J Fluoresc (2008) 18:487–497
DOI 10.1007/s10895-007-0290-1

ORIGINAL PAPER

Improvement in Retinol Analysis by Fluorescence and Solid Phase Extraction (SPE) in Micellar Medium

M. Torre · M. Sánchez-Hernández · S. Vera · M. P. San Andrés

Received: 26 September 2007 / Accepted: 14 November 2007 / Published online: 16 January 2008

Abstract In this paper, the determination of retinol, the structure with the most activity as vitamin A, was carried out in an aqueous micellar medium with a low quantity of a short-chain alcohol. The analytical technique used in this work was fluorescence, which gave us very much information qualitative and quantitative. The sensitivity of the method is higher than that obtained in other media; the detection limit is 0.03 mg L^{-1} and retinol was stable in solution for at least 5 days. The use of solid phase extraction (SPE) for organic samples, allowed us to change the organic matrix by a mixture CTAB 5%/*n*-butanol 10%/water 85% w/w/w with recoveries in retinol spiked samples close to 100%. In addition, the combination of SPE and fluorescence is a good preconcentration technique, sensitive and fast for the identification and determination of retinol, simultaneously.

Keywords All-*trans*-retinol · Micellar medium · Fluorescence · SPE extraction · Quantitative analysis

Introduction

Retinol is the main structure of the broad group of lipophilic related compounds known as vitamin A. The vitamin activity of other retinoids and carotenoids are calculated respect to the corresponding effect of retinol, that posses 100% vitamin A activity, after their transformation. Retinol plays an important role in vision, skin protection, bone growth, and cell division and differentiation [1].

Nowadays, the vitamin A activity of foods and pharmaceutical preparations is measured as retinol equivalents (RE) in micrograms instead of international unities (IU) [2]. The human body obtains the vitamin A from food as preformed retinol or as provitamin carotenoids that can be converted to retinol in the body. It was determined that 6 μg of β-carotene are necessary for the production of 1 μg of retinol or 1 RE.

The most often used analytical technique for determination of retinol and other vitamins has been high-performance liquid chromatography (HPLC) [3, 4]. HPLC determination of retinol has been applied to food products [5–10], plants [11], biological fluids, tissues or livers [12–23], etc. Given the fact of the high hydrophobicity of most vitamin A compounds, the employ of reversed-phase HPLC requires the use of mobile phases containing methanol, acetonitrile or its mixtures with a small or null proportion of water [5, 8–11, 13, 15, 17, 20, 21–23]. On the other hand, the use of normal-phase HPLC, with mobile phases such as *n*-hexane/isopropanol, *n*-heptane/tert-butyl methyl ether, etc., improved the chromatographic separation of non-polar retinoid isomers [6, 7, 18, 24].

One important characteristic of the retinol molecule is its native fluorescence. Very few works have studied the possibilities of retinol determination based on the measurement of this analytical property. The retinol determination by fluorescence measurements has been applied previous saponification and extraction of the sample and measurements in hexane solutions [4, 25–27]. One recent and quite different application of the fluorescence technique is the discrimination of different cheeses based on the fluorescence spectra of thin slides of cheeses recorded at the excitation wavelengths of tryptophan, riboflavin and vitamin A [28–30].

M. Torre · M. Sánchez-Hernández · S. Vera · M. P. San Andrés (✉)
Department of Analytical Chemistry, Faculty of Chemistry, University of Alcalá,
Ctra. Madrid-Barcelona km 33,6,
28871 Alcalá de Henares, Madrid, Spain
e-mail: mpaz.sanandres@uah.es

It is well known that all-*trans*-retinol is almost insoluble in water, chemically labile and susceptible to photo-oxidation [31]. For this reason, stabilization of this vitamin by polymeric micelles [32], cyclodextrins [33], liposomes [34] or even lipid particles [35] has been proposed.

Our investigation group has studied the influence of organized media on the stability of vitamins and their analytical determination by fluorescence. Different water-soluble and fat-soluble vitamins have been determined in different micellar media. The water-soluble vitamins were determined in bis-2-ethylhexylsulfosuccinate sodium salt (AOT) aqueous medium by synchronous fluorescence [36]. The fat-soluble vitamins A and E were determined in a total aqueous micellar medium of sodium dodecylsulfate (SDS), hexadecyltrimethylamonium bromide and chloride (CTAB and CTAC), and polyoxietylen(23)lauryl ether (Brij 35). The presence of cationic micelles (CTAB) and nonionic micelles (Brij 35) increased the sensitivity and allowed the simultaneous quantification of vitamins A (retinol) and E in milk samples; however, in the presence of anionic micelles of SDS the fluorescence signal of retinol was much lower [37].

In addition, we have developed an analytical screening method for fat-soluble vitamins, including vitamin A [38]. This screening method made possible to verify the presence of vitamin A (retinol), E, B_1, B_2 and B_6, in a medium of direct micelles of CTAC in pure water. The method was validated in qualitative terms and it was applied to the detection of the vitamins in pharmaceuticals, isotonic drinks and fruit juices samples.

In order to improve the signal of retinol obtained in CTAB solutions in pure water, our objective in this work was to study the influence of the addition of little concentrations of a short chain alcohol to the micellar solution upon the fluorescence signal of retinol. This way, it would be possible the easy and fast analysis of retinol in samples fortified or prepared with this vitamin.

In addition, the high quantity of water in the medium of analysis reduced the employ of high amounts of organic solvents normally used in these methods (chloroform, hexane, petroleum ether, dichloromethane, etc). Given the fact of the high water quantity of the measurement media, for samples of organic matrices, the solid-phase extraction (SPE) was proposed for both vitamin preconcentration as well as a solvent change technique.

Experimental

Reagents

All reagents used in this work were of analytical grade. The standard used was all-*trans*-retinol, from Fluka (Madrid, Spain). Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

The surfactant hexadecyltrimethylamonium bromide (CTAB) was from Merck (Darmstadt, Germany). Methanol, *n*-propanol, *n*-butanol and hexane, from Scharlab (Barcelona, Spain), were of HPLC grade.

Stock solutions of retinol were prepared by dissolving the appropriate quantity of this standard in methanol. These solutions were stored in dark flask at −18 °C.

The working solutions of retinol for calibration were prepared daily by diluting suitable volumes of stock solutions of retinol into the optimum micellar medium of measurement. These solutions were stored in the refrigerator at 4 °C until its use.

In addition, diluted solutions of retinol used for the recovery studies with solid phase extraction were prepared by diluting appropriate aliquots of the retinol stock solution into hexane.

Two samples of vegetable oil were studied: sunflower oil (Emile Noël, Biological product, Pont-Saint-Esprit, France) and sesame oil (Luz de Vida, Ecological product, Gerona, Spain). These oils did not contain retinol in their composition, according to the label of these products. For solid phase extraction studies, oil solutions were prepared by accurately weighing of appropriate amounts of each oil and dilution to 25 mL with hexane.

