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Pressurized liquid extraction and dispersive liquid–liquid microextraction for determination of tocopherols and tocotrienols in plant foods by liquid chromatography with fluorescence and atmospheric pressure chemical ionization-mass spectrometry detection

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ABSTRACT

Pressurized liquid extraction (PLE) and dispersive liquid–liquid microextraction (DLLME) were used to isolate and preconcentrate tocopherols and tocotrienols from plant foods. The Taguchi experimental method was used to optimize the six factors (three levels for each factor), affecting DLLME, namely: carbon tetrachloride volume, methanol volume, aqueous sample volume, pH of sample, sodium chloride concentration and time of the centrifugation step. The influencing parameters selected were 2 mL of methanol:isopropanol (1:1) (disperser solvent), 150 μL carbon tetrachloride (extraction solvent) and 10 mL aqueous solution. The organic phase was injected into reversed-phase liquid chromatography (LC) with an isocratic mobile phase composed of an 85:15 (v/v) methanol:water mixture and a pentafluorophenyl stationary phase. Detection was carried out using both fluorescence and atmospheric pressure chemical ionization mass spectrometry (APCI-MS) in negative ion mode. Quantification was carried out by the standard addition method. Detection limits were in the range 0.2–0.3 ng mL^{-1} for the vitamers with base-line resolution. The recoveries obtained using the optimized DLLME were in the 90–108% range, with RSDs lower than 6.7%. The APCI-MS spectra, in combination with fluorescence spectra, permitted the correct identification of compounds in the vegetable and fruit samples. The method was validated according to international guidelines and using two certified reference materials.

1. Introduction

Pressurized liquid technology (PLE) is an emerging greener technique based on the use of liquid solvents at elevated temperature and pressure, thus improving the extraction performance of classical techniques [1]. The extraction of bioactive compounds, which are sensitive, thermolabile and found in low concentrations in foods, leads to low yields with traditional techniques. However, PLE enhances the extraction efficiency by increasing solubility and mass transfer properties [2–3].

The perspective of hyphenation and combination of different sample preparation techniques is one recent strategy in analytical chemistry. Thus, by combining PLE with miniaturized analytical techniques, it would be possible to extract compounds from samples and high preconcentration through chemical processes which use low quantities of solvents for dissolving or extracting analytes, in line with the priorities of green chemistry [4]. Dispersive liquid–liquid microextraction (DLLME) is a very simple and rapid technique [5] using a ternary component solvent system formed by an aqueous solution containing the analytes, a water-immiscible extraction solvent and a water miscible disperser solvent.

The vitamin E group includes eight liposoluble vitamers or tocols. Its structure is comprises two primary parts: a chromanol ring and a hydrophobic side chain [6], which are divided into two fundamental groups, four tocopherols (T), with saturated isoprenoid side chains, and four tocotrienols (T3) with isoprenyl side chains with three double bonds. Furthermore, each group includes four vitamers (α-, β-, γ- and δ), which differ in the number and position of the methyl substitutes in the chromanol ring [7]. The most widely distributed and biologically active as a vitamin is α-T, while the activity of β-T is 30% of that of α-T; γ-T has 15%; and δ-T only 3%. The activity of α-T3 is 25% of that of α-T.

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The vitamin E content in animal foods is very variable, depending on the animal diet. In contrast, it is mainly found in plant foods, especially in oilseeds, green parts of plants, and oils of wheat germen, sunflower, corn and olive. Because of its antioxidant role, the vitamin E content in foods also has a technological significance, since these compounds react with free radicals, and are reduced during the manufacture and storing of foods [8].

Liquid chromatography (LC) is the most widely used technique for vitamin E determination in foods [8,9]. It uses different detection systems, such as UV–vis [10–16], fluorescence [17–32], electrochemical [33–35] or mass spectrometry (MS), which combines the resolution of LC with the detection specificity of MS, mainly using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) [11,12,36–43].

The food sample preparation is the main source of error and the most commonly used methods to extract vitamin E are solvent extraction or alkaline hydrolysis [7–9]. Natural foods would not need to be hydrolyzed because the vitamers occur mainly as free compounds, but fortified foods should be saponified because they are generally added as esters [10]. As an alternative, tocols may be extracted by PLE techniques [33–35,40], thus achieving shorter extraction times and decreasing solvent volumes. The Standardized Method of analysis for vitamin E includes the determination of α-, β-, γ- and δ-tocopherol by LC separation and subsequent photometric (UV) or preferably fluorimetric detection. In most cases, a saponification of the material followed by an extraction is necessary [44].

