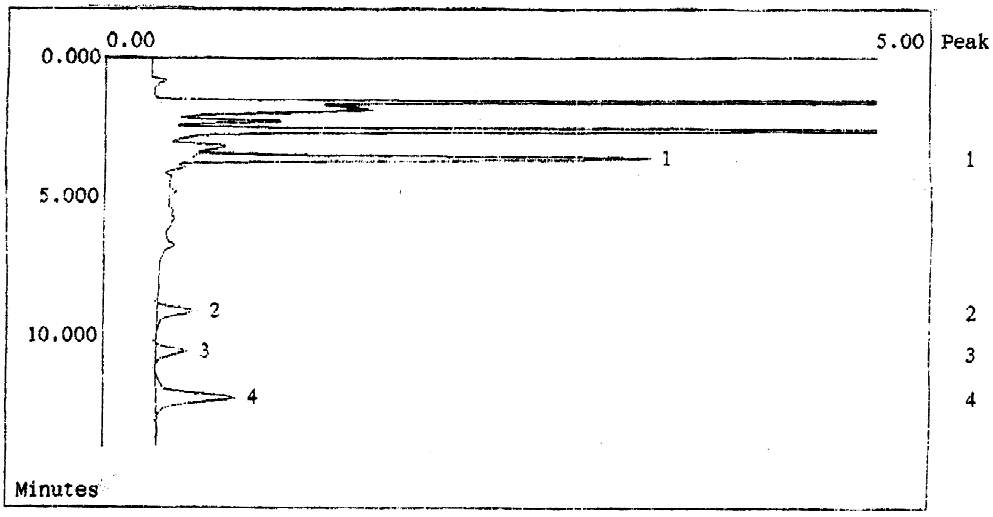

Fig. 2. Chromatogram of a milk extract. Peaks: 1=retinol; 2=δ-tocopherol; 3=β+γ-tocopherol; 4=α-tocopherol.

vial containing a standard solution of retinol (10 mg/l) and tocopherols (50 mg/l) at a distance of about 10 cm from a lamp emitting at 350 nm. Thermal degradation was investigated by placing a standard solution of 10 mg/l of retinol and 20 mg/l of each tocopherol isomer in a oven regulated at 45°C±2°C. An aliquot of each type of solution was withdrawn at various time intervals and analysed by HPLC using a DAD. Peak purity testing was assessed applying spectral overlay (SO), spectral suppression (SS) and absorbance ratio (AR) techniques on a DAD.

The linearity of the peak area versus retinol or total tocopherol concentration was assessed using five mixed standard solutions of: 0.5 to 50 mg/l of retinol and 1 to 50 mg/l of each of the three fractions of tocopherol α, (β+γ), δ-tocopherol. The linearity of the analytical procedure and recovery studies were assessed using the method of standard additions. On each day, spiked samples of milk and flour were prepared as follows: an accurately weighed amount of about 1.25 g of milk and soy flour was spiked with retinol (250–1000 μg/100 g) and tocopherol (2–25 mg/100 g) at four different concentration levels. Quantitation of retinol and total tocopherol in spiked and unspiked matrices was carried out on each of the three days by reference to a mixed standard solution of retinol (10 mg/l) and of tocopherol (20 mg/l each). The determination of retinol and total tocopherol content of a commercial infant powdered milk, with labelled doses of vitamin A and E, was also carried out.

The precision was appreciated by inter and intra serial determination method. Six successive injections of mixed standard solutions at three concentration levels corresponding to respectively 2, 10 and 20 mg/l of retinol and 6, 30 and 60 mg/l of total tocopherol were performed. The same procedure was applied to milk and soy flour extracts. In order to verify the repeatability of the procedure, unspiked samples of milk and flour were analysed by applying the whole analytical procedure six times successively (six independent extractions). The vitamin A and E content were determined by reference to a mixed standard solution of retinol and tocopherol. The mean area duplicate injections was used for calculations. Inter-day precision was estimated from the linearity graphs of the procedure ($n=3$ days)

Limit of detection (LOD) was evaluated as the lowest concentration of a mixed standard solution of retinol and tocopherol which gave a signal-to-noise ratio of three.