Equipment

The fluorescence spectra of retinol solutions and samples were obtained in a luminescence spectrophotometer Perkin-Elmer model LS-50B, equipped with a quartz cell of 1 cm light pass thermostatized with a bath Thermomix BU at 25 °C. The acquisition and data treatments were realized with the Perkin-Elmer Flwin Lab software.

For solid-phase extraction, a twelve cartridges capacity Visiprep™ from Supelco (Madrid, Spain) was used. The vacuum pump was KNF Lab Laboport, from Sharlab (Barcelona, Spain). Several different SPE cartridges were tested: Discovery DSC-Si solid-phase cartridges, from Supelco (Madrid, Spain), of 1, 3 and 6 mL and HLB-Oasis cartridges from Waters (Milford, Mass, USA).

Methodology

Influence of alcohol percentages in fluorescence intensity of retinol in CTAB aqueous micellar media

First, the effect on the retinol fluorescence intensity of different short-chain alcohols added to a micellar aqueous medium of CTAB with a retinol concentration of 1.0 mg L^{-1} was studied. The CTAB concentration was 5% w/w/w and

the alcohols studied were methanol, *n*-propanol and *n*-butanol. The percentage of the alcohols was varied from 0% v/v to 100% v/v. The retinol fluorescence intensity was measured at the maxima excitation and emission wavelengths.

The fluorescence spectra for retinol in the CTAB/*n*-butanol/water mixtures, were obtained in three-dimensions: emission intensity as a function of the excitation wavelength and emission wavelength, simultaneously. These wavelengths were ranged from 250 to 490 nm (excitation) and from 280 to 600 nm (emission). The fluorescence intensity measurements were carried out with an excitation slit of 2.5 nm and emission slit of 5 nm.

Analytical characteristics of the fluorescence method employed for retinol determination

Retinol determination was accomplished by the external standard calibration method in the medium chosen: CTAB 5%/*n*-butanol 10%/water 85% w/w/w. With this purpose, data obtained by measuring the fluorescence emission intensity, at the maxima excitation and emission wavelengths, as a function of the retinol concentration (mg L^{-1}) had been fitted to a regression line by the mathematical method of least squares. In this study, retinol concentration was ranged between 0.1 and 1.0 mg L^{-1}. The fluorescence intensity of each calibration standard was measured at least in triplicate. This calibration curve was also obtained in pure *n*-butanol.

The method sensitivity was calculated as the slope of the straight line, with its corresponding units. The detection and quantification limits had been obtained from the straight line, as the retinol concentration which produces a fluorescence intensity whose value is the blank fluorescence signal (in CTAB 5%/*n*-butanol 10%/water 85% w/w/w without retinol) plus three or ten times, respectively, the standard error of the estimate. The estimate means the statistics that explains the value for the standard deviation of the residuals [39].

The precision of the method was evaluated in terms of repeatability and reproducibility. The repeatability was calculated as the relative standard deviation (RSD or % CV) of the fluorescence intensity values measured, in the same day, for ten independent solutions of retinol with an intermediate concentration of the calibration curve (0.42 mg L^{-1}). The reproducibility of the method (between-day repeatability) had been determined at three different retinol levels (0.2, 0.5 and 0.7 mg L^{-1}) by measuring (in triplicate) the fluorescence signal of independent standard solutions of retinol prepared in six different days.

The robustness was the relative standard deviation of the slope of the calibration curve for thirteen calibration curves obtained in different days during 1 month. All data used for these studies were under statistical control, according to the Individuals Charts Analysis realized with Statgraphics Plus 5.1 for Windows (Statistical Graphics Corporation, 1994–2000).

The stability of retinol in the micellar medium used in this method was studied by measuring the fluorescence intensity of a retinol solution (0.42 mg L^{-1}) during fifteen consecutive days.

The accuracy of this method was studied by calculating the retinol recovery of the vegetable oil samples analyzed by the optimised method: SPE and fluorescence measurement in CTAB 5%/*n*-butanol 10%/water 85% w/w/w. With this purposed, oil samples were analyzed both without retinol addition and after the spiking of known retinol concentrations, ranging from 0.1 to 0.3 mg L^{-1}.

Preconcentration and solvent change by solid phase extraction (SPE) of retinol in organic samples using the proposed analytical method

Solid phase extraction was used as a strategy for both preconcentration of retinol and as a technique that allowed a change from an organic medium (sample) to an aqueous solution (micellar system employed for retinol analysis).

Different types of solid phases were tested in order to found the most appropriate one for the retention of all-*trans*-retinol onto the SPE cartridge. It was necessary to take into account the non-polar character of this vitamin, the solvent in which the standard of retinol and the oil were dissolved (hexane) and the elution medium (CTAB 5%/*n*-butanol 10%/water 85% w/w/w). The solid phase extraction phases tested were: normal phase cartridges from Supelco (DSC-Si), in three different sizes: 1 mL (100 mg), 3 mL (500 mg) and 6 mL (500 mg), and hydrophilic-lipophilic phases, HLB-Oasis (Waters).

The DSC-Si cartridges were activated with 1, 3 or 5 mL, of hexane. After retinol standard solution application (retinol concentration varied from 0.1 to 0.4 mg L^{-1}; solution volumes of 10 or 25 mL), the sorbent was washed with 1, 3 or 5 mL hexane and allowed to run dry. Retinol was eluted with increased volumes (from 2 to 25 mL) of the CTAB 5%/*n*-butanol 10%/water 85% w/w/w solution.

The same working protocol was applied for the extraction of retinol from vegetable oils (sunflower and sesame oil). With this purpose, solutions of different oil weights in hexane (25 mL), spiked with 0, 0.1, 0.2 and 0.3 mg L^{-1} were prepared and passed through the SPE cartridge.

The SPE recovery percentages of retinol were calculated based on the retinol concentration in the sample and the retinol concentration eluted from the cartridge.