As regards new clean sample preparation techniques, solid-phase microextraction (SPME) has been used to the extraction of vitamins A, D_3 and E [45], microextraction of vitamin E with hexane and fluorescence detection [46]. A liquid-phase microextraction (LPME) procedure using solidification of a floating drop has been proposed for determination of fat-soluble vitamins [47], and DLLME has recently been applied in the determination of α-tocopherol [48] and tocopherols and tocotrienols using fluorescence detection [49].

When designing an optimization model, the multiple factors affecting DLLME can be considered together by a balanced orthogonal array design (OAD) based on the Taguchi method [50]. Depending on the number of parameters, the OAD approach made it possible to run experiments, analyze data, identify the optimum conditions and perform confirmation runs with the optimum levels of all the parameters.

This study proposes a procedure using LPE and DLLME such as green sample preparation techniques for the efficient determination of tocopherols and tocotrienols in plant foods with LC using fluorescence and APCI-MS detection. The Taguchi experimental method is applied to study the possible influence on the performance of the method of six factors. The method is validated according to international guidelines. The main contribution of this study is that is the first time that vitamin E forms are preconcentrated by PLE and DLLME and unequivocally identified by MS.

2. Experimental

2.1. Reagents and samples

Chromatographic quality acetonitrile, methanol and carbon tetrachloride were obtained from Sigma (St. Louis, MO, USA). The water used was previously purified in a Milli-Q system (Millipore, Bedford, MA, USA). DL-α-tocopherol, rac-β-tocopherol, γ-tocopherol and δ-tocopherol, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (1000 μg mL^{-1}) were prepared in ethanol and stored in amber vials at −20 °C. The stock solutions were checked for concentration and purity by UV spectroscopy using the known absorption coefficient of each isomer [35]. Working standard solutions were prepared daily in ethanol and stored at 4 °C. Because tocotrienols were not available, refined palm oil was obtained from Fluka (Spain). The oil (2 g) was extracted by a PLE treatment [35] using methanol/isopropanol (50 mL, 1:1, v/v) as solvent at a temperature of 50 °C and a pressure of 1600 psi, with one cycle of extraction during a static time of 5 min. The extract contained α-, γ- and δ-tocotrienol and was used to provide reference retention times for tocotrienols. The calibration graphs of tocopherols were used to quantify both tocopherols and their corresponding tocotrienols, according to the literature [35]. Other reagents were hydromatrix celite (Agilent), sodium chloride, ascorbic acid and potassium hydroxide (Merck).

Samples of fruits and vegetables were commercially obtained and just analyzed. Samples were spiked with a mixture containing the standards and extracted after 30 min.

2.2. Instrumentation for LC-fluorescence

The LC-fluorescence system consisted of an Agilent 1100 (Agilent, Waldbronn, Germany) quaternary pump (G1311A) operating at room temperature. The solvents were degassed using an on-line membrane system (G1379A). The fluorescence detector was an Agilent FLD (G1321A) operating at an excitation wavelength of 298 nm and an emission wavelength of 345 nm.

The analytical column used for the reversed-phase technique was Ascentis® Express F5 filled with dimethylpentafluorophenylpropyl (15 cm × 0.46 cm × 5 μm) (Sigma). The mobile phase was a 85:15 methanol:water (v/v) mixture under isocratic conditions. The flow-rate was 1 mL min^{-1}. Aliquots of 20 μL were injected manually using a Model 7125-075 Rheodyne injection valve (Rheodyne, Berkeley, CA, USA). Solutions were stored in 2 or 10 mL amber glass vials. 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. Vegetable samples were homogenized using an IKA A 11 homogenizer (Staufen, Germany).

Extractions were performed with a Dionex (Germany) 200 Accelerated solvent extractor system, equipped with 22 mL stainless steel extraction cells and 60 mL Dionex glass vials for extract collection.

2.3. LC–APCI-MS system

The LC system consisted of an Agilent 1200 (Agilent, Waldbronn, Germany) binary pump (G1312A) operating at a flow-rate of 1 mL min^{-1}. The solvents were degassed using an on-line membrane system (G1379A). The column was maintained in a thermostated compartment at room temperature (G1316A), and injection (20 μL) was performed using an autosampler (G1329A). The column and the mobile phase were the same as those optimized for fluorescence detection. The LC system was coupled to an ion-trap (IT) mass spectrometer (1036 model) equipped with an APCI interface operating in negative ion mode. The selected ion monitoring (SIM) mode was applied. The instrument parameters were: drying temperature 350 °C, APCI temperature 400 °C, drying gas flow 5 L min^{-1} and nebulizer gas pressure 60 psi.

