Talanta 103 (2013) 166–171

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

An evaluation of *cis*- and *trans*-retinol contents in juices using dispersive liquid–liquid microextraction coupled to liquid chromatography with fluorimetric detection

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ARTICLE INFO

Article history:
Received 7 June 2012
Received in revised form
27 September 2012
Accepted 6 October 2012
Available online 12 October 2012

Keywords:
Dispersive liquid–liquid microextraction
Liquid chromatography
Saponification
cis-Retinol
trans-Retinol
Fruit juices

ABSTRACT

This study describes a method for coupling dispersive liquid–liquid microextraction (DLLME) and normal-phase liquid chromatography (NP-LC) with fluorescence detection for vitamin A determination with the view to developing a new green sample preparation technique. Parameters affecting DLLME, including the nature and volume of both extractant and disperser solvents, salt addition and time and speed of the centrifugation step, were optimized. The sample was saponified according to European Standards to convert all forms of vitamin A to retinol. For microextraction, 8 mL water were placed in a glass tube with conical bottom and the saponified sample consisting of 2 mL of the methanolic extract containing 100 μL tetrachloroethane was rapidly injected by syringe, thereby forming a cloudy solution. Phase separation was performed by centrifugation, and a volume of 20 μL of the sedimented phase was analyzed by NP-LC. The enrichment factor, calculated as the ratio between the slopes of DLLME-LC and direct LC, was 50 ± 3. The matrix effect was evaluated for different juice samples, and it was concluded that sample quantification can be carried out by aqueous calibration when the standards are also submitted to saponification. The proposed method was applied for determining both *cis*- and *trans*-retinol isomers in commercial juices of different types. The intraday and interday precisions were lower than 6% in terms of relative standard deviation. The method was validated using two certified reference materials.

1. Introduction

Some functional foods in the drinks sector are marketed as so-called ACE juices. These are soft drinks based on fruit and vegetable juices to which the antioxidant vitamins A, C and E have been added [1]. Food products enriched with vitamins are assumed to present health benefits against oxidation and the ageing [2]. All the main antioxidants are found in plant foods, which explains the need to include fruits, legumes, vegetables and cereals in the diet [3]. When a food product is enriched with vitamins, a quality control programme must be established to ensure that the product contains the declared levels. However, an excess of several vitamins may have harmful effects.

Vitamin A plays an essential role in vision, and also acts in the body as an antioxidant, providing protection against the risk of certain cancers. There are two sources of dietary vitamin A. Active forms are obtained from animal products and include retinal, retinol and retinyl esters, whose hydrolysis results in retinol, which may exist in *trans*- or *cis*-configuration. Precursors, also known as provitamins, are obtained from fruits and vegetables containing pigments, known as carotenoids, the most well-known being β-carotene [4]. For this reason, amounts of vitamin A are measured in retinal equivalents (RE). Pure retinol is extremely sensitive to oxidization and, when prepared as a dietary supplement, it is stabilized as the ester derivatives. Preform vitamin A is a nutrient of high toxic potential when consumed in excess [5]. The EU Commission [6] established the requisite relative to the nutrient vitamin A in vegetable juices as a final content in the product no lower than 100 μg RE/100 kcal (annex II). The compounds permitted to be added as vitamin A are retinol, retinyl acetate, retinyl palmitate and β-carotene (annex IV). The maximum content of vitamin A in cereal-based products and baby foods is 180 μg RE/100 kcal (annex VI).

Vitamin A has mainly been analyzed in food samples by liquid chromatography (LC) using different detectors, such as diode array, fluorescence, electrochemical or mass spectrometry [7–29]. It was sometimes analyzed together with other liposoluble vitamins. The complex matrix of food samples and the low vitamin concentrations expected mean it is necessary to include isolation and preconcentration steps in the analytical procedure.

