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Dispersive liquid–liquid microextraction for the determination of vitamins D and K in foods by liquid chromatography with diode-array and atmospheric pressure chemical ionization-mass spectrometry detection

Pilar Viñas, María Bravo-Bravo, Ignacio López-García, Manuel Hernández-Córdoba*

Department of Analytical Chemistry, Faculty of Chemistry, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, E-30071 Murcia, Spain

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ABSTRACT

A simple and rapid method was developed using reversed-phase liquid chromatography (LC) with both diode array (DAD) and atmospheric pressure chemical ionization mass spectrometric (APCI-MS) detection, for the simultaneous analysis of the vitamins ergocalciferol (D_2), cholecalciferol (D_3), phylloquinone (K_1), menaquinone-4 (K_2) and a synthetic form of vitamin K, menadione (K_3). The Taguchi experimental method, an orthogonal array design (OAD), was used to optimize an efficient and clean preconcentration step based on dispersive liquid–liquid microextraction (DLLME). A factorial design was applied with six factors and three levels for each factor, namely, carbon tetrachloride volume, methanol volume, aqueous sample volume, pH of sample, sodium chloride concentration and time of the centrifugation step. The DLLME optimized procedure consisted of rapidly injecting 3 mL of acetonitrile (disperser solvent) containing 150 μL carbon tetrachloride (extraction solvent) into the aqueous sample, thereby forming a cloudy solution. Phase separation was performed by centrifugation, and the sedimented phase was evaporated with nitrogen, reconstituted with 50 μL of acetonitrile, and injected. The LC analyses were carried out using a mobile phase composed of acetonitrile, 2-propanol and water, under gradient elution. Quantification was carried out by the standard additions method. The APCI-MS spectra, in combination with UV spectra, permitted the correct identification of compounds in the food samples. The method was validated according to international guidelines and using a certified reference material. The validated method was applied for the analysis of vitamins D and K in infant foods and several green vegetables. There was little variability in the forms of vitamin K present in vegetables, with the most abundant vitamer in all the samples being phylloquinone, while menadione could not be detected. Conversely, cholecalciferol, which is present in food of animal origin, was the main form in infant foods, while ergocalciferol was not detected.

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1. Introduction

Vitamin D is a group of fat-soluble secosteroids which is needed to maintain strong bones and to help the body absorb calcium from foods, and has two major forms, ergocalciferol (D_2) and cholecalciferol (D_3). Dietary sources include fatty fish species, egg, beef liver and fish liver oils [1,2]. In most foodstuffs, vitamin D is naturally present as cholecalciferol, while ergocalciferol is sometimes present in fortified foodstuffs, and some foods contain both forms [3]. Vitamin K is a group of structurally similar naphtoquinones, which are needed for blood coagulation and in metabolic pathways in bone and other tissues. It includes two natural homologs, vitamin K_1 (phylloquinone), found in green leafy vegetables and in soybean oil [4] and vitamin K_2 (menaquinone), found in meat, eggs and dairy products [5]. Although the natural K_1 and K_2 forms are non-toxic, the synthetic form K_3 (menadione) has shown toxicity. For certain vitamins, excessive intakes are associated with toxicity or a health risk [6].

Vitamins are usually present at low levels and are accompanied by other compounds. Extraction from foods requires specific conditions, depending on the stability of each vitamin with pH, temperature, light, and oxygen, and its bond to the food matrix. Classical methods of vitamin D analysis include saponification to remove triglycerides and/or liquid–liquid extraction (LLE) in low polarity organic solvents, as is the case of the Standardized Method of Analysis [7]. The sample extract solution is analyzed

* Corresponding author. Tel.: +34 868887406; fax: +34 868887682.
E-mail address: hcordoba@um.es (M. Hernández-Córdoba).

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by normal-phase LC followed by reversed-phase LC. However, the method is not applicable to foods containing both vitamin D_3 and D_2, because one vitamer is used as internal standard when the other is to be determined [7]. Because vitamin K is destroyed by strong alkali, the removal of triglycerides before extraction requires enzymatic hydrolysis and extraction into non-polar solvents [8].

LC is the technique most widely used for vitamin determination in foods [9,10]. Some studies for vitamins D and K used ultraviolet–visible (UV–vis) [11–15], fluorescence [16–20] or electrochemical [21–25] detection. The coupling of LC and mass spectrometry (MS) combines high resolution and specificity, and several papers have proposed the determination of vitamins D and/or K in foods by LC–MS using particle beam [26,27], electrospray ionization (ESI) [28–31] and atmospheric pressure chemical ionization (APCI) [32–43], leading to better selectivity than when using UV–vis or fluorescence detection systems.