2.4. PLE procedure for food samples

The fruit and vegetable foods consisted of different types, such as spinach, corn, cranberry, pomegranate and mango juice, all commercially obtained from street markets. The samples were cut

into small pieces and homogenized. Three grams of crushed food was weighed and mixed with 1.5 g of the drying agent (hydromatrix celite) in order to prevent the aggregation of sample particles and was placed in a 22 mL extraction cell with double glass fiber (Dionex Corp.) inside. The sample was extracted by a PLE treatment using methanol:isopropanol (1:1 v/v, 50 mL) as solvent at an oven temperature of 50 °C and a pressure of 1600 psi, with one cycle of extraction during a static time of 5 min. After the extraction, a volume of clean solvent was pumped into the sample cell, and the solvent was then purged from the cell with nitrogen with a purge time of 60 s. The extracts were diluted to 50 mL and filtered through 0.45 μm PVDF filters.

2.5. DLLME procedure

For DLLME, a 0.2–2 mL-volume organic fraction resulting from the PLE extraction (depending on the analyte concentration) was recovered and used as dispersant solvent, to which methanol: isopropanol (1:1, v/v) up to 2 mL and 150 μL of carbon tetrachloride (extractant solvent) was added. The mixture was then rapidly injected into 10 mL of water using a micropipette, and gently shaken manually for several seconds. After centrifugation at 3000 rpm for 3 min, the extraction solvent was sedimented at the bottom of the conical tube (volume recovered 50 ± 10 μL). The sedimented phase was collected and evaporated to dryness under argon flow. The residue was reconstituted with 50 μL of methanol, and 20 μL was injected into the LC.

2.6. Analysis of certified reference materials

The method was validated using two reference materials: 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). The samples were saponified (according to the procedure indicated by suppliers) in duplicate. All operations were performed in subdued light. Fifty milligrams of the sample was weighed and 25 mg 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). Aliquots were filtered using 0.45 μm PVDF filters for subsequent DLLME, using 0.2 mL of the extract plus 1.8 mL 1:1 methanol:isopropanol, as dispersant solvent.

3. Results and discussion

3.1. Chromatographic separation

The optimization of the chromatographic separation was carried out using a palm oil sample extracted by PLE and fortified with the four tocopherols. Several reversed-phase (RP) stationary phases including C_8 and C_{18} (both endcapped and non-endcapped), such as Zorbax Eclipse XDB-C_8 (15 cm × 0.46 cm × 5 μm), Zorbax ODS endcapped (15 cm × 0.46 cm × 5 μm) and Zorbax Eclipse ODS non-endcapped (25 cm × 0.46 cm × 5 μm) were compared. T and T3 were separated into two groups because of their different polarity and saturation degree of the side chain. In RP-LC, the tocopherols eluted later than tocotrienols because of their lower polarity. With all these stationary phases, good separation was achieved for all tocols with the exception of the band pair corresponding to β- and γ-T, for which none of the assayed mobile and stationary phases provided good resolution. An Ascentis® Express F5 filled with dimethylpentafluorophenylpropyl (15 cm × 0.46 cm × 5 μm) was then tested. This stationary phase is packed with electron-deficient phenyl rings due to the presence of electronegative fluorines. In addition, F5 phases also retain compounds by polar interactions. As a result of their having both polar and non-polar character, F5 phases retain hydrophobic compounds less than C18, and are ideal for the separation of closely related compounds, such as the isomers β- and γ-T.

Several mobile phases corresponding to mixtures of methanol and water in different percentages were assayed. A 85:15 (v/v) methanol:water mixture in isocratic mode led to the elution of the tocols with appropriate retention times, between 5.2 and 15.5 min, leading to complete resolution of the isomers band pair of β- and γ-T, which cannot be resolved in most published procedures. The mobile phase flow-rate was maintained at 1.0 mL min^{-1}. Table 1 summarizes the order of elution, retention times and retention factors for the analytes in the selected conditions.