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http://dx.doi.org/10.1016/j.talanta.2012.10.027

Saponification and/or solvent extraction in relatively low polarity organic solvents, as in the case of the standardized method of analysis [30], are classical methods of vitamin A analysis. However, these methods are tedious and also require large amounts of organic solvents, as the samples are first saponified using methanol, before being extracted with organic solvents and the procedure is repeated 3–4 times. The combined extracts are washed with water and then evaporated.

Some innovative extraction techniques, such as cloud point extraction [31] and microwave-assisted extraction [32] have been used for the preconcentration of vitamins.

Liquid-phase microextraction (LPME) includes several miniaturized techniques based on the extraction of analytes in a liquid phase using very low amounts of organic solvents [33]. Dispersive liquid–liquid microextraction (DLLME) is a very simple and rapid technique which uses very low volumes of extraction solvent and has successfully been applied to the extraction and determination of organic compounds in aqueous samples, providing high enrichment factors [34]. Among its advantages is the absence of cross-memory effects and the fact the analyses take less time. A small amount of a water-immiscible extraction solvent is dissolved in a water-soluble solvent and is then rapidly injected with a syringe into the aqueous sample. The fast injection of the mixture of organic solvents into the water disperses the water-immiscible solvent in the aqueous mass as small micro-drops, from which the target analytes can be rapidly extracted. The enriched organic phase is then separated from the aqueous sample by centrifugation and directly subjected to chromatography.

As regards the new green sample preparation techniques, no reference has been found to the preconcentration of retinol using DLLME, or to the coupling of LC and DLLME for the vitamin determination. The present study describes a fast and direct method for the sensitive determination of the *cis*- and *trans*-retinol isomers in fruit juices using DLLME-LC-Fluorescence.

2. Experimental

2.1. Reagents

All-*trans*-retinol was obtained from Sigma-Aldrich (St. Louis, MO, USA). A stock solution (100 μg mL^{-1}) was prepared in hexane and stored in amber vials at −20 °C. Working standard solutions were freshly prepared in hexane and stored at 4 °C. Ascorbic acid, potassium hydroxide and hydrochloric acid were purchased from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany). Chromatographic quality ethanol, methanol, *n*-hexane, 2-propanol and 1,1,2,2-tetrachloroethane were obtained from Sigma. Water used was previously purified in a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The LC system consisted of an Agilent 1100 (Agilent, Waldbronn, Germany) quaternary pump (G1311A) operating at room temperature with a flow-rate of 2 mL min^{-1}. The solvents were degassed using an on-line membrane system (Agilent 1100, G1379A). The fluorescence detector was an Agilent FLD (Agilent 1100, G1321A) operating at an excitation wavelength of 325 nm and an emission wavelength of 480 nm. The analytical column used for the normal-phase technique was an LiChrospher Si 60 (25 cm × 0.4 cm × 5 μm) (Agilent). The mobile phase was a 98:2 (v/v) *n*-hexane: 2-propanol mixture. Aliquots of 20 μL were injected manually using a Model 7125-075 Rheodyne injection valve (Rheodyne, Berkeley, CA, USA).

To filter the samples, PVDF filters (0.45 μm) (Teknokroma, Barcelona, Spain) were used. An EBA 20 (Hettich, Tuttlingen, Germany) centrifuge was used at a speed near to the maximum supported by the conical glass tubes, 3000 rpm. The ultrasonic processor UP 200H (Dr. Hielscher, Germany) was used for the experiments carried out using the ultrasound-assisted emulsification microextraction (USAEME) technique.