Limit of quantitation (LOQ) was evaluated as the lowest concentration of a mixed standard solution of retinol and tocopherol which gave a signal-to-noise ratio of ten. The repeatability of measurement of a mixed standard solution at the LOQ concentration was assessed by performing six successive injections of the same solution.

3. Results and discussion

Analysis of the data concerning the stability of solutions (Table 1) showed that standard solutions of retinol and tocopherol can be kept at ambient temperature for at least 24 h without noticeable degradation. More than 98% of the concentration of

Table 1
Stability data (expressed as RF[a]) of standard solutions of retinol and tocopherol kept at ambient temperature

	$t=0$ h	$t=2$ h	$t=3$ h	$t=6$ h	$t=24$ h
Retinol	0.17	0.18	0.18	0.18 (98.88%)[b]	0.19
δ-Tocopherol	1.10	1.10	1.09	1.10 (100.09%)[b]	1.11
β,γ-Tocopherol	0.70	0.70	0.70	0.70 (99.43%)[b]	0.71
α-Tocopherol	1.28	1.29	1.29	1.29 (99.53%)[b]	1.30

[a] Response factors=concentration (mg/l)/peak area.
[b] Percentage of vitamin concentration at $t=6$ h/$t=0$ h.

all the components was found after 6 h at ambient temperature. The same standard solution kept in the dark at 4°C did not show any noticeable degradation for at least 7 days. Extracts of milk and flour were stable up to 5 h at ambient temperature with 90 to 99% of the initial content of the different vitamins found after 5 h (Table 2).

The study of selectivity showed a dramatic decrease of the retinol peak observed after only 3 h of irradiation at 350 nm. Very little retinol remained after 24 h of irradiation. Tocopherols were less sensitive to light with a decrease of less than 10% after 24 h of irradiation at 350 nm. Solutions kept at 45°C for 6 h showed a small degradation (about 10 to 15%) with an increased absorption of the dead volume. The same observation was found for test solutions (milk extracts) submitted to the same stress conditions. This showed that recommendations related to light and heat protection should be included in the procedure for sample handling, particularly for retinol and α-tocopherol in test solutions.

Peak homogeneity is also a way to determine selectivity. The overlay of retinol spectra captured automatically at the apex, up slope and down slope from a test solution was good. Absorbance ratios recorded across the chromatographic peak with two pairs of wavelengths did not give any significant deviation in the ratio under the conditions used. However, because one difficulty of this technique is in discriminating between impurities and spikes at the edges of the chromatographic peak (due to the low absorbance measured), spectral suppression is a useful complementary technique: any residual peak, which may be observed as a positive or negative incursion in the chromatogram gives an indication of the presence of another component. In our study, the spectral suppression constants were calculated from the spectrum of retinol and each tocopherol isomers in the mixed standard solution, respectively for two pairs of wavelengths. The difference absorbance for each nutrient was measured on the standard solution and then on test solutions of milk or flour. Applying the same difference functions to a test solution of milk or flour did not give any significant deviation in the baseline at the retention time of all components, which means that there was no detectable interference from the matrix under the analyte peak.

Table 2
Stability data of retinol and tocopherols (expressed in mg) of test solutions from milk kept at ambient temperature

	$t=0$ h	$t=2$ h	$t=5$ h	
Retinol	3.53	3.49	3.23	(91.50%)[a]
δ-Tocopherol	7.63	7.48	7.36	(96.46%)[a]
β,γ-Tocopherol	6.55	6.32	6.30	(96.18%)[a]
α-Tocopherol	33.99	33.80	33.75	(99.29%)[a]

[a] Percentage of vitamin concentration at $t=5$ h/$t=0$ h.

In this study, the linearity of peak area versus retinol and tocopherol concentration (mg/l) were examined. For retinol, the regression line calculated using the least squares method was: Area= $0.20\pm3.33+(6.04\pm0.16)$ conc. with the confidence intervals calculated at $P=0.05$. The determination coefficient was $r^2=0.9996$. An ANOVA was used to confirm the linearity which was highly significant. $F_{cal}=13504.09$ ($P<0.0001$). Because the graph passed through the origin, a single concentration can be used as the standard solution in the procedure. For tocopherol, the regression line calculated with the same method was: Area= $-0.29\pm0.69+(0.35\pm0.01)$ conc. The determination coefficient was $r^2=0.9997$. The linearity, confirmed by an ANOVA was significant $F_{cal}=15829.39$ ($P<0.0001$). In this case also, a single concentration can be used as the standard solution in the procedure because the graph passed through the origin.