3.2. APCI-MS detection

APCI was selected for ionization as vitamin E forms are neutral and non-volatile compounds of low polarity, thus giving higher signals in APCI than in ESI. The optimal ionization mode was studied using a full-scan of each analyte, and maximum sensitivity was obtained operating in negative ion mode for all isomers, obtaining the deprotonated molecular ion $[M–H]^-$ as base peak. Then, optimization of the mass-to-charge ratio values was carried out in the selected ion monitoring (SIM) mode and the *m/z* corresponding to the ions were *m/z* 429.5 for α-T, *m/z* 415.5 for β-T and γ-T, *m/z* 401.4 for δ-T, *m/z* 423.5 for α-T3, *m/z* 409.5 for γ-T3 and *m/z* 395.4 for δ-T3.

3.3. Optimization of the PLE technique

Vitamin E is generally extracted from a food matrix using organic solvents or by alkaline hydrolysis, which improves extractability although significant losses may occur unless protected from oxidation [7,9]. As an alternative, tocols may be extracted by a PLE technique, which decrease solvent volume, extraction time and facilitates automation. Several extraction organic solvents, such as acetonitrile, methanol and a methanol:isopropanol mixture were tried and slightly higher recoveries were obtained using the 1:1 (v/v) methanol:isopropanol mixture, which was selected. Extractions were performed at 50, 70, 80 and 90 °C at a pressure of 1600 psi. However, high temperatures might affect thermo-labile compounds and, due to the thermal instability of vitamin E, a value of 50 °C was selected to avoid losses. The effect of elevated pressure was tried. Thus, extractions were carried out at 1400, 1600 and 1800 psi. However, the effect of pressure on recovery was not significant, as previously reported [2], and a pressure of 1600 psi was selected. The optimization of sample amount was performed between 1 and 5 g sample. Recovery was highest for a sample of 3 g, which was mixed with the drying agent hydromatrix celite to prevent the aggregation of sample particles. The static time was optimized with extractions at 5, 8 and 10 min and recoveries did not show significant differences, so a static time of

Table 1
LC parameters for the vitamin E forms.

Analyte	Retention time (min)	Retention factor
δ-T3	5.2	3.9
γ-T3	6.9	5.5
α-T3	8.1	6.6
δ-T	9.4	7.9
β-T	11.8	10.1
γ-T	12.9	11.1
α-T	15.5	13.7

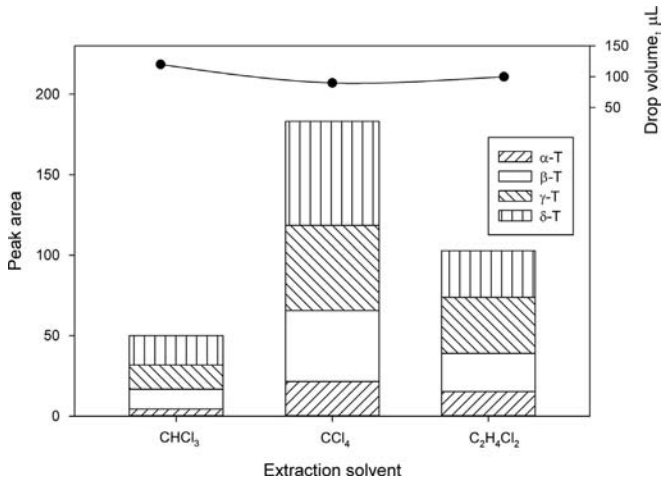

Fig. 1. Influence of the extractant solvent on the extraction efficiency for tocopherol isomers and on the organic drop volume.

Table 2
Variables selected for the Taguchi design for DLLME.

Factors	Level 1	Level 2	Level 3
Extractant organic volume (μL)	50	100	150
Aqueous phase volume (mL)	3	6	10
Dispersant volume (mL)	0.5	1	2
pH	3	6	9
Sodium chloride (% m/v)	0	10	25
Centrifugation time (min)	1	2	3

5 min was selected. The static process can be repeated and a number of 1, 2 and 3 extraction cycles were assayed. No significant differences were obtained for several cycles and one single extraction cycle was applied to decrease the total treatment time.