An extraction step such as solid phase extraction (SPE) or liquid–liquid extraction (LLE) [44], is normally required for sample preparation. An innovative technique, cloud point extraction [45] has been used for the preconcentration of vitamins. Miniaturized techniques (solid-phase microextraction, SPME or liquid-phase microextraction, LPME), which permit high preconcentration factors for analytes with high partition coefficients, have also been used for the purpose. These techniques share the priorities of green chemistry with respect to the environment through the use of chemical processes involving low amounts of solvents for dissolving or extracting analytes [46]. Thus, SPME has been used for the extraction of vitamins A, D_3 and E [47]. An LPME procedure based in solidification of a floating drop has been proposed for the determination of fat-soluble vitamins [48]. Dispersive liquid–liquid microextraction (DLLME) is a simple and rapid technique, that uses a very low volume of extraction solvent and has been successfully applied for the extraction and determination of mainly organic compounds in aqueous samples, providing high enrichment factors [49]. DLLME has only been used three times as a clean-up sample preparation technique for the determination of vitamin A [50,51] and α-tocopherol [52].

The present study describes a sensitive determination of vitamins D_2, D_3, K_1, K_2 and K_3 in different foods by coupling DLLME with LC using diode-array and APCI-MS detection. The Taguchi experimental method [53] was applied to study the possible influence on performance of six factors (each factor at three levels, 27 experiments) using a balanced orthogonal array design (OAD). The novelty of this study is based on the use for the first time of the technique DLLME for the determination of vitamins D and K using green chemistry principles coupled to LC-MS, avoiding the use of high amounts of solvents and the generation of residues.

2. Experimental

2.1. Reagents

Chromatographic quality acetonitrile, ethanol, isopropanol and carbon tetrachloride were obtained from Sigma (St. Louis, MO, USA). The water used was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Ergocalciferol (vitamin D_2), cholecalciferol (vitamin D_3), phylloquinone (vitamin K_1), menaquinone-4 (vitamin K_2) and menadione (vitamin K_3) 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. They were stable for at least one month. Working standard solutions were prepared daily in ethanol and stored at 4 °C. 0.2 M sodium formiate/formic acid and ammonium chloride/ammonia buffer solutions were used.

2.2. Instrumentation for LC–DAD

The LC–DAD 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 diode-array detector (Agilent 1200, G1315D) was operated at 245 nm and 265 nm for vitamins K and D, respectively. The UV–vis spectra, recorded in the 200–600 nm range, were used for the tentative identification of vitamins at the corresponding retention times.

The analytical column was a Zorbax Eclipse ODS non-endcapped (25 cm x 0.46 cm x 5 μm) (Agilent). The mobile phase consisted of acetonitrile, isopropanol and water, operating under gradient elution conditions with the optimized program being: an isocratic step with a 70:30 acetonitrile:water (v/v) mixture for 2 min, then a linear gradient from 70:30 acetonitrile:water (v/v) to 60:40 acetonitrile:isopropanol (v/v) in 0.1 min, which was then held for 12 min. Finally, the initial conditions were re-established in 1 min and held for 10 min. 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). Vegetable samples were homogenized using a IKA A 11 homogenizer (Staufen, Germany).

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 25 °C (G1316A), and injection (20 μL) was performed using an autosampler (G1329A). The column and the gradient program for the mobile phase were the same as those optimized for DAD detection. The LC system was coupled to an ion-trap mass spectrometer (1036 model) equipped with an APCI interface operating in negative ion mode for 8 min (vitamin K_3) and in positive ion mode until the end of analysis. The selected ion monitoring (SIM) mode was applied using the ions m/z 172 from 0 to 8 min (K_3), m/z 445 from 8 to 10.6 min (K_2), m/z 397 from 10.6 to 12.3 min (D_2), m/z 385 from 12.3 to 13.1 min (D_3) and m/z 451 from 13.1 to 16 min (K_1). 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. Sample preparation and DLLME procedure

The foods of plant origin consisted of different types as spinach, cos lettuce, iceberg lettuce, and lamb's lettuce, all commercially obtained from markets. The vegetables were cut into small pieces and homogenized. An amount of 0.2–2 g (depending of the content of the vitamin) of crushed vegetable was weighed in a polypropylene tube with conical bottom and 3 mL of acetonitrile were added. The suspension was centrifuged at 6000 rpm for 10 min. The supernatant was recovered and used as dispersive solvent, to which 150 μL of carbon tetrachloride (extractant solvent) was added. The infant foods and certified reference material, infant/adult nutritional formula SRM 1849a supplied by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA), were analyzed in the same way but using 50–250 mg of the sample. 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 3000 rpm, which is near the maximum speed supported by the conical glass tubes.

For DLLME procedure, the 3 mL-volume organic fraction resulting from the sample extraction was recovered and used as dispersive solvent, to which 150 μL of carbon tetrachloride (extractant solvent) was added. Then, the mixture was rapidly injected into 6 mL of water

using a micropipette, and gently shaken manually for several seconds. After centrifugation at 3000 rpm for 2 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 a nitrogen flow. The residue was reconstituted with 50 μL of acetonitrile, and a volume of 20 μL was injected into the LC.