2.3. Concentration and purity test for vitamin A

The mass concentration of all-*trans*-retinol was calculated considering a molar absorptivity value of 1830 mol L^{-1} cm^{-1}. For an additional check, the absorbance of the standard solution at different wavelengths was measured with 2-propanol as reference. As the ratios between molar absorptivity values at each wavelength and 325 nm did not exceed the permitted values of 0.602 (300 nm), 0.452 (350 nm) and 0.093 (370 nm), the standard substance is suitable for use. On the other hand, the purity test was carried out by injecting a standard solution of all-*trans*-retinol in *n*-hexane into the LC and calculating the correction factor for purity (*P*) using the ratios of the peak area with the standard solution and the sum of both peak areas. Values for *P* were 0.9960 and 0.0040 for all-*trans*-retinol and 13-*cis*-retinol, respectively. Retention times were 4.61 min for 13-*cis*-retinol and 5.76 min for all-*trans*-retinol. Quantification of *cis*-retinol in the juices was carried out considering a value of 1686 mol L^{-1} cm^{-1} for the molar absorptivity of this compound [30].

2.4. Samples and saponification procedure

The samples were different type of juice enriched with vitamin A (ACE juices) containing orange, pineapple, pear, apple–mango, banana–apple and multifruits, soft orange drinks, milk-containing fruit juices and concentrated fruit juices with milk. The method was validated using two reference materials, an infant/adult nutritional formula SRM 1849a supplied by the National Institute of Standards and Technology (NIST) and whole milk powder ERM®-BD600 supplied by the Institute for Reference Materials and Measurements (IRMM).

Samples were submitted to a saponification procedure, as described by the European Standard Committee [30]. All operations were performed in subdued light. Amounts of 0.2–2 mL were measured (depending of the content of the vitamin) and 0.25 g of ascorbic acid, 50 mL of methanol and 5 mL of potassium hydroxide solution (50 g/100 mL) were added. Saponification was carried out in the absence of light, at room temperature overnight (approximately 16 h). Then, the pH of the mixture was adjusted to 6.5–7 using 37% hydrochloric acid. Aliquots were filtered using 0.45 μm PVDF filters for subsequent DLLME-LC, using 2 mL of the methanolic extract containing 100 μL tetrachloroethane. The certified reference materials were analyzed in the same way but using 50 mg of the sample. All samples were saponified in duplicate.

2.5. DLLME procedure

For DLLME, 8 mL water were placed in a 15-mL screw cap glass tube with conical bottom. Then, 2 mL of the methanolic extract from the saponified standard solution or juice sample (dispersive solvent) containing 100 μL of tetrachloroethane (extractant solvent) was rapidly injected into the water solution using a micropipette, and the mixture was again gently shaken manually for several seconds. A cloudy solution consisting of very fine droplets of tetrachloroethane dispersed through the sample solution was formed, and retinol was extracted into the fine droplets. After centrifugation for 2 min at 3000 rpm, the extraction solvent was sedimented at the bottom of the conical tube (volume about 40 ± 10 μL). Twenty microlitres of the sedimented phase were removed with a microsyringe and injected into the NP-LC.

2.6. Recovery assays

Juice samples free of vitamin A were enriched at two concentration levels, 5 and 25 ng mL^{-1}. The spiked samples were set aside for 1 h in the closed vessels and submitted to the above described saponification and preconcentration procedures. Samples were analyzed in duplicate.

3. Results and discussion

3.1. Chromatographic separation

The normal-phase technique was selected for the separation using a LiChrospher Si 60 analytical column. The mobile phase was selected according to the European Standard [30]. Under these conditions, retention times were 4.61 min for 13-*cis*-retinol and 5.76 min for all-*trans*-retinol. The flow-rate was 2 mL min^{-1}.

3.2. DLLME parameters

The parameters affecting the DLLME procedure, the extraction and disperser solvents, as well as their volumes, the addition of salt, pH and both the speed and time of the centrifugation step, were optimized. For this purpose, an aqueous standard solution or a diluted juice sample (2 mL saponified juice and 8 mL water) containing analyte concentrations of about 20 ng mL^{-1} were used and 20 μL of the settled phase was injected into the LC.