3.4. Optimization of the DLLME procedure using a Taguchi design method

The experimental variables affecting DLLME procedure were optimized using the Taguchi method. These experiments were carried out using diluted mango–apple juice fortified with 100 ng mL^{-1} of tocols. First, the solvents used as extractant and dispersant were selected, given that they should have a low boiling temperature because the sedimented organic phase must be evaporated and reconstituted using a solvent compatible with RP-LC. The extractant solvents assayed were carbon tetrachloride, chloroform and 1,2-dichloroethane (150 μL volume), using 2 mL of dispersant solvent. Fig. 1 gives the results obtained for tocopherols, showing higher extraction efficiency for all the isomers when using carbon tetrachloride. The volume obtained for the organic drop collected after centrifugation of the dispersion was smaller for this solvent. Similar experiments were repeated to select the optimal dispersant solvent by rapidly injecting 2 mL of each dispersant (acetone, methanol:isopropanol, ethanol and acetonitrile) containing 150 μL of CCl_4 into 10 mL of both a fortified diluted juice sample and a spinach extract. The volume of the collected organic drop was similar for all dispersant solvents, while the extraction efficiency was higher for all analytes using acetone and 1:1 methanol:isopropanol, so the latter was selected.

The rest of the experimental variables affecting DLLME were optimized using an orthogonal array design (OAD), the Taguchi method, which was applied for six factors (each factor at three levels), as shown in Table 2. The proposed OAD considers the

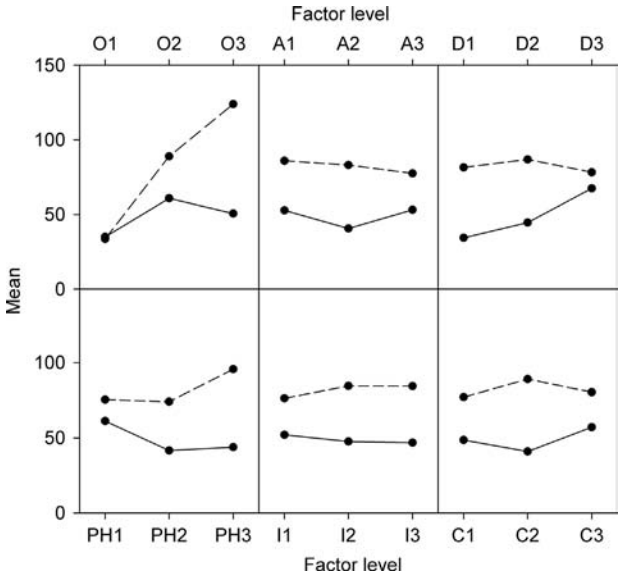

Fig. 2. Effects of factor levels of the extractant organic volume (O), aqueous phase volume (A), disperser volume (D), pH of the aqueous phase (pH), sodium chloride concentration (I) and centrifugation time (C) on the mean response for the extraction efficiency of tocopherols (solid lines) and the mean response for the drop volume (dotted lines).

factors without their interactions with 27 different trials. On the other hand, the temperature, extraction time and stirring were not considered as variables, as equilibrium was rapidly reached. The centrifugation speed was fixed at 3000 rpm.

Fig. 2 shows the effects of the six factor levels on the mean response for the extraction efficiency of tocopherols (solid lines) and the mean response for the drop volume (dotted lines). The extraction efficiency increased up to 100–150 μL of carbon tetrachloride volume (depending on the sample), while it decreased for higher volumes due to the dilution effect; therefore, a 150 μL volume was selected. The variation of the aqueous phase volume led to an almost constant peak area for all the range studied, and a volume of 10 mL was chosen. The increase in the volume of the dispersant solvent produced a continuous increase in the sensitivity and a 2 mL volume was selected. The variation of the pH between 3 and 9 with 0.01 M buffer solutions shows that optimal results were obtained at pH 3. The volume of the sedimented organic solvent did not vary and no significant differences in the analytical signals were observed. Extraction efficiency slightly decreased when the salt concentration was increased and, thus, the addition of NaCl to the extraction solution was discarded. The centrifugation time necessary to disrupt the cloudy solution and collect the sedimented phase caused higher peak areas for higher values and a time of 3 min at 3000 rpm was selected.

The mean response for the drop volume shows that no significant variations were produced when all the parameters were varied, with the exception of the extraction solvent volume, where the drop volume continuously increased in the range studied.

A statistical analysis of variance (ANOVA) was performed to discriminate which parameters significantly affect extraction efficiency (Table 3). From the calculated variance ratios, *F*, it can be deduced that only three factors considered in the experimental design were statistically significant at a 95% confidence level (in all cases the calculated *F* was greater than the critical value). These variables were the dispersant volume, the extractant volume and the pH of the aqueous solution. The most influential factors were the volumes of both the dispersant and the extractant solvents. The contribution of the residual error to the signal variability

Table 3
Results of the analysis of variance for mean response and volume drop (into brackets).