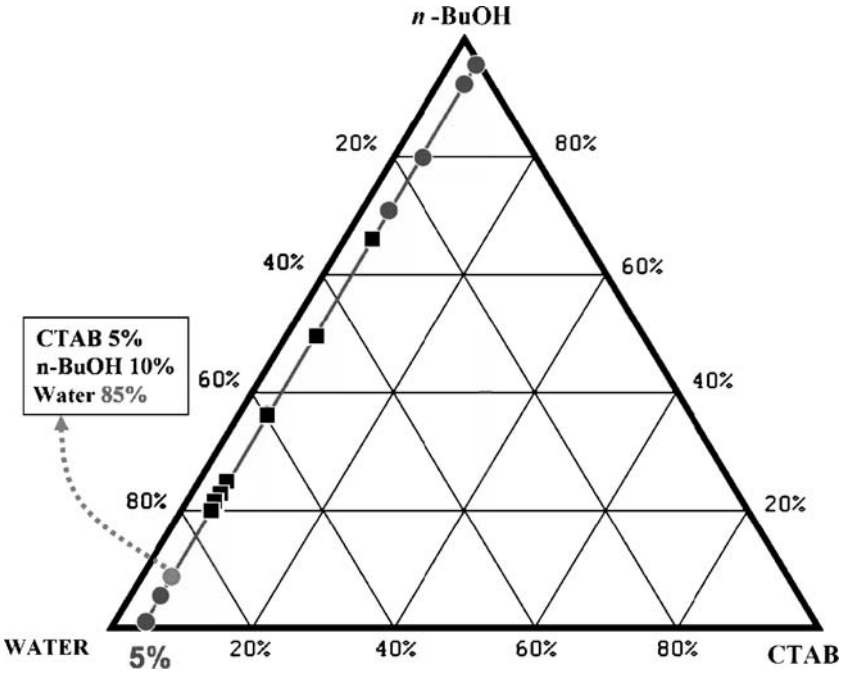

Fig. 1 Composition of the different mixtures alcohol/water containing CTAB 5% weight over the ternary phase diagram

Results and discussion

Study of the fluorescence intensity of retinol in a CTAB/alcohol/water medium

The variation of the fluorescence intensity of retinol in presence of an aqueous anionic (SDS), cationic (CTAB) and non-ionic surfactant (Brij 35) solution had been studied by us in a previous work [37]. It was found that the fluorescence intensity was higher in CTAB and Brij 35 media than in water or methanol. The intensity increased in the aqueous solutions when increasing the surfactant concentration until the critical micellar concentration (CMC) was reached. From this CMC value up to 0.1 mol L^{-1}, the fluorescence intensity practically remained constant.

The fluorescence intensity in CTAB increased due to the long hydrophobic chain of the retinol structure, which produced a great interactivity in the aqueous micelles. At CTAB concentrations higher than the CMC value (9.4

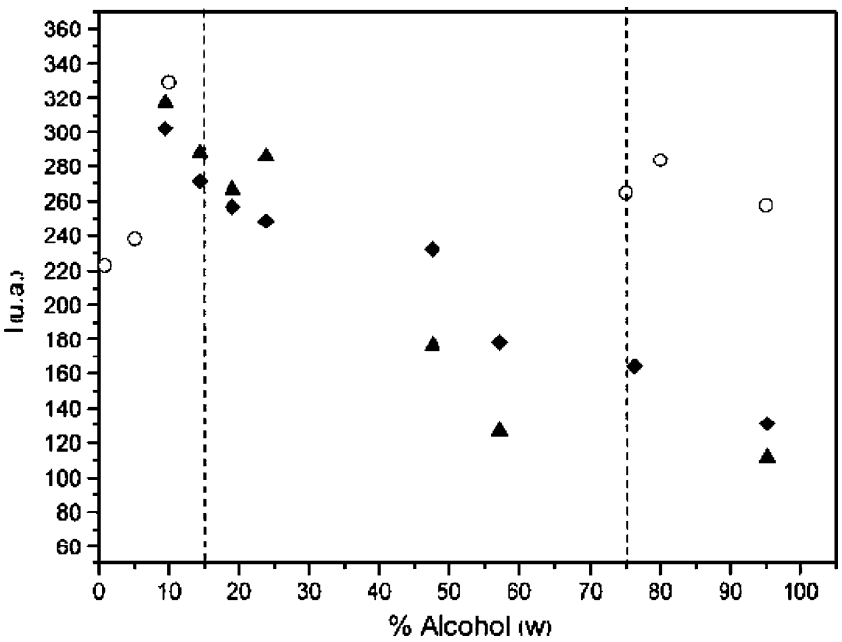

Fig. 2 Variation of the fluorescence intensity of retinol (1.0 mg L^{-1}) *vs* alcohol percentage added to aqueous solutions of CTAB 5% weight (λ_{exc} =330.0 nm; λ_{em}=472.5 nm). *Filled triangle* Methanol; *filled diamond* *n*-propanol; *empty circle* *n*-butanol

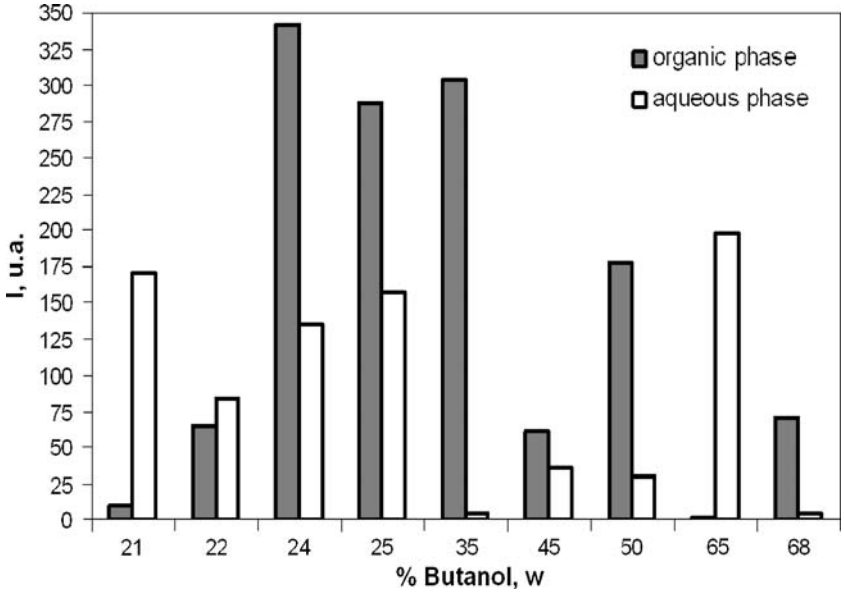

Fig. 3 Fluorescence intensities found for different mixtures CTAB 5%/*n*-BuOH/water w/w/w *vs n*-butanol percentages in the zone of separation phases (λ_{exc}=330.0 nm; λ_{em}= 472.5 nm)

10^{-4}mol L^{-1}) the retinol solubility was increased at the same time as a stabilization in the fluorescence signal was observed.

To increase even more the high fluorescence signal of retinol obtained in aqueous CTAB solutions, the influence of the addition of a short chain alcohol to the medium was studied. With this aim, a surfactant concentration higher than the minimum CTAB concentration producing the maximum fluorescence intensity in aqueous solution (5% w) was chosen.

The alcohols added were: methanol and *n*-propanol, which are miscible with water, and *n*-butanol, which formed different phases and structures in ternary mixtures with CTAB and water. The CTAB/*n*-butanol/water ternary phase diagram [40] shows three different zones for CTAB 5% w: two isotropic mixtures at very low and very high percentages of *n*-butanol, respectively, and an intermediate zone of two immiscible phases. En effect, this alcohol is miscible with CTAB 5% and water only in percentages lower than 10% w and higher than 75% w of *n*-butanol.