The extraction solvent must have the following characteristics: high extraction capability, a higher density than water, low solubility in water and good chromatographic behaviour. Several solvents with a lower density than water (decanol, 1-undecanol and 2-octanone) were tested, but extraction was almost negligible for all these solvents. When extraction solvents of higher density than water were assayed, well-defined settled volumes were recovered with 1,1,2,2-tetrachloroethane, chloroform and carbon tetrachloride, using 100 μL of the extraction solvent and 0.5 mL methanol as the disperser solvent. Values for peak areas were, respectively, 18.4, 6.4 and 3.3. The sedimented phase was not discernible when using dichloromethane due to its high solubility in water. The best extraction efficiency was obtained using tetrachloroethane as extraction solvent.

The influence of the $C_2H_2Cl_4$ volume was first studied in the 50 to 110 μL range for a 20 ng mL^{-1} aqueous standard solution. Volumes of 50–60 μL led to very small volumes for the sedimented organic drop. Fig. 1A shows that peak areas increased with increasing extraction solvent volumes from 80 to 90 μL. Any further increase in the volume of the extraction solvent led to the peak areas decreasing as a consequence of dilution, and so 90 μL appeared the most appropriate volume for the aqueous solution (the volume of the sedimented phase was 40 ± 10 μL after extraction and centrifugation). These experiments were repeated using the diluted juice sample (2 mL saponified juice and 8 mL water) spiked with 20 ng mL^{-1} retinol. Fig. 1B shows that in this case the volume of the sedimented phase was too low for extractant volumes below 100 μL, while the sensitivity decreased for higher volumes. Therefore, a 100 μL extractant volume was selected, which also permitted to obtain a volume of the sedimented phase of 40 ± 10 μL for the juice sample.

The disperser solvent must be miscible in the extraction solvent and the aqueous phase. Acetone, methanol, ethanol, acetonitrile and diethyl ether were tested by rapidly injecting 0.5 mL of each disperser containing 100 μL of $C_2H_2Cl_4$ into the aqueous solution. The extraction efficiency was highest when using methanol (Fig. 2A), which was therefore selected. The volumes assayed for the disperser solvent were in the range

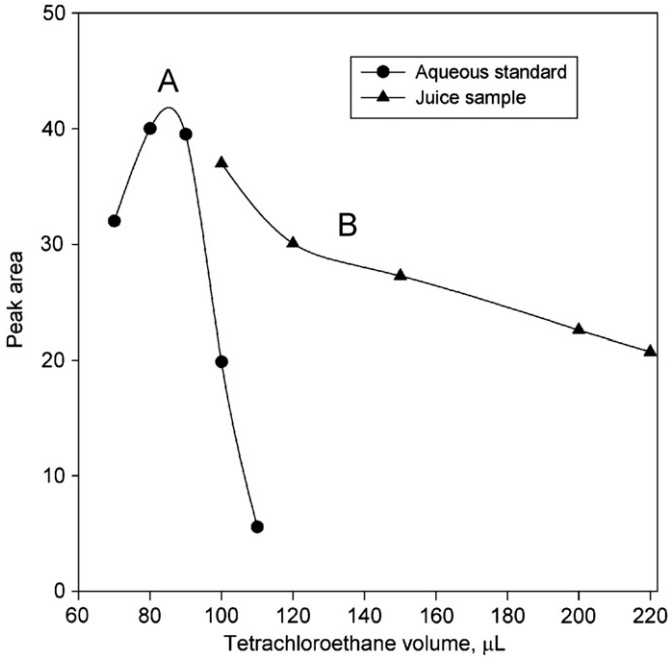

Fig. 1. Influence of the volume of tetrachloroethane on the peak area of all-*trans*-retinol by DLLME-LC for (A) an aqueous standard solution (20 ng mL^{-1}) and (B) a juice sample.