Variation source	Degrees of freedom	Sum of squares	Mean of squares	*F*	*P*	Contribution (%)
Extractant volume (μL)	2	3038.9 (37243.6)	1519.4 (18621.8)	4.95 (35.6)	0.024 (0)	24.3 (89.6)
Aqueous volume (mL)	2	910.8 (333.4)	455.4 (166.7)	1.48 (0.32)	0.26 (0.73)	7.3 (0.8)
Dispersant volume (mL)	2	5156.5 (336.1)	2578.3 (168)	8.41 (0.32)	0.004 (0.73)	41.2 (0.8)
pH	2	2076.6 (2598.3)	1038.3 (1299.1)	3.39 (2.49)	0.063 (0.119)	16.6 (6.2)
NaCl (% w/v)	2	141.9 (389.4)	71 (194.7)	0.23 (0.37)	0.796 (0.696)	1.1 (0.9)
Centrifugation time (min)	2	1184.9 (680.5)	592.5 (340.3)	1.93 (0.65)	0.182 (0.536)	9.5 (1.6)
Error	14	4293.9 (7314.1)	306.7 (522.4)			
Total	26	16803.4 (48895.4)				

Table 4
Calibration parameters for tocopherols.

	δ-T	β-T	γ-T	α-T
Fluorescence detection				
Linear range (ng mL^{-1})	1–50	1–50	1–50	5–100
Intercept	-0.95 ± 0.31	-0.52 ± 0.32	0.42 ± 0.28	-0.41 ± 0.22
Slope (mL ng^{-1})	2.02 ± 0.01	1.22 ± 0.06	1.68 ± 0.03	0.31 ± 0.01
Sy/x	0.26	0.32	0.46	1.6
Detection limit (ng mL^{-1})	0.15	0.21	0.32	1.1
APCI-MS detection				
Linear range (ng mL^{-1})	1–50	1–50	1–50	1–50
Intercept ($\times 10^3$)	5.2 ± 0.9	4.2 ± 0.6	1.7 ± 0.7	-2.3 ± 1.0
Slope ($\times 10^3$ mL ng^{-1})	11.9 ± 0.48	15.1 ± 0.28	16.0 ± 0.37	15.1 ± 0.46
Sy/x	0.41	0.35	0.23	0.48
Detection limit (ng mL^{-1})	0.21	0.20	0.25	0.32

indicates the goodness of the experimental design used. On the other hand, the only parameter having a significant effect on the drop volume was the organic extractant volume, as can be deduced from the variable values in brackets.

3.5. Validation of the method

The method was validated for linearity, detection and quantification limits, selectivity, recovery, accuracy, precision and robustness, according to international guidelines [51]. Calibration graphs were obtained by the external standard procedure using DLLME and LC-fluorescence by least-squares linear regression analysis of the peak area versus analyte concentration using 10 levels (1–100 ng mL^{-1}) in duplicate experiments. The linearity of the method was assessed from 1–50 ng mL^{-1} for the isomers β-, γ- and δ-T and in the range 5–100 ng mL^{-1} for α-T, due to the lower fluorescence intensity for this isomer. The results obtained are summarized in Table 4. The sensitivity of the method was evaluated by calculating the limits of detection (LOD, for a signal-to-noise ratio of 3), which are also shown in Table 4.

Calibration graphs were also obtained by using DLLME and LC–APCI-MS in the SIM mode of the negative fragment ions and the results obtained are also summarized in Table 4, as well as the LOD values. The linearity of the method was in the range 1–50 ng mL^{-1} for all the tocopherols. Tocotrienols were quantified using the calibration equations of the corresponding tocopherols.

The selectivity of the method was judged from the absence of interfering peaks at the elution times of the tocols for chromatograms of different samples. The performance criteria from EU Commission Decision (2002/657/EC) [51] established the use of co-chromatography to improve the identification of analytes. Thus, the extract prior to LC was divided into two parts, one being directly chromatographed while the other was fortified with the standards and analyzed. To comply with the EU Decision [51], the variability in the fluorescence spectra of the tocols in the samples were not visibly different from the spectra of the calibration standards. Consequently, no matrix compounds existed that might cause interference in the samples. The selectivity was also confirmed by the LC–APCI-MS spectra.

A precision study was carried out on the basis of repeatability, calculated by using the relative standard deviation (RSD) from a series of ten consecutive DLLME followed by LC analyses of a sample spiked with all the analytes at 25 ng mL^{-1}. The RSD values ranged between 5.4% and 7.5%. These values indicate that the precision of the method was satisfactory for control analysis purposes.