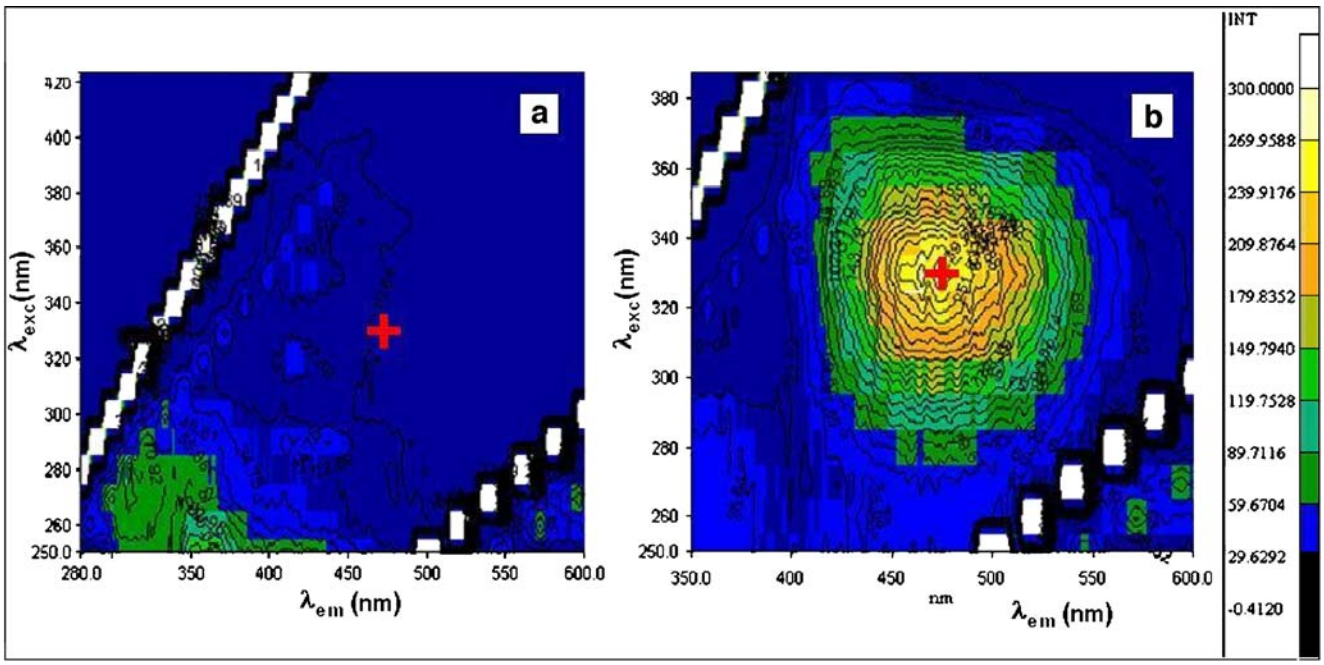

Fig. 4 Three-dimensional fluorescence spectrum of CTAB 5%/*n*-butanol 10%/water 85% w/w/w. **a** Without retinol and **b** with 1.0 mg L^{-1} of retinol

Table 1 Analytical characteristics of the retinol determination by fluorescence at 330/474 nm excitation and emission wavelengths, respectively

Characteristics of the retinol	
Linear range[a] (mg L^{-1})	0.11–0.80
Correlation coefficient (% RSD), n=13	0.9985 (0.14)
Sensitivity (L mg^{-1}) (% RSD), n=13	462.5 (1.1)
Detection limit (mg L^{-1}) (SD), n=11	0.03 (0.01)
Quantification limit (mg L^{-1}) (SD), n=11	0.11 (0.04)
Repeatability (% RSD), n=10 ([Retinol]=0.42 mg L^{-1})	0.7
Reproducibility (% RSD)	
n=6 ([Retinol]=0.20 mg L^{-1})	4.2
n=6 ([Retinol]=0.50 mg L^{-1})	3.9
n=6 ([Retinol]=0.70 mg L^{-1})	3.8
Robustness (% RSD), n=13	1.1

Calculations was carried out with those data under statistical control
[a] Lower linearity limit calculated as the quantitation limit (LOQ)

Figure 1 shows all the different mixtures CTAB 5%/*n*-butanol/water in which standard solutions of 1.0 mg L^{-1} of retinol had been prepared.

The fluorescence intensity variation found in presence of the three alcohols studied is shown in Fig. 2. As can be seen in this figure, the addition of increasing percentages of methanol and *n*-propanol to an aqueous solution of CTAB 5% w produced an important decrease in the fluorescence intensity of retinol.

The behaviour of *n*-butanol was quite different. There was an increase in the retinol fluorescence intensity between 0.8 and 10% w, being this intensity almost constant at percentages of *n*-butanol higher than 75% w. Moreover, the retinol fluorescence intensity obtained at high percentages of *n*-butanol was much higher than those corresponding to methanol and *n*-propanol.

The zone with intermediate percentages of *n*-butanol, was studied by adding retinol and separating both phases and measuring the retinol fluorescence intensity in the organic and in the aqueous phases. Figure 3 shows the intensity values obtained in both phases. The mixture CTAB 5%/*n*-Butanol 35%/water 60% w/w/w seemed to be adequate to measure all the retinol quantity in the organic phase, with the corresponding preconcentration factor. However, the distribution of retinol between both phases was very irreproducible and showed different retinol quantities in organic and aqueous phases whenever the analysis was repeated.

From this study, it may be deduced that the best medium for the retinol determination by fluorescence was CTAB 5%/*n*-butanol 10%/water 85% w/w/w. In this system, only one phase was observed; moreover, the fluorescence intensity signal of retinol in this medium had a very high value. This fact may be explained taking into account that the presence of an alcohol in the CTAB micellar aqueous system has a strong effect on both the solubilization and stabilization of a lipophilic molecule such as retinol, thus exerting an increase in its fluorescence intensity measured.

Figure 4 shows the spectra of a CTAB 5%/*n*-butanol 10%/water 85% solution (a) and a retinol solution (1.0 mg L^{-1}) in the same medium (b). As can be seen in this figure, the system CTAB 5%/*n*-butanol 10%/water 85% (w/w/w) exhibited a very low fluorescence intensity (close to zero) in all the spectral zones recorded and, specially, at the maxima excitation and emission wavelengths of retinol in this medium: 330.0 and 474.0 nm, respectively.