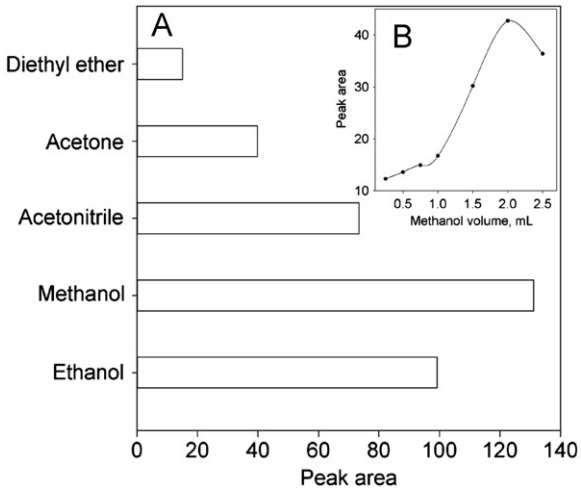

Fig. 2. Effect of the type (A) and volume (B) of the disperser solvent on the peak area of an aqueous standard solution (20 ng mL^{-1}) of all-*trans*-retinol by DLLME-LC.

0.25–2.5 mL, containing in all cases the optimized extraction solvent volume. At low disperser volumes, the cloudy state was difficult to reach, meaning that extraction recovery was low. According to Fig. 2B, the extraction efficiency increased up to 2 mL and then decreased with higher volumes. Highest sensitivity was attained with 2 mL of methanol for both the aqueous standard and the diluted juice sample.

The influence of ionic strength was evaluated at 0–30% (m/v) sodium chloride concentrations in the aqueous phase, in order to reduce the solubility of retinol and increase the solubility in the organic phase. However, peak area decreased with increasing salt concentration throughout the range studied and, so, the addition of salt was discarded. To evaluate the influence of pH in the aqueous phase, different samples were adjusted to pH values ranging between 4 and 10 with a 0.01 M phosphate buffer solution. The volume of the sedimented organic solvent did not

vary and no significant differences in the analytical signal were appreciated for all-*trans*-retinol. Consequently, samples were analyzed without pH adjustment in the aqueous phase.

Extraction time in DLLME is defined as the time between injecting the mixture of disperser and extraction solvents and before starting the centrifugation step. As expected, no differences in sensitivity for retinol were attained in the interval 30 s to 5 min, demonstrating that DLLME is practically time-independent, one of its most important advantages. Therefore, the mixture was shaken for a few seconds and then centrifuged. Nevertheless, the centrifugation time and speed necessary to disrupt the cloudy solution and collect the sedimented phase were evaluated. When the centrifugation time was varied between 1 and 5 min, extraction efficiency increased up to 2 min and decreased with longer times, and so a time of 2 min is recommended (Fig. 3A). The centrifugation speed was modified in the 1000–3000 rpm range and sensitivity increased up to 3000 rpm, which was selected (Fig. 3B).

The DLLME procedure was compared with the ultrasound-assisted emulsification microextraction (USAEME) technique, which provided very lower sensitivity.

3.3. Analytical characteristics of the method

The method was validated for linearity, detection and quantification limits, selectivity, accuracy and precision. For comparison purposes, the direct LC method without preconcentration was also evaluated. The calibration curve using DLLME-LC was obtained by least-squares linear regression analysis of the peak area versus retinol concentration, using six levels in duplicate experiments, after saponification of the aqueous standards. When the aqueous standards were not saponified, the calibration graph showed a higher slope. The validation results obtained are summarized in Table 1. The value of r^2 was good ($r^2 > 0.99$), demonstrating excellent linearity for the range studied. The limit of detection (LOD, calculated for a signal-to noise ratio of 3) and the limit of quantification (LOQ, calculated for a signal-to noise ratio of 10) are also shown in Table 1. The selectivity of the method was judged