The accuracy and linearity of the procedure was assessed using the method of standard additions. In our study, the linearity graph (peak area vs concentration added) for vitamin A and E allowed the calculation of their initial content in the test sample from the extrapolated graph. Constructing the graph on each of three days allowed the evaluation of inter-day precision. Statistical analysis was carried out as follows: Variance homogeneity was assessed on recovery data to see if there was a systematic day-to-day effect on the results. As such effect was not observed, a common graph $1X-3Y$ was plotted for retinol and tocopherol for both milk and flour with X=concentration added of vitamin A (μg/100 g) or concentration added of vitamin E (mg/100 g), and Y=peak area. The determination coefficient was

Table 3
Precision of the method

Standard	Retinol standard solution (mg/l)	RSD (%)	Tocopherol standard solution (mg/l)	RSD (%)
Repeatability of the chromatograhic system (n=6)				
	2	0.42	6	0.70
	10	0.20	30	0.55
	20	0.13	60	0.62
Sample	Retinol (μg/100 g)		Tocopherol (mg/100 g)	
milk	758.60	0.60	7.37	1.94
flour	–	–	31.05	1.20
Repeatability of the entire procedure (n=6)				
milk	788.67	4.42	7.40	2.29
flour	–	–	30.10	4.48

calculated for each graph ($r^2 \geq 0.9950$) and the ANOVA used ($F_{cal} > 9000$ for $P < 0.0001$) confirmed the linearity of retinol and tocopherol in each case.

A common graph of accuracy for each component was also plotted in milk and in flour. All these graphs went through the origin and the confidence intervals for the slopes show that the slopes are very close to unity ($P = 0.05$). Recoveries at each concentration, more than 90%, were acceptable for the intended application of the method. These results demonstrate that samples of milk and flour can be analysed against a mixed standard solution of retinol and tocopherol in ethanol and that standard additions are not required.

The determination of vitamin A and E levels of a commercial infant milk powder by this method gave 554.39 μg/100 g of vitamin A and 3.84 mg/100 g of vitamin E for a labelled dose of 574 μg/100 g of vitamin A and 3.8 mg/100 g of vitamin E.

The repeatability of the chromatographic system was better than 2% for standard and test solutions. The repeatability of the entire procedure for milk and flour extraction was better than 5% (Table 3). In this study, inter-day precision was evaluated on the whole concentration range and for each of the two nutrients by performing standard addition method on each of the three days. This approach was found to be sound.

The LOD (signal-to-noise ratio = 3) of each nutrient, determined from a mixed standard solution of retinol and tocopherol isomers, was 0.02 mg/l (4 μg/100 g) for retinol and respectively 0.036 mg/l ($7.2 \cdot 10^{-3}$ mg/100 g), 0.026 mg/l ($5.2 \cdot 10^{-3}$ mg/100 g) and 0.037 mg/l ($7.4 \cdot 10^{-3}$ mg/100 g) for δ, β+γ and α-tocopherol isomers.

The LOQ (signal-to-noise = 10), determined from a mixed standard solution was 0.06 mg/l (12 μg/100 g) for retinol with a RSD of 9.6% ($n = 6$ injections) and of respectively 0.08 mg/l (0.016 mg/100 g), 0.07 mg/l (0.014 mg/100 g) and 0.11 mg/l (0.023 mg/100 g) for δ, β+γ and α-tocopherol isomers. The RSD values were respectively 10.18%, 9.96% and 11.8% ($n = 6$ injections).

The chromatographic method developed, allows resolution of retinol and tocopherols in a single chromatographic run. The results of the validation procedure show that the method proposed is specific, precise, accurate and sensitive for the routine determination of vitamins A and E in ivorian food consumed by children in Ivory Coast.

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