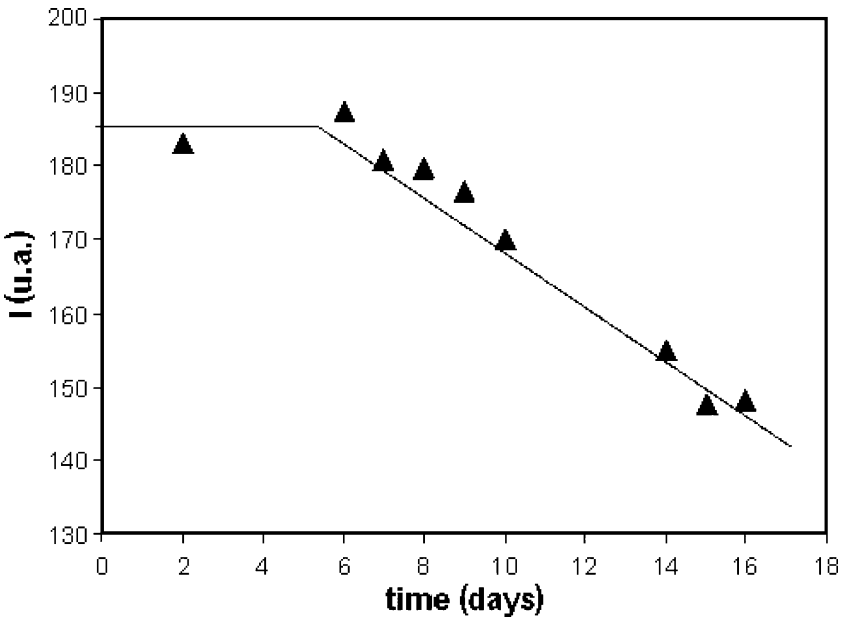

Fig. 5 Variation of fluorescence intensity (330/474 nm) of a 0.42 mg L^{-1} retinol solution in CTAB 5%/*n*-butanol 10%/water 85% w/w/w *vs* time

Analytical characteristics of the quantitative determination method of retinol by fluorescence in a CTAB 5%/*n*-butanol 10%/water 85% w/w/w medium

The aggregates formed in the mixture CTAB 5%/*n*-butanol 10%/water 85%, w/w/w, gave the most appropriate medium to quantify the vitamin A as retinol by setting the linear relationship between the fluorescence intensity of retinol and the retinol concentration. Table 1 summarized the analytical characteristics of this fluorescence method.

The method was linear from 0.11 up to 0.80 mg L^{-1}. The correlation coefficients of thirteen calibration lines were ranged from 0.9950 to 0.9999. The sensitivity of this method (462.5 L mg^{-1}) was high enough compared to that obtained in *n*-butanol (255 L mg^{-1}) or in media such as aqueous solutions of CTAB or Brij 35 (approximately four times lower) [37]. It can be said that the micellar solution composed by CTAB 5%/*n*-butanol 10%/water 85%, w/w/w provided us a very sensitive determination of retinol by fluorescence. The detection limit was low: 0.03 mg L^{-1}. The limit of quantification (0.11 mg L^{-1}) allowed us the determination of this vitamin at the usual concentrations in which all-*trans*-retinol is presented in different samples [6, 7, 9, 12, 13].

The repeatability for a concentration of 0.42 mg L^{-1} measured for ten different solutions in the same day was 0.7%. This value indicated a good stability of the prepared diluted solutions along one day. The reproducibility

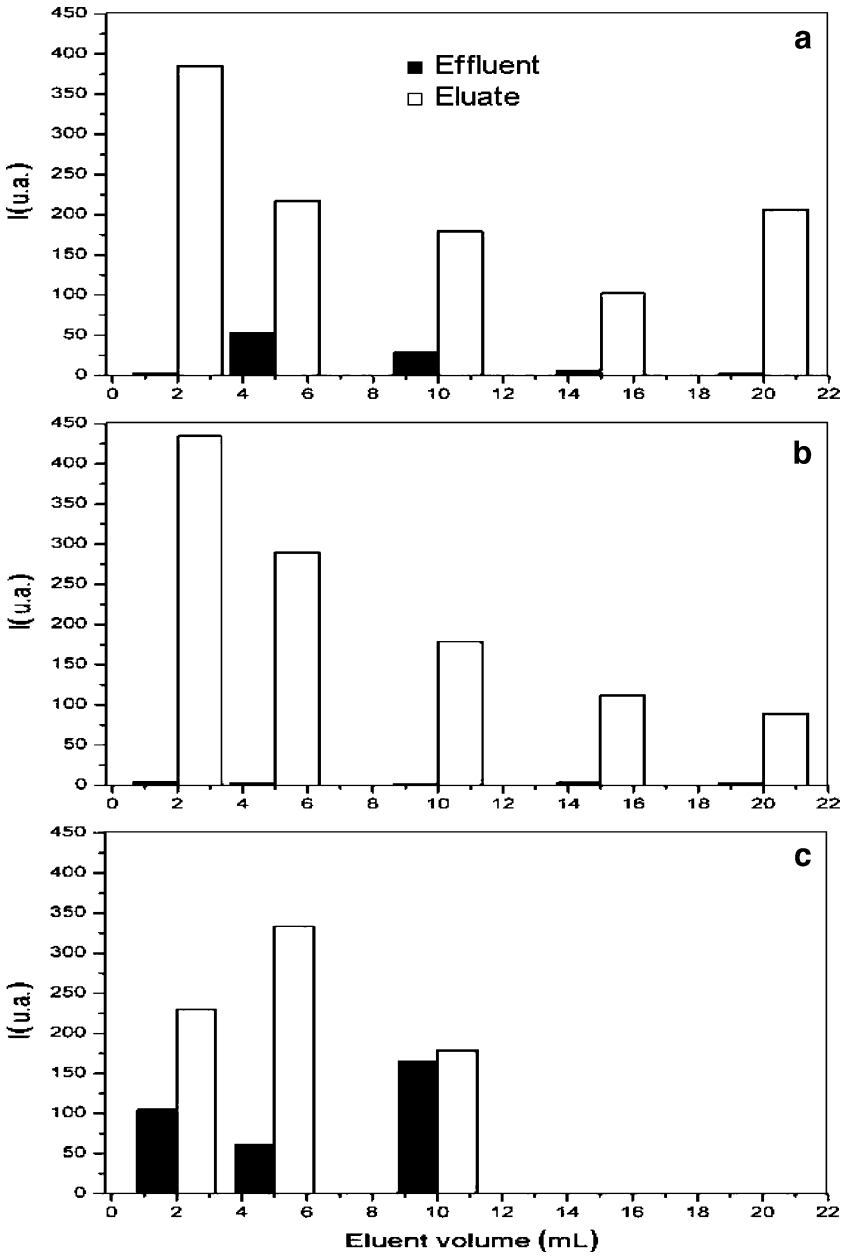

Fig. 6 Influence of the size of the SPE DSC-Si cartridges on the retinol (0.5 mg L^{-1} in hexane) fluorescence intensity (330/474 nm) in the effluent and in the eluate. **a** 6, **b** 3 and **c** 1 mL

obtained for three different retinol concentration levels was less than 4.2%, and it was similar for the three concentrations studied. The robustness was also very good, because the variability of the calibration curve slopes obtained in thirteen different days was 1.1%.