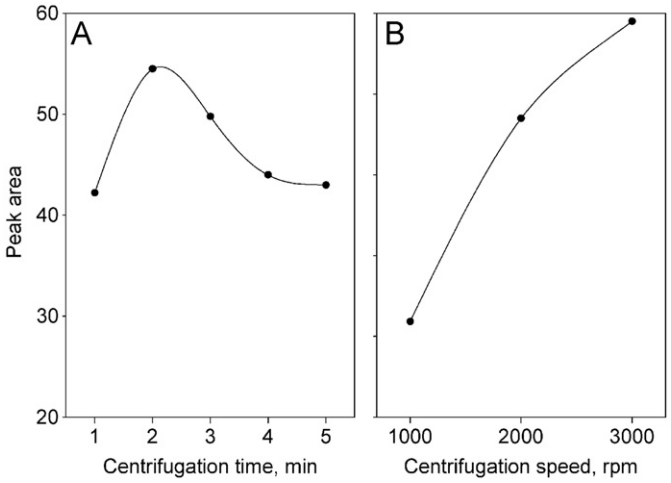

Fig. 3. Influence of the centrifugation time (A) and centrifugation speed (B) on the sensitivity obtained for an aqueous standard solution (20 ng mL^{-1}) of all-*trans*-retinol by DLLME-LC.

Table 1
Validation results for all-*trans*-retinol by DLLME-LC.

Parameter	Value
Linearity (ng mL^{-1})	0.5–50
Slope ± SD (mL ng^{-1})	0.767 ± 0.012
Regression coefficient (r^2)	0.9997
LOD (ng mL^{-1})	0.23
LOQ (ng mL^{-1})	0.76
Intraday RSD (%)	1.2
Interday RSD (%)	5.7
Enrichment factor	50 ± 3

Table 2
Slopes of calibration graphs for all-*trans*-retinol by DLLME-LC.

Sample	Slope ± SD (mL ng^{-1})	r^2
Aqueous standards without saponification	1.51 ± 0.019	0.9995
Aqueous standards with saponification	0.767 ± 0.012	0.9997
Pinneapple juice with saponification	0.753 ± 0.013	0.9998
Orange drink with saponification	0.822 ± 0.013	0.9994
Concentrated juice with saponification	0.798 ± 0.007	0.9995