3. Results and discussion

3.1. Chromatographic separation

For selecting the stationary phase, different C_8 and C_{18} columns, including a Zorbax Eclipse XDB-C_8 (15 cm × 0.46 cm × 5 μm), Supelco Discovery HS C_{18} (15 cm × 0.21 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. From these stationary phases, the best retention and resolution (when using different mobile phases of acetonitrile, isopropanol and water mixtures) were obtained with C_{18} rather than with C_8 stationary phases. One drawback was the band pair of vitamins D_2 and D_3, for which none of the mobile phases assayed provided good separation, poor peak shapes, peak overlap and deficient resolution being observed. Columns of different lengths, diameters and chemical properties were compared in an attempt to overcome the problem. When the Discovery HS C_{18} and the endcapped Zorbax ODS columns were assayed using different organic solvent-water mixtures, an increase in the percentage of organic solvent led to reduced retention for all compounds, giving wide peaks that proved inappropriate for the chromatographic purpose. The non-endcapped Zorbax Eclipse ODS was finally selected since narrow peaks and improved tailing factors were achieved.

Several mobile phases corresponding to mixtures of methanol, isopropanol or acetonitrile and water in different percentages were assayed. A 70:30 (v/v) acetonitrile:water mixture in isocratic mode led to the elution of vitamin K_3 (the least well-retained) with a retention factor of 2.5, while vitamins D_2, D_3 and K_1 eluted with very high retention factors. Vitamin K_3 eluted at the void time for higher acetonitrile percentages. Consequently, several mixtures using isopropanol were assayed. After several experiments involving isocratic elution with different solvent proportions, gradient elution was investigated, with the optimized gradient program being included in the Experimental section. The mobile phase flow-rate was investigated over the range from 0.8–1.5 mL min^{-1} to determine the effect on the separation and resolution of fat-soluble vitamins. The optimum value of 1.0 mL min^{-1} was selected because lower flow rates resulted in longer retention times. Table 1 summarizes the order of elution, retention times and retention factors for the analytes with the selected program. Values for the selectivity factor (α) and resolution (R_s) were in the 1.1–2.5 and 1.1–13 ranges, respectively.

3.2. APCI-MS detection

Protonation of liposoluble vitamins is difficult, and the sensitivity obtained using electrospray ionization was very low. APCI was selected for ionization as vitamins D and K are neutral and non-volatile compounds of low polarity, thus giving high signals in APCI. The optimal ionization mode was studied using a full-scan of each analyte, and maximum sensitivity was obtained operating in negative ion mode for vitamin K_3 and in positive ion mode for all the other vitamins, which showed the protonated molecule $[M+H]^+$ as the base peak. Then, optimization of the m/z values was carried out in the selected ion monitoring (SIM) mode. The mass spectrum of vitamin D_2 yielded a protonated molecule $[M+H]^+$ at m/z 397, a fragment obtained through the loss of one molecule of water generating the less intense ion $[MH–H_2O]^+$ at m/z 379, and the fragment at m/z 271 corresponding to the loss of its side chain. The vitamin D_3 spectrum contained protonated molecule $[M+H]^+$ at m/z 385, and the dehydrated protonated ion $[MH–H_2O]^+$ at m/z 367. Vitamins K_1 and K_2 produce protonated molecular ions $[M+H]^+$ at m/z 451 and 445, respectively, and both homologs lose their side chain producing the ion at m/z 187. Finally, the experiments in APCI(-) showed that the negative ion m/z 172, which corresponds to the loss of the side chain, was the most abundant for vitamin K_3, while no molecular ion was observed.

Thus, the ion m/z 172 in negative mode was used from 0 to 8 min. However, the protonated ions using APCI(+) were more intense and had higher signal-to-noise (S/N) ratios for the rest of the vitamins. Consequently, the positive mode was used for the ion m/z 445 from 8 to 10.6 min (K_2), the ion m/z 397 from 10.6 to 12.3 min (D_2), the ion m/z 385 from 12.3 to 13.1 min (D_3) and the ion m/z 451 from 13.1 to 16 min (K_1). Table 1 summarizes the MS parameters selected for the vitamins using this fragmentation study. Other MS parameters were: plate potential, 500 V; skimmer between −17 and 29 V; target trap 30,000; maximum accumulation time, 300 ms; scan from 100 to 500 m/z.

Fig. 2A shows the elution profile obtained using DLLME–LC–DAD, and Fig. 2B shows the corresponding chromatogram obtained by DLLME–LC–APCI-MS in SIM mode for a standard solution of the vitamins D and K in the selected conditions, as well as the mass spectra of the extracted ions for each one of the analytes (Fig. 2C).

Table 1
LC-APCI-MS parameters for the vitamins.