3.6. Matrix effect and recovery study

The matrix effect was first evaluated for the DLLME and LC-fluorescence procedure by comparing the slopes of aqueous standards and standard additions calibration graphs for the different food samples, obtained by plotting concentration (at six levels) against peak area and following linear regression analysis. A statistical study was carried out using one-way variance analysis (ANOVA) and the presence of a matrix effect was discarded because the "*P*" values obtained were higher than 0.05 for all the analytes. Consequently, calibration and analysis of the samples using fluorescence detection must be performed using aqueous standards.

Moreover, matrix interferences are very important in quantitative analysis with APCI and can produce the signal suppression of the analytes due to co-eluting compounds. This phenomenon may affect the reproducibility, linearity and accuracy of the method. However, since blank samples were not available, quantification was carried out using the standard additions method [43]. Standard additions were performed according to EU [51] by analyzing one portion of the sample as such, while known amounts of the standard analytes were added to the other test portions before analysis by DLLME followed by LC.

The accuracy of the method was tested by fortifying three samples (spinach, corn and mango–apple juice) with tocopherol amounts of 50 and 100 ng g^{-1}. The recoveries obtained were in the range 90–108%, with RSD lower than 6.7% in all cases (Table 5).

3.7. Analysis of food products and validation using certified reference materials

The proposed method was used for the determination of tocopherols and tocotrienols in different fruits and vegetables. Food samples were extracted by PLE and submitted to analysis. Fig. 3 shows the chromatograms obtained using DLLME and LC with APCI-MS detection in SIM mode for (A) a mixture containing the tocopherol standards and tocotrienols (from the palm oil extract), (B) a spinach extract, (C) a corn sample and (D) the 1849a CRM, as well as the mass spectra of the extracted ions for each one of the peak isomers. Similar chromatograms were obtained for the other samples. The elution profiles obtained demonstrated the absence of interfering compounds eluting at the retention times of the different tocols. Comparison of the retention times for the compounds in the standard mixture and the fortified samples and, especially, the MS spectra, allowed the identification of the vitamin E forms. On the other hand, the chromatograms obtained using fluorescence detection also demonstrated the agreement between spectra for the standards, the samples and the fortified samples.

Table 5
Recoveries[a] from different samples (%).

Sample	Spike level (ng g^{-1})	α-T	β-T	γ-T	δ-T
Spinach	50	108 ± 7	94 ± 4	102 ± 3	101 ± 6
	100	100 ± 5	97 ± 5	107 ± 5	105 ± 4
Corn	50	101 ± 6	93 ± 5	96 ± 5	108 ± 7
	100	96 ± 5	101 ± 6	92 ± 4	98 ± 7
Mango-apple juice	50	98 ± 6	98 ± 5	99 ± 4	103 ± 6
	100	104 ± 7	90 ± 5	102 ± 5	93 ± 5

[a] Mean value ± standard deviation (n=3).

Table 6 shows the tocopherol and tocotrienol contents obtained using the standard additions method to the food samples when using APCI-MS detection. The results were similar when the fluorescence detector was used. Among vegetables and fruits, α-T levels were relatively high in spinach, cranberry and mango. γ-T was higher than α-T in some products, including corn and pomegranate. T3s were found in some plant foods but usually at levels lower than those of Ts. However, γ-T3 was the predominant vitamin E form in corn, cranberry and pomegranate, as indicated by other authors [52].

Finally, the accuracy and reliability of the method was further checked by analyzing two certified reference materials, infant/adult nutritional formula SRM 1849a (NIST) and whole milk powder ERM®-BD600 (IRMM). Table 6 shows the results obtained. The contents for the vitamins obtained by the proposed DLLME and LC–APCI-MS methods were in agreement with the certified contents. The statistical study using the paired t-test showed that there was no significant difference (95% confidence interval)