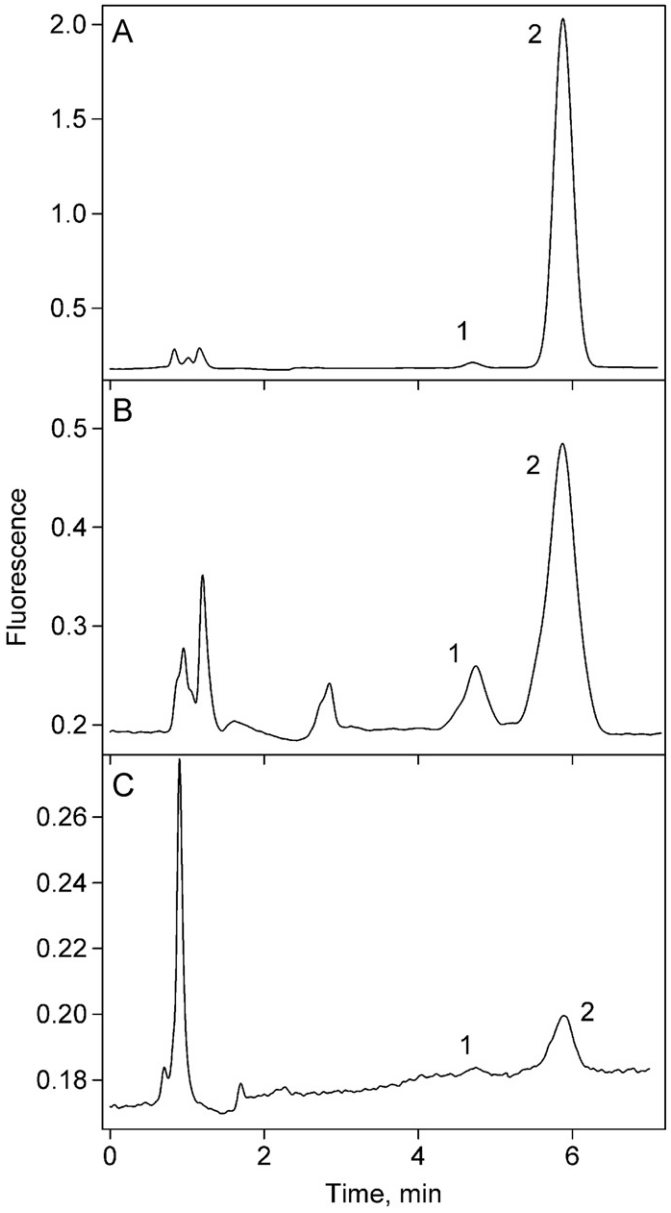

Fig. 4. Elution profiles obtained from a concentrated juice (A), an orange juice (B) and the whole milk powder ERM®-BD600 reference material (C) by DLLME-LC. The sample taken was 0.2 mL (A), 2 mL (B) and 50 mg (C). Peaks correspond to: 1, 13-*cis*-retinol and 2, all-*trans*-retinol.

from the absence of interfering peaks at the retinol isomer elution times for blank chromatograms of different non-spiked juices. No matrix compounds were observed that might give a false positive signal in the blank samples. The repeatability was calculated by using the relative standard deviation (RSD) from a series of ten consecutive DLLME-LC analyses of one juice sample containing retinol at 10 ng mL^{-1} The enrichment factor (EF) was calculated as the ratio between the slope value obtained by DLLME and the slope obtained by LC. In this case, a value of about 50 ± 3 was attained.

The matrix effect was studied by comparing the slopes of aqueous standards and standard additions calibration graphs for the different juice samples, obtained by plotting concentration (at six different levels) against peak area and following linear regression analysis. For the direct LC procedure, the presence of a matrix effect was discarded because "p" value obtained from application of a one-sample t-test was higher than 0.05 ($p=0.919$). In addition, the matrix effect was studied for the proposed DLLME-LC procedure (Table 2), by obtaining the slopes for the different juice samples and again the matrix effect was discarded because the "p" value obtained from the one-sample t-test was higher than 0.05 ($p=0.836$). Consequently, calibration and analysis of the samples must be performed using saponified aqueous standards.

3.4. Analysis of samples

The juice samples were saponified according to the European Standard using 50 mL methanol and 5 mL KOH for sample volumes of 2–10 mL, depending on the vitamin content. However, after saponification, DLLME was performed to avoid the tedious liquid–liquid extraction procedure with large amounts of organic solvents (100 mL, 3–4 times), washing with water (2–4 times, 50–150 mL) and then evaporation at low temperature.

Fig. 4 shows the elution profiles obtained using DLLME-LC with fluorescence detection for two juice samples and a certified reference material. Similar chromatograms were obtained for the other samples. The elution profiles obtained demonstrated the absence of interfering compounds eluting at the retention times of retinol isomers. Identification was carried out by comparing the retention time and the fluorescence spectra while the analyte passed through the flow cell. Table 3 shows the results obtained. The contents of all-*trans*-retinol obtained were in accordance with the contents reported by the manufacturers when the juices analyzed were freshly prepared. However, the contents decreased as the storage time increased. Similar values were also obtained for all-*trans*-retinol when using the proposed DLLME-LC procedure and the direct method using LC. The *cis*-retinol isomer was also found when using the DLLME-LC procedure in most of the juice samples analyzed, but always at concentrations considerably lower than those for the *trans*-isomer. However, the direct method using LC cannot be applied to the quantification of this isomer because the concentrations were below the detection limit when no preconcentration step was applied, thus confirming the usefulness of the DLLME procedure for low levels of retinol.