Analyte	Retention time (min)	Retention factor	Ionization mode	m/z
Menadione (K_3)	5.3	2.8	Negative	172
Menaquinone-4 (K_2)	9.8	6.0	Positive	445
Ergocalciferol (D_2)	12.1	7.6	Positive	397
Cholecalciferol (D_3)	12.6	8.0	Positive	385
Phylloquinone (K_1)	14.1	9.1	Positive	451

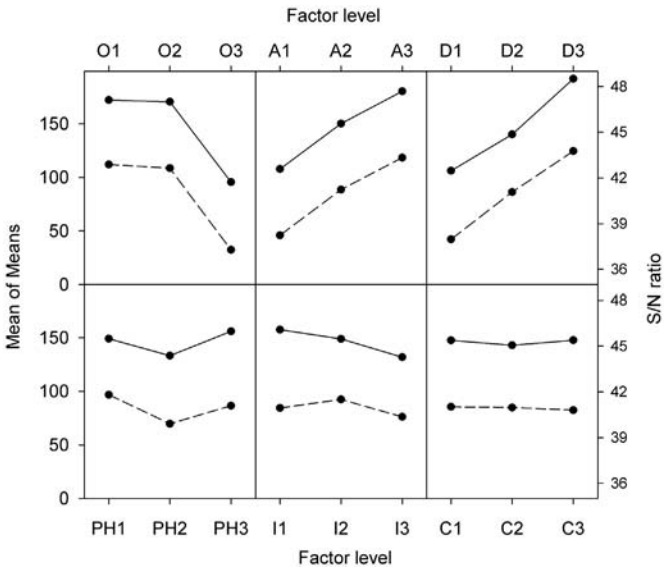

Fig. 1. Effects of factor levels of the extractant organic volume (O), aqueous phase volume (A), disperser volume (D), pH of the aqueous phase (pH), sodium chloride concentration (I) and centrifugation time (C) on the mean response (solid lines) and the signal-to-noise ratio (dotted lines) for the vitamins.

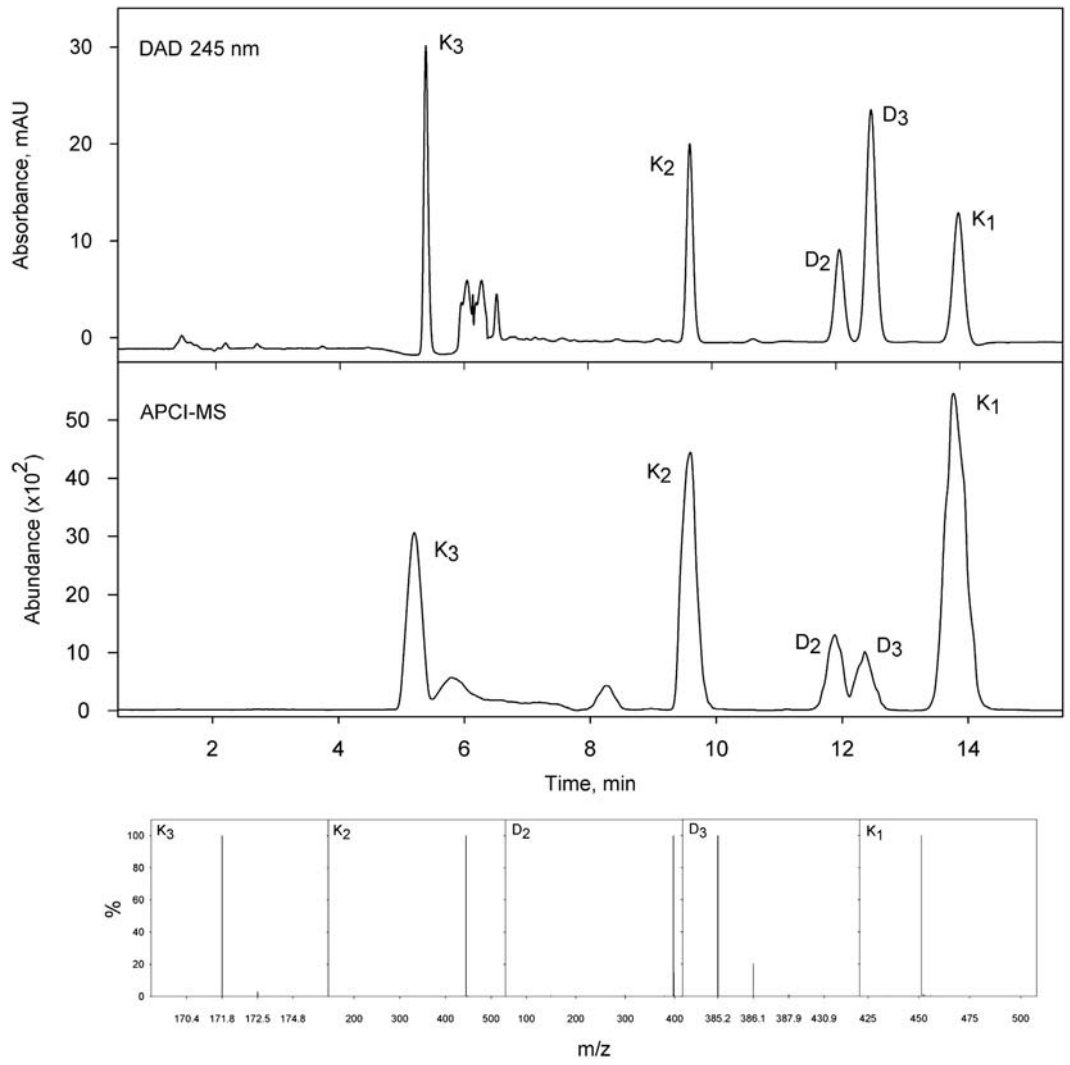

Fig. 2. Elution profile using DLLME–LC–DAD (A) and DLLME–LC–APCI-MS in SIM mode (B) for a standard solution of the vitamins D and K in the selected conditions. (C) APCI mass spectra of the extracted ions for each one of the analytes.