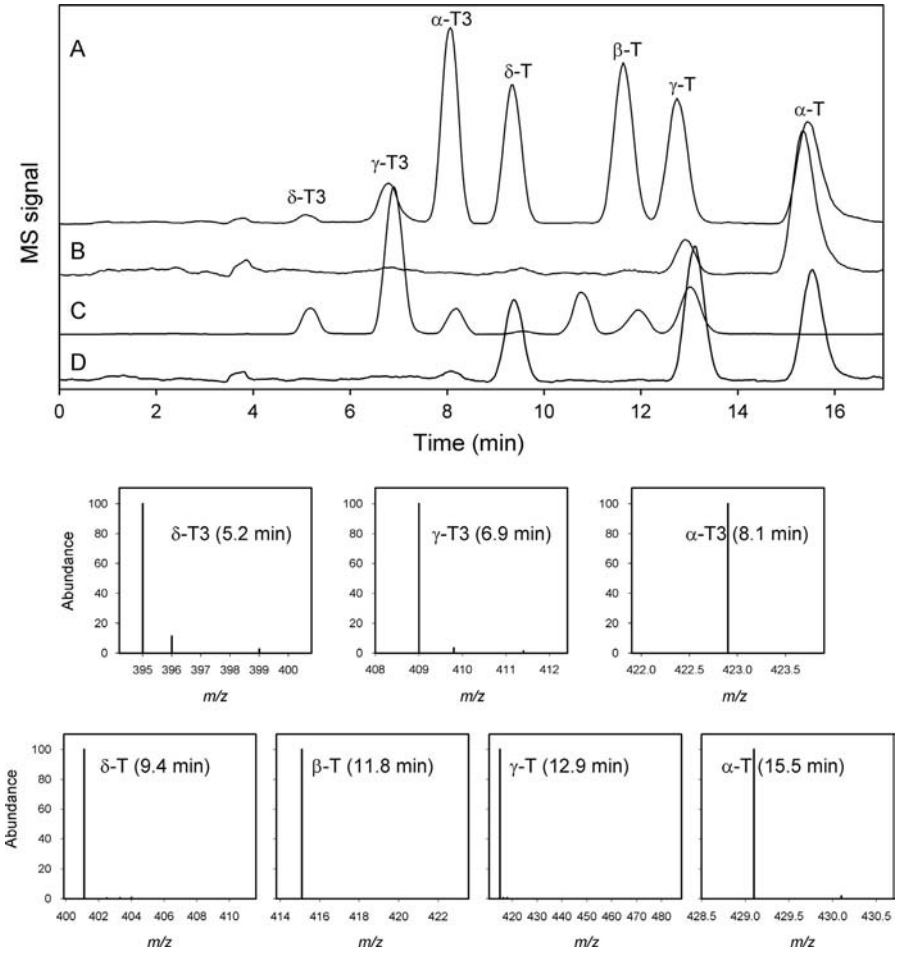

Fig. 3. Chromatograms obtained using DLLME followed by LC–APCI-MS in SIM mode for (A) a mixture containing the tocopherol standards and tocotrienols (from the palm oil extract), (B) a spinach extract, (C) a corn sample and (D) the 1849a CRM, and the mass spectra of the extracted ions for the peak isomers.

Table 6
Content of tocopherols and tocotrienols in plant foods and certified reference materials determined by DLLME and LC–MS.

Food sample	Content ($\mu g\ kg^{-1}$)						
	α-T	β-T	γ-T	δ-T	α-T3	γ-T3	δ-T3
Spinach	284 ± 13	8 ± 0.1	83 ± 3	ND	ND	ND	ND
Corn	ND	143 ± 10	408 ± 20	81 ± 4	753 ± 21	2630 ± 79	744 ± 31
Cranberry	117 ± 5	ND	74 ± 3	ND	ND	334 ± 12	93 ± 3
Pomegranate	ND	ND	7 ± 1	21 ± 2	9 ± 0.7	56 ± 4	ND
Mango–apple juice	319 ± 19	20 ± 2	10 ± 1	39 ± 3	16 ± 1	ND	ND
α-T ($mg\ kg^{-1}$)							
CRM	DLLME and LC-MS	Certified value					
SRM 1849a	204 ± 6	177 ± 47 (added as α-T acetate)					
ERM-BD600	72 ± 4	86 ± 15 (added as α-T)					

between the results obtained and the certified values (*P* value obtained was 0.805). These data also confirm the efficacy of the extraction procedure for recovering both free supplemented and endogenous tocopherols in the samples.

4. Conclusion

The hyphenation of different sample preparation techniques is a recent strategy in analytical chemistry. The combination of PLE, which is an emerging greener technique based on extraction using liquid solvents at elevated temperature and pressure, with a miniaturized analytical technique as DLLME, which uses low amounts of solvents for extracting analytes, makes it possible to extract and determine tocopherols and tocotrienols, according to the priorities of green chemistry. The combination with LC using a dimethylpentafluorophenylpropyl stationary phase permits the separation of all the isomers with good resolution. The agreement between fluorescence spectra, the expected retention time and APCI-MS spectra allows a reliable identification of different forms of vitamin E in food samples.

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