The determination of the retinol solution stability in the measurement medium is very important, since it is well known the instability of retinol solutions prepared in most solvents. In fact, these diluted solutions must be stored at −18 °C in inner atmosphere and light protected. At this respect, Fig. 5 shows the fluorescence intensity of one solution of retinol 0.42 mg L^{-1} measured in a range of thirteen days. As can be seen in this figure, the vitamin A decomposition in this micellar medium was observed starting from the five day after its preparation.

Solid phase extraction (SPE) of retinol from standard solutions and real samples

The proposed method for retinol determination had a very good sensitivity and analytical characteristics. The application of this fluorescence method to real samples that contain retinol implies a solvent change if the samples to be analyzed have an organic matrix, since the determination of this vitamin is carried out in a CTAB 5%/*n*-butanol 10%/water 85% w/w/w. In addition, solid phase extraction (SPE) can be a very good preconcentration technique in this case, because the analyte is usually found in a low concentration level in the samples.

The solvent used in the study was hexane since it is one of the most employed solvents for vitamin analysis in food samples, biological fluids, etc. [4, 6]. The SPE technique had been applied to standards of retinol in hexane as well as to real samples dissolved in the same solvent. The fluorescence signal in the effluent was measured in order to verify if there was any quantity of retinol. The elution was carried out with the micellar medium chosen in this work.

The HLB-Oasis cartridge did not produce recuperation percentages higher than 48%. Taking into account the hydrophobic character of retinol, a solid normal phase as DSC-Si was tested in order to increase the recuperation percentages of retinol in the eluate. The retinol was eluted with different volumes of CTAB 5%/*n*-butanol 10%/water 85% w/w/w. These results are summarized in Fig. 6. As can be seen in this figure, the only cartridge that retained quantitatively all retinol quantity was the DSC-Si; in fact, the retinol concentration in the effluent was below to the quantification limit of the analytical method. In addition, when using the cartridge of 3 mL/500 mg, the recovery in the eluate leads to 95% with an eluent volume of 10 mL (preconcentration factor is 1). Using this cartridge, the highest recovery obtained was for the following conditions: 10 mL of the sample and an elution volume of 10 mL of CTAB 5%/*n*-BuOH 10%/water 85% w/w/w.

Next, it was studied the influence on retinol recovery of different quantities of retinol (from 2.0 to 12.5 μg) passed through the chosen cartridge and the volume of the eluent used (Table 2). According to these results, the minimum volume of eluent necessary to obtain a recovery close to 100% was 5 mL. On the other hand, when the quantity of retinol fixed onto the phase was higher than 5.0 μg, the

Table 2 Recovery studies of retinol standards from hexane using solid-phase extraction (SPE) with a cartridge DSC-Si of 3 mL

Retinol concentration (mg L^{-1})	Hexane solution volume (mL)	Retinol quantity (μg)	V eluent (mL)	Retinol quantity eluted (μg)	Recovery (%)
0.2	10.00	2.00	2.00	1.15±0.02	58±1
			5.00	1.96±0.01	98.0±0.5
			10.00	2.0±0.2	101±8
			15.00	2.16±0.01	108.0±0.1
			20.00	2.10±0.04	105±2
0.5	10.00	5.00	5.00	4.95±0.1	99±2
			10.00	4.5±0.1	90±2
			15.00	4.81±0.01	96.26±0.01
			20.00	5.28±0.08	106±2
0.2	25.00	5.00	10.00	5±2	100.0±0.4
			15.00	5.1±0.0	101.5±0.7
			20.00	5.0±0.2	100±4
0.3	25.00	7.50	15.00	2.56±0.03	34.2±0.4
0.4	25.00	10.0	15.00	1.77±0.05	17.7±0.5
0.5	25.00	12.5	5.00	9.0±0.2	71.80±0.01
			10.00	10.0±0.1	80.0±0.8
			15.00	4.71±0.05	38.0±0.4
			20.00	0.82±0.02	6.6±0.2

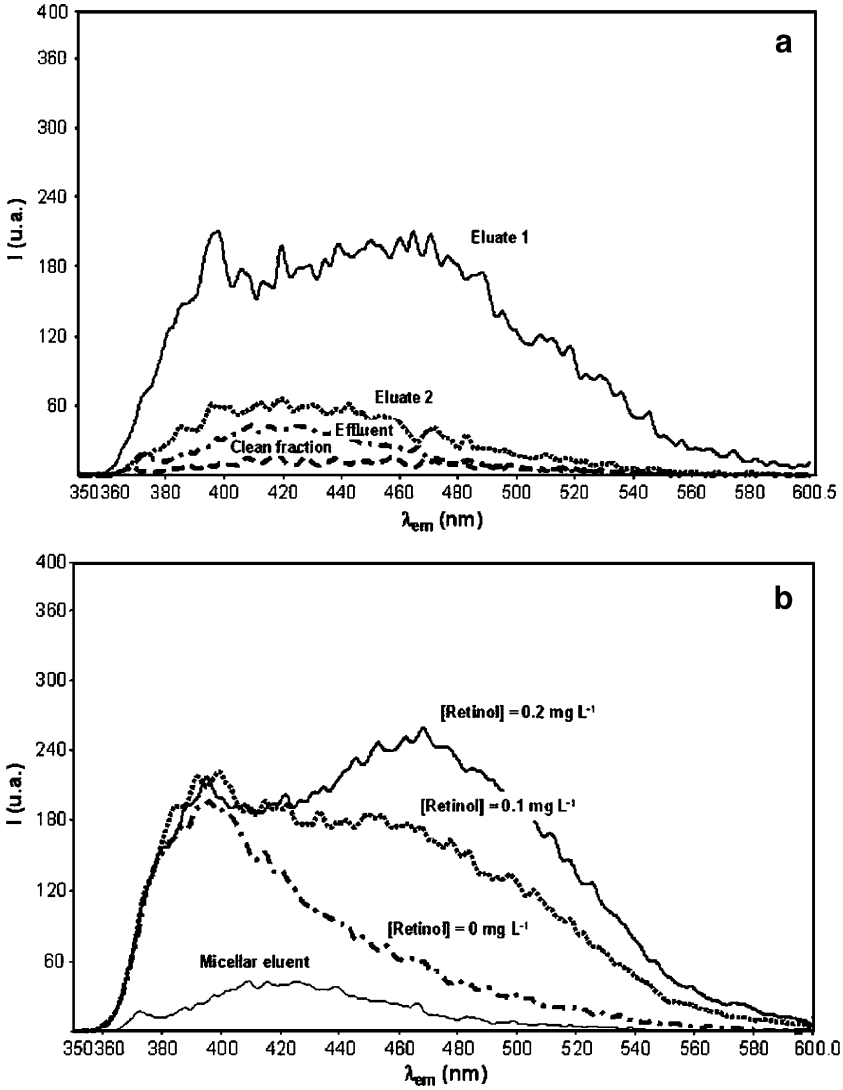

Fig. 7 Fluorescence emission spectra (λ_{exc}=330 nm) obtained for the SPE retinol extraction from sesame oil. **a** CTAB 5%/*n*-butanol 10%/water 85% w/w/w, effluent and two eluates (10 mL) for retinol 0.20 mg L^{-1} added to 0.20 g oil/25 mL hexane. **b** First eluate (10 mL) for different retinol concentrations added to 0.50 g oil/25 mL hexane

retinol was found both in the effluent and in the eluate, and the recoveries in these cases were not quite reproducible. Thus, if the retinol quantity in the sample is higher than 5.0 μg, a cartridge with higher capacity should be used to avoid losses of retinol in the effluent.