3.3. Optimization of the Taguchi design method for the DLLME procedure

The DLLME step was optimized by studying the experimental variables which affect the extraction efficiency, using the Taguchi method. For this purpose, an aqueous standard solution containing analyte concentrations of about 100 ng mL^{-1} was used. First, the solvents used as extractant and dispersant were selected. The solvents should have a low boiling temperature because the organic phase recovered has to be evaporated and reconstituted using a solvent compatible with reversed-phase LC. Experiments were carried out using carbon tetrachloride, chloroform, 1,2-dichloroethane and dichloromethane and a solvent volume of 150 μL (in the presence of 2 mL acetonitrile as disperser solvent). Higher peak areas were obtained using both chloroform and carbon tetrachloride. The volume obtained for the organic drop collected after centrifugation of the dispersion was similar for all extractants. For most analytes, the best extraction was achieved with carbon tetrachloride, which was selected. The disperser solvent was selected by rapidly injecting 2 mL of each disperser containing 150 μL of CCl_4 into 10 mL of the aqueous standard solution. Acetone, methanol and acetonitrile were assayed because they are miscible in the extraction solvent and in the aqueous solution. The volume recovered for the organic drop was again similar for all disperser solvents, while the extraction efficiency was higher for all vitamins when acetonitrile was used, and so it was selected for the optimized method.

The Taguchi experimental method, an orthogonal array design, was applied to study the possible influence on the performance of the DLLME method of six factors (each factor at three levels), namely extractant organic volume, disperser volume, aqueous phase volume, pH of aqueous phase, sodium chloride concentration and duration of the centrifugation step. The temperature, extraction time and stirring were not considered as variables, as equilibrium was reached rapidly. The centrifugation speed was fixed at 3000 rpm. Table 2 shows the three levels selected for each of the six factors in the Taguchi design proposed. An OAD that considers the factors without their interactions with 27 different trials was selected.

Fig. 1 shows the effects of the six factors on the mean response (A) and the signal-to-noise ratio (B). The signal-to-noise ratio was calculated as a higher-is-better response ($S/N = -10\log(1/n\sum_{i=1}^{n} 1/y_i^2)$), where n was the number of repetitions in each of the 27 trials ($n=3$), and y_i were the experimental response values obtained for each trial.

The extraction efficiency was nearly constant up to 150 μL of extractant volume but decreased for high carbon tetrachloride volume due to the dilution effect; therefore a 150 μL volume was

selected. When varying the volume of the disperser solvent, it was found that for volumes assayed below 1 mL, dispersions were deficient and peak areas were very low. For higher volumes between 1 and 3 mL, the sensitivity gradually increased, and a volume of 3 mL was selected. Similarly, higher volumes of the aqueous solution led to greater sensitivity, and thus 6 mL was selected for the aqueous phase.

To evaluate the influence of the pH, different samples were adjusted to pH values between 3 and 9 with 0.01 M buffer solutions. The volume of the sedimented organic solvent did not vary, and no significant differences in the analytical signals were observed. To study the effect of the ionic strength, the experiments were carried out at different sodium chloride concentrations ranging from 0% to 25% (m/v). The results showed that extraction efficiency slightly decreased for all compounds when the salt concentration was increased. Therefore, the addition of NaCl to the extraction solution was discarded. The centrifugation time necessary to disrupt the cloudy solution and collect the sedimented phase was not relevant, and thus a medium value of 2 min at the maximum speed recommended for the glass conic tubes, 3000 rpm, was selected.

A statistical analysis of variance (ANOVA) was performed to judge which design parameters significantly affect the optimal procedure by calculating the sum of squared deviations, the mean square and the *F* value (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). The three variables with a significant effect on the extraction efficiency were the volume of the organic extractant, the disperser volume and the volume of the aqueous solution. The most influential factors were the volumes of both the extractant and the disperser solvents. The contribution of the residual error to the signal variability indicates the validity of the experimental design used. Consequently, it can be asserted that the experimental design used in this work takes into account all the variables affecting the response to be optimized, and that the levels tested are fit for the purpose.

Table 2
Variables selected for the Taguchi design.

Factors	Level 1	Level 2	Level 3
Extractant organic volume (μL)	100	150	200
Aqueous phase volume (mL)	3	6	10
Disperser volume (mL)	0.5	1	3
pH	3	6	9
Sodium chloride (%) (m/v)	0	10	25
Centrifugation time (min)	1	2	3

3.4. Validation of the method

The method was validated for linearity, detection and quantification limits, selectivity, recovery, accuracy, precision and robustness, according to international guidelines [54].