To test the accuracy of the proposed method, three juice samples free of vitamin A were enriched at two concentration levels (5 and 25 ng mL^{-1}) and analyzed by DLLME-LC after saponification. The results showed a mean recovery $\pm$ standard deviation of $94.7 \pm 5.3\%$ ($n=12$).

Finally, the reliability of the method was further checked by using two certified reference materials: infant/adult nutritional formula SRM 1849a and whole milk powder ERM®-BD600. Table 4 shows the results obtained. The values obtained by the proposed DLLME-LC method were in excellent agreement with the certified contents. The statistical study using the paired t-test showed that there was no significant difference (95% confidence interval) between the results obtained and the certified values (p values obtained were 0.481 for all-*trans*-retinol and 0.453 for all-*trans*+*cis*-retinol). Such data also confirm the efficacy of the extraction procedure for recovering both free supplemented and endogenous vitamin A in foods.

4. Conclusion

The vitamin A content of several juices was evaluated using a new green sample preparation technique, in this case a miniaturized

Table 3
Vitamin A content in juice samples.

Sample	DLLME-LC (μg mL^{-1})		LC (μg mL^{-1})	Declared
	All-*trans*-retinol	13-*cis*-retinol	All-*trans*-retinol	Vitamin A (μg mL^{-1})
Orange drink sunny delight	1.11 ± 0.12	ND	1.03 ± 0.17	1.2
Pineapple juice 1	1.29 ± 0.21	0.227 ± 0.043	1.21 ± 0.20	1.2
Pineapple juice 2	1.26 ± 0.11	0.244 ± 0.012	1.13 ± 0.02	1.2
Orange juice	1.49 ± 0.02	0.169 ± 0.005	1.27 ± 0.07	1.2
Multifruits	ND	ND	ND	1.2
Concentrated pineapple juice and milk 1	4.67 ± 0.12	0.108 ± 0.017	4.5 ± 0.54	4.8
Concentrated pineapple juice and milk 2	6.30 ± 0.23	0.249 ± 0.003	6.52 ± 0.2	6.6
Pear juice	0.9 ± 0.022	0.082 ± 0.004	0.87 ± 0.01	1.2
Apple–mango juice	1.07 ± 0.02	0.085 ± 0.001	1.2 ± 0.013	1.2
Banana–apple juice	0.93 ± 0.06	0.081 ± 0.003	0.82 ± 0.01	1.2
Milk-containing multifruit juice	1.64 ± 0.09	0.385 ± 0.021	1.74 ± 0.07	1.2

Table 4
Vitamin A content in certified reference materials.

Reference material	DLLME-LC (μg g^{-1})		Certified value
	All-*trans*-retinol	13-*cis*-retinol	Vitamin A (μg g^{-1})
Infant/adult nutritional formula SRM 1849a	7.94 ± 0.73	ND	7.68 ± 0.23 mg/kg retinol equivalents, total (*trans*+*cis*)-retinol, added as retinol palmitate
Whole milk powder ERM®-BD600	3.78 ± 0.21	0.34 ± 0.1	3.8 mg kg^{-1} all-*trans*-retinol and 4.1 mg kg^{-1} all-*trans*-retinol and 13-*cis*-retinol

preconcentration procedure based on DLLME coupled to normal phase LC-Fluorescence. In addition to the low detection limits as a result of the high enrichment power of DLLME, very low quantities of solvent were used, meaning that the procedure can be described as environmentally friendly. The juice samples were saponified according to the European Standard and quantification could be carried out by aqueous calibration. The USAEME technique was also tried, but the sensitivity was lower than that of the DLLME procedure.

Acknowledgements

The authors acknowledge the financial support of the Comunidad Autónoma de la Región de Murcia (CARM, Fundación Séneca, Project 15217/PI/10) and the Spanish MICINN (Project CTQ2009-08267/BQU). M. Bravo-Bravo acknowledges a fellowship from Fundación Séneca, CARM.

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