The SPE technique was also applied to samples with very complex matrices: sunflower and sesame oils. As commented above, it was necessary to change the medium for measuring the retinol in the oil matrix. With this purpose, 25 mL of oil solutions in hexane spiked with the appropriate amounts of retinol were passed through the cartridge and the retinol was eluted with two independent volumes of 10 mL of CTAB 5%/*n*-butanol 10%/water 85% w/w/w. The retinol in the effluent and the two eluates was analysed by the proposed method. As an example, it was shown in Fig. 7a the spectra of all these fractions for sesame oil spiked with 5.0 μg of retinol, in comparison with the spectrum of the micellar solution. From these results, it was evident that most the retinol was recuperated

Table 3 Recovery studies for oil samples spiked with different quantities of retinol using solid-phase extraction (SPE)

Oil quantity (g)	Retinol quantity added (μg)	Retinol quantity eluted (μg)	Recovery (%)
Sunflower oil			
0.5000	2.50	3.1±0.1	123±4
0.5092	5.00	5.1±0.4	108±9
0.5035	7.50	8.2±0.2	109±3
0.2024	2.50	3.0±0.3	119±10
0.2138	5.00	6.4±0.4	128±7
Sesame oil			
2.0105	2.50	3.64±0.01	145.6±0.4
1.0130	2.50	3.5±0.1	140±4
0.5200	2.50	2.7±0.6	108±2
0.5083	5.00	5.3±0.2	106±4
0.5089	7.50	6.1±0.1	82±1
0.2121	2.50	3.1±0.2	123±8
0.2221	5.00	5.2±0.1	104±2

in the first eluate. Similar results were obtained for sunflower oil. Figure 7b shows the spectra obtained for the sesame oil at the maximum excitation and emission wavelengths of retinol, when different amounts of this vitamin were added to hexane solution of this oil.

The results obtained in the studies of SPE extraction of retinol from the oils spiked with different quantities of retinol are summarized in Table 3. Both for sunflower and sesame oil, it was found that oil quantities in hexane solution lower than 0.5 g, gave retinol recoveries close to 100%. Higher amounts of oil implied a distribution of retinol between the effluent and the eluate, and the recovery values found were anomalous (higher than 140%). This behaviour had also been found at high amounts of retinol in the studies realized with standards. On the other hand, it seemed to be that the nature of the oil had not influence on the amounts of retinol eluted.

The oils were also analysed in absence of added retinol. In these cases, the matrix of the oils in the two eluates analyzed gave a signal of fluorescence intensity at the excitation and emission wavelengths of retinol. This effect was much more important in the case of sunflower oil. In fact, as can be seen in Figure 7b, the spectra of the first eluate of a solution of sesame oil in hexane was quite different to that obtained for the oil spiked with retinol. This effect could explain the fact that there was almost no influence of the oil matrix in the retinol analysis in samples spiked with this vitamin and thus, recovery values close to 100% could be obtained.

To summarize, the application of the SPE technique to the oil samples, and the following analysis by the fluorescence method using the micellar system CTAB 5%/ *n*-butanol 10%/water 85% w/w/w allowed the easy determination of retinol at concentration levels usually found in food products with good recovery values.

Conclusions

The proposed SPE-fluorescence method is very sensitive, easy and fast for the determination of retinol in comparison with other methods found in the literature, which involve the retinol separation by means of saponification, extraction with organic solvents or the application of chromatographic techniques, previous its analysis. The analytical characteristics of the method are good enough: detection limit of 0.03 mg L^{-1}, quantification limit of 0.11 mg L^{-1} and sensitivity of 462.5 L mg^{-1}.

This method can be applied to very complex samples such as vegetable oils with quantitative recovery values. In addition, the micellar measurement medium used has a very low quantity of organic solvent (*n*-butanol), being a practically aqueous micellar medium.

The solvent change is easy by SPE with the preconcentration of the retinol in oil samples, when necessary.

Acknowledgments The authors gratefully acknowledge to University of Alcalá (Project GC 2006-008) and to University of Alcalá and Community of Madrid (Project CCG06-UAH/MAT-0423) for supporting this work.

References

1. Dietary Supplements Fact Sheet (2007) Vitamin A and carotenoids. Office of Dietary Supplements. National Institutes of Health, Belthesda, Maryland, USA
2. Thurnham DI (2007) Bioequivalence of β-carotene and retinol. J Sci Food Agric 87(1):3–39
3. Ball GFM (2000). In: Nollet ML (Ed) Food Analysis by HPLC, Marcel Dekker, New York, p.321
4. Rodríguez-Bernaldo de Quirós A, Costa HS (2006) Analysis of carotenoids in vegetable and plasma samples. A review. J Food Compos Anal 19(1):97–111
5. Hulshof PJM, van Roekel-Jansen T, van de Bovenkamp P, West CE (2006) Variation in retinol and carotenoid content of milk products in the Netherlands. J Food Compos Anal 19(1):67–75
6. Heudi O, Trisconi M-J, Blake C-J (2004) Simultaneous quantification of vitamins A, D3 and E in fortified infant formulae by chromatography-mass spectrometry. J Chromatogr A 1022 (1–2):115–123
7. Salo-Väänänen P, Ollilainen V, Mattila P, Lehikoinen K, Salmela-Mölsä E, Piironen V (2000) Simultaneous HPLC analysis of fat-soluble vitamins in selected animal products after small-scale extraction. Food Chem 71:535–543
8. Kuhnlein HV, Barthet V, Farren A, Falahi E, Leggee D, Receveur O, Berti P (2006) Vitamins A, D, and E in Canadian artic traditional food and adult diets. J Food Compos Anal 19(6–7):495–506
9. Herrero-Barbudo MC, Granado-Lorencio F, Blanco-Navarro I, Olmedilla-Alonso B (2005) Retinol, α and γ-tocopherol and carotenoids in natural and vitamin A and E fortified dairy products commercialized in Spain. Int Dairy J 15(5):521–526
10. Mathiasson L, Turner C, Berg H, Dahlberg L, Theobald A, Anklam E, Ginn R, Sharman M, Ulberth F, Gaberning R (2002) Development of methods for the determination of vitamins A, E and β-carotene in processed foods based on supercritical fluid extraction: a collaborative study. Food Addit Contam 19(7): 632–646
11. Lee BL, Su J, Ong ChN (2004) Monomeric C_{18} chromatographic method for the liquid chromatographic determination of lipophilic antioxidants in plants. J Chromatogr A 1048(2):263–267
12. Ortega H, Coperías JL, Castilla P, Gómez-Coronado D, Lasunción MA (2004) Liquid chromatographic method for the simultaneous determination of different lipid-soluble antioxidants in human plasma and low-density lipoproteins. J Chromatogr B 803(2): 249–255
13. Urbánek L, Solichová D, Melichar B, Dvorak J, Svobodová I, Solich P (2006) Optimization and validation of a high performance liquid chromatography method for the simultaneous determination of vitamins A and E in human serum using monolithic column and diode-array detection. Anal Chim Acta 573–574:267–272
14. Nierenberg DW, Nann SL (1992) A method for determining concentrations of retinol, tocopherol, and five carotenoids in human plasma and tissue samples. Am J Clin Nutr 56:417–426