The linearity of the method was assessed from the calibration graphs obtained using DLLME–LC–DAD by least-squares linear regression analysis of the peak area versus analyte concentration using ten levels (1–100 ng mL^{-1}) in duplicate experiments. Quantification was performed by the external standard procedure. The results obtained are summarized in Table 4. The r^2 values were good ($r^2 > 0.99$), demonstrating the excellent linearity for the range studied. The sensitivity of the method was evaluated by quantifying the limits of detection (LOD, calculated for a signal-to-noise ratio of 3) and the limits of quantification (LOQ, calculated for a signal-to-noise ratio of 10), which are also shown in Table 4. The enrichment factor (EF) was calculated as the ratio between the slope obtained by DLLME and the slope obtained by direct LC-DAD without preconcentration. Consequently, the EF values were 21.8, 23.9, 26.1, 24.4 and 21.9 for the vitamins K_3, K_2, D_2, D_3 and K_1, respectively. The selectivity

Table 3
Results of the analysis of variance for mean response.

Variation source	Degrees of freedom	Sum of squares	Mean of squares	*F*	*P*	Contribution, (%)
Extractant volume (μL)	2	34,508	17,254	5.59	0.016	33.2
Disperser volume (mL)	2	33,617	16,808	5.44	0.018	32.4
Aqueous volume (mL)	2	23,926	11,963	3.87	0.046	23.0
pH	2	2445	1222	0.4	0.680	2.4
NaCl (%) (w/v)	2	3063	1531	0.5	0.619	2.9
Centrifugation time (min)	2	135	67	0.02	0.978	0.1
Error	14	43,231	3088			
Total	26	140,924				

Table 4
Method validation data for LC–DLLME.

DAD detection	K_1	K_2	K_3	D_2	D_3
Linear range (ng mL^{-1})	1–100	1–100	1–100	1–100	1–100
Regression coefficient (r^2)	0.9915	0.9916	0.9997	0.9923	0.9906
Slope (mL ng^{-1})	0.963 ± 0.037	0.860 ± 0.003	0.817 ± 0.004	0.435 ± 0.017	1.19 ± 0.05
Detection limit (ng mL^{-1})	0.3	0.3	0.2	0.6	0.4
Quantification limit (ng mL^{-1})	0.9	0.9	0.8	2.0	1.3
APCI-MS Detection	K_1	K_2	K_3	D_2	D_3
Linear range (ng mL^{-1})	1–500	1–500	1–500	10–500	10–500
Regression coefficient (r^2)	0.9978	0.9942	0.9990	0.9997	0.9967
Slope (mL ng^{-1})	2602 ± 49	685 ± 30	503 ± 7	84.9 ± 1.1	70.8 ± 2.5
Detection limit (ng mL^{-1})	0.3	0.2	0.5	3.1	4.0
Quantification limit (ng mL^{-1})	0.9	0.8	1.6	10	13

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

Sample	Spike level (ng g^{-1})	K_1	K_2	K_3	D_2	D_3
Cos lettuce	10	94 ± 5 (5.3)	89 ± 7 (7.9)	92 ± 6 (6.5)	93 ± 4 (4.3)	95 ± 5 (5.3)
	20	96 ± 3 (3.1)	105 ± 4 (3.8)	97 ± 4 (4.1)	96 ± 4 (4.2)	88 ± 6 (6.8)
	30	97 ± 4 (4.1)	91 ± 6 (6.6)	101 ± 5 (5.0)	91 ± 6 (6.6)	98 ± 4 (4.1)
Infant cereals	10	92 ± 5 (5.4)	90 ± 5 (5.6)	89 ± 5 (5.6)	90 ± 5 (5.6)	103 ± 5 (4.9)
	20	101 ± 4 (4.0)	97 ± 6 (6.2)	95 ± 5 (5.3)	92 ± 4 (4.3)	93 ± 5 (5.4)
	30	96 ± 7 (7.3)	92 ± 5 (5.4)	103 ± 7 (6.8)	98 ± 3 (3.1)	95 ± 6 (6.3)

[a] Mean value ± standard deviation ($n=3$), Values into brackets correspond to the RSD (%).

Table 6
Content of vitamin K and D homologs in food samples and a certified reference material.

Sample	K_1 (μg g^{-1})	K_2 (μg g^{-1})	D_3 (μg g^{-1})	
Spinach	0.54 ± 0.04	0.013 ± 0.006	ND	
Iceberg lettuce	0.34 ± 0.07	ND	ND	
Cos lettuce	0.07 ± 0.01	0.046 ± 0.01	ND	
Lamb's lettuce	1.16 ± 0.08	ND	ND	
Infant formula	0.206 ± 0.03	ND	0.082 ± 0.01	
Infant cereals (no-gluten)	ND	ND	0.071 ± 0.01	
	K_1 (μg g^{-1})		D_3 (μg g^{-1})	
Certified reference material	DLLME–LC–MS	Certified value	DLLME–LC–MS	Certified value
Infant/adult nutritional formula SRM 1849a	1.19 ± 0.06	1.06 ± 0.17	0.114 ± 0.014	0.111 ± 0.017

of the method was judged from the absence of interfering peaks at the elution times of the vitamins for chromatograms of different samples. The performance criteria from EU Commission Decision (2002/657/EC) [54] established the use of co-chromatography to improve the identification of analytes, especially when no suitable internal standard can be used. Thus, the extract prior to LC was divided into two parts, one of them being directly chromatographed, while the other was fortified with the standards and analyzed. To comply with the EU Decision [54], the variability in the spectra caused by the sample matrix and the detector performance should be verified. The absorption maxima in the spectrum of the analyte shall be at the same wavelengths as those of the calibration standard within a margin determined by the resolution of the detection system. For DAD, this is typically within ± 2 nm. The spectra of the vitamins in the samples were not visibly different from the spectra of the calibration standards. Consequently, no matrix compounds existed that might give a false positive signal in the samples.

Calibration graphs were also obtained by using DLLME–LC–APCI-MS and the results obtained are also summarized in Table 4. SIM of the *m/z* 172 ion was used for the quantitative analysis of vitamin K_3, because it was the base peak of the negative ion APCI mass spectra of the compound. For the vitamins K_2, D_2, D_3 and K_1 the APCI mass spectra show higher abundance of the positive ions with *m/z* 445, 397, 385 and 451, respectively. Table 4 shows the values of r^2, which again demonstrated good linearity for the range studied, as well as the LOD and LOQ values. The selectivity was also confirmed with the LC–APCI-MS data by comparing the variability in the MS spectra caused by the sample matrix and the detector performance.

A precision study was conducted on the basis of repeatability for the vegetable samples, calculated by using the relative standard deviation (RSD) from a series of ten consecutive DLLME–LC analyses of a sample spiked with all the analytes at 50 ng mL^{-1}. The RSD values were 4.1, 8.2, 7.6, 7.7 and 8.4 for K_3, K_2, D_2, D_3 and K_1, respectively. These values indicate that the precision of the method was satisfactory for control analysis purposes.

Quantitative analysis with APCI can be affected by signal suppression due to unknown matrix interferences produced by compounds co-eluting compounds from the sample, which affect analyte ionization [36]. This can be attributed to deprotonation, the presence of non-volatile components and, in complex samples, large amounts of competing species of the available ions. 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 [34]. Standard additions were performed according to EU [54] by analyzing one portion of the native sample, while known amounts of the standard analytes of between two and five times the mean estimate of the analytes in the samples were added to the other test portions before analysis. A study of the relative recovery values for different samples is shown in Tables 5 and 6.

3.5. Sample preparation

Extraction of the food samples was complex because the fat-soluble vitamins have different structures and chemical properties. Vitamins D_2 and D_3 are quickly destroyed by light, oxygen and acids, and that they are normally extracted from foods using alkaline saponification and subsequent extraction with an organic solvent. On the other hand, vitamin K_1 is degraded by light, oxygen and alkaline reagents, and is extracted from foods using organic solvents.

A direct liquid-phase extraction was used for the extraction of vitamins using different organic solvents, such as acetonitrile, ethanol, methanol, ethyl acetate and their mixtures. The best extractant leading to maximum recoveries of all analytes was acetonitrile. A second extraction step proved unnecessary. Sample preparation was conducted in dim light and at room temperature.

3.6. Analysis of food products and validation using a certified reference material

Food samples were treated as described in the Experimental section and were analyzed. Fig. 3 shows the elution profiles