15. Moulas AN, Zervos IA, Taitzoglou IA, Tsantarliotou MP, Botsoglou NA (2003) Simultaneous determination of retinoic acid, retinol, and retinyl palmitate in ram plasma by liquid chromatography. J Liq Chromatogr Relat Technol 26:559–572
16. Miyagi M, Yokoyama H, Shiraishi H, Matsumoto M, Ishii H (2001) Simultaneous quantification of retinol, retinal, and retinoic acid isomers by high-performance liquid chromatography with a simple gradiation. J Chromatogr B 757:365–368
17. Hosotani K, Kitagawa M (2003) Improved simultaneous determination method of β-carotene and retinol with saponification in human serum and rat liver. J Chromatogr B 791(1–2):305–313
18. Casal S, Macedo B, Oliveira MBPP (2001) Simultaneous determination of retinol, β-carotene and α-tocopherol in adipose tissue by high-performance liquid chromatography. J Chromatogr B 763(1–2):1–8
19. Chatzimichalakis PF, Samanidou VS, Papadoyannis IN (2004) Development of a validated liquid chromatography method for the simultaneous determination of eight fat-soluble vitamins in biological fluids after solid-phase extraction. J Chromatogr B 805(2):289–296
20. Majchrzak D, Fabian E, Elmadfa I (2006) Vitamin A content (retinol and retinyl esters) in livers of different animals. Food Chem 98(4):704–710
21. Siluk D, Oliveira RV, Rodriguez-Rosas ME, Ling S, Bos A, Ferrucci L, Wainer IW (2007) A validated liquid chromatography method for the simultaneous determination of vitamins A and E in human plasma. J Pharm Biomed Anal 44(4):1001–1007
22. Alvarez JC, de Mazancourt P (2001) Rapid and sensitive high-performance liquid chromatographic method for simultaneous determination of retinol, α-tocopherol, 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 in human plasma with photodiode-array ultraviolet detection. J Chromatogr B 755(1–2):129–135
23. Karpińska J, Mikołuć B, Motkowski R, Piotrowska-Jastrzębska J (2006) HPLC method for simultaneous determination of retinol, α-tocopherol and coenzyme Q_{10} in human plasma. J Pharma Biomed Anal 42(2):232–236
24. Nöll GN, Becker C (2000) High-performance liquid chromatography of non-polar retinoid isomers. J Chromatogr A 881(1–2):183–188
25. Thompson JN, Erdody P, Maxwell WB, Murray TK (1972) Fluorometric determination of vitamin A in dairy products. J Dairy Sci 55(8):1077–1080
26. Thompson JN, Erdody P (1974) Destruction by light of vitamin A added to milk. Can Inst Food Sci Technol J 7:157–158
27. Parrish DB, Moffitt RA, Noel RJ, Thompson JN (1985) In: J. Augustin, B.P. Klein, D.A. Becker y P.B. Venugopal (Eds.), Methods of Vitamin Assay. John Wiley and Sons, New York, pp 153
28. Kulmyrzaez A, Dufour E, Noël Y, Hanafi M, Karoui R, Qannari EM, Mazerolles G (2005) Investigation at the molecular level of soft cheese quality and ripening by infrared and fluorescence spectroscopy and chemometrics-relationships with rheology properties. Int Dairy J 15(6–9):669–678
29. Karoui R, Dufour E, Schoonheydt R, De Baerdemaeker J (2007) Characterisation of soft cheese by front face fluorescence spectroscopy coupled with chemometric tools: effect of the manufacturing process and sampling zone. Food Chem 100(2):632–642
30. Karoui R, Dufour E, De Baerdemaeker J (2006) Common components and specific weights analysis: a tool for monitoring the molecular structure of semi-hard cheese throughout ripening. Anal Chim Acta 572(1):125–133
31. Sxuts EZ, Harosi FI (1991) Solubility of retinoids in water. Arch Biochem Biophys 287(2):297–304
32. Kim D-G, Jeong Y-I, Choi C, Roh S-H, Kang S-K, Jang M-K, Nah J-W (2006) Retinol-encapsulated low molecular water-soluble chitosan nanoparticles. Int J Pharm 319(1–2):130–138
33. Semenova EM, Cooper A, Wilson CG, Converse CA (2002) Stabilization of all-trans-retinol by cyclodextrins: a comparative study using HPLC and fluorescence spectroscopy. J Incl Phenom Macrocycl Chem 44(1–4):155–158
34. Singh AK, Das J (1998) Liposome encapsulated vitamin A compounds exhibit greater stability and diminished toxicity. Biophys Chemist 73(1–2):155–162
35. Han S-H, Lee JS, Kim YJ, Kim J, Chang I-S, Chung DJ, Suh KD, Kim J-W (2007) Quantitative characterization of degradation behaviors of antioxidants stabilized in lipid particles. Talanta 71 (5):2129–2133
36. García L, Blázquez S, San Andrés MP, Vera S (2001) Determination of thiamine, riboflavin and pyridoxine in pharmaceuticals by synchronous fluorescence spectrometry in organized media. Anal Chim Acta 434(2):193–199
37. Ramos-Lledó P, Vera S, San Andrés MP (2001) Determination of vitamins A and E in milk samples by fluorescence in micellar media. Fresenius J Anal Chem 369:91–95
38. León V, Vera S, San Andrés MP (2005) Validation of a screening method for the simultaneous identification of fat-soluble and water-soluble vitamins (A, E, B_1, B_2 and B_6) in an aqueous micellar medium of hexadecyltrimethylammonium chloride. Anal Bioanal Chem 381(8):1568–1575
39. Miller JN, Miller JC (2000) Statistics and chemometrics for analytical chemistry. Prentice Hall, Dorchester, Dorset, England, p 120
40. Ekwall P, Khan A, Lindstrom B, Maciejewski D, Puang-Ngern S (1991) Phase equilibria and structures in ternary systems of a cationic surfactant (C_{16} TABr or $(C_{16}$ TA$)_2SO_4$), alcohol, and water. Colloid Polym Sci 269(7):727–742