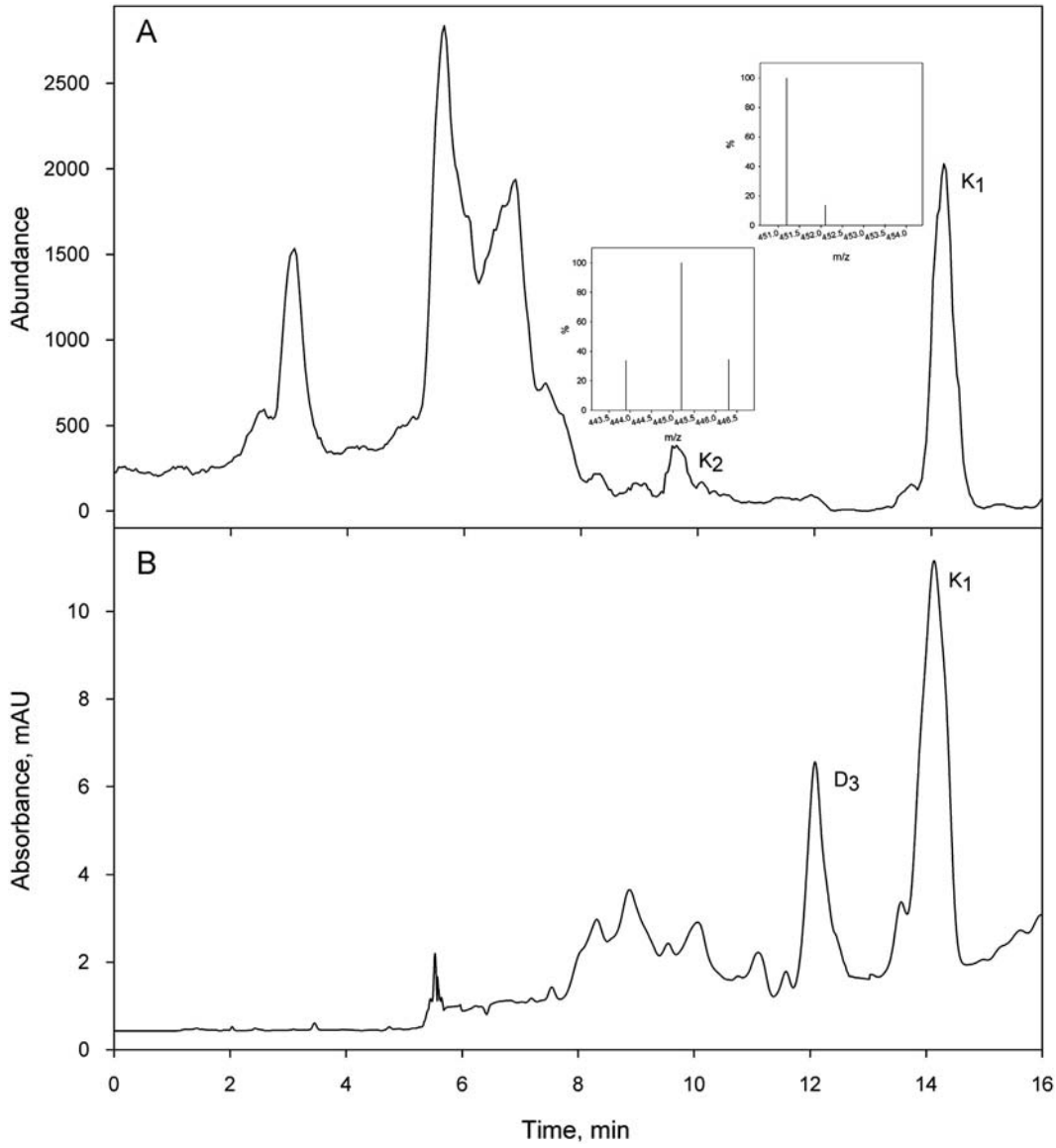

Fig. 3. Elution profiles obtained using DLLME–LC with APCI-MS detection in SIM mode for (A) a lettuce sample (as well as the mass spectra of the extracted ions) and DAD detection for (B) the certified reference material.

obtained using DLLME-LC with APCI-MS detection in SIM mode for (A) a lettuce sample (as well as the mass spectra of the extracted ions for each one of the peaks) and DAD detection for (B) the certified reference material. Similar chromatograms were obtained for the other samples. The elution profiles demonstrate the absence of interfering compounds eluting at the retention times of the different vitamins. 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 vitamins. The chromatograms obtained using DAD detection also demonstrated the agreement between UV–vis spectra (200–650 nm) for the standards and the analytes in the samples. Table 5 shows the vitamin contents determined in the food samples when using APCI-MS detection. The results were similar when the DAD detector was used. Quantification was performed using the standard additions method.

There was little variability in the forms of vitamin K present in vegetables, and in all the samples the most abundant vitamer was phylloquinone (0.07–1.2 $\mu g\, g^{-1}$). Menaquinone-4 was only found in some samples and in lower amounts (0.01–0.05 $\mu g\, g^{-1}$), and menadione and vitamin D were not detected. In contrast, cholecalciferol was the main form in infant formula and infant cereals, ergocalciferol was not detected, and vitamin K was present as phylloquinone. Some losses of vitamins were detected after storage, although the behavior of each vitamin varied strongly among samples [35].

Finally, the accuracy and reliability of the method was further investigated by analyzing a certified reference material, infant/adult nutritional formula SRM 1849a. Table 5 shows the results obtained. The contents for the vitamins obtained by the proposed DLLME-LC–APCI-MS method were in excellent agreement with the certified contents. The statistical study using the one-sample *t*-test showed that there was no significant difference (95% confidence interval) between the results obtained and the certified values (*P* values obtained were 0.746 for D_3 and 0.0642 for K_1). Such data also confirm the efficacy of the extraction procedure for recovering both free supplemented and endogenous vitamins D and K in the samples. Vitamin D fortified milk contains vitamin D added in the form of D_3 [3], which agrees with the form found in the certified reference material.

4. Conclusion

DLLME is proposed as an environmentally friendly sample-preparation technique for the determination of vitamins D and K since preconcentration is achieved involving a low amount of organic solvent. As a new solvent fraction of only a few microlitres is used for each extraction, no memory effect exists and high enrichment factors are achieved. The combination with LC permits the separation of two forms of vitamin D and three K homologs. The agreement between UV–vis spectra, the expected retention time and APCI-MS spectra allows a reliable identification of these vitamins in foods. The successful validation according to standard guidelines indicates that this method shows considerable potential for routine analysis of vitamin D and K in foods.

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