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Analysis of vitamin E isomers in seeds and nuts with and without coupled hydrolysis by liquid chromatography and coulometric detection

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Abstract

A method is described for the determination of vitamin E isomers [α-, (β+γ)- and δ-tocopherols] in seeds and nuts by reversed-phase HPLC with coulometric detection. Three methods of sample treatment were compared. The first method included alkaline hydrolysis, extraction of analytes from unsaponifiable and injection into the chromatographic system. In the second method, alkaline hydrolysis and later continuous membrane extraction of isomers were coupled with the HPLC system. The third method involved direct extraction of the analytes through a silicone membrane coupled on-line with the chromatographic system. The three methodologies used for the determination of vitamin E isomers in these samples afforded good results for α-tocopherol. However for (β+γ)- and δ-tocopherols the best results were obtained with the third method. The method without hydrolysis was the simplest one to carry out and analysis took no longer than 40 min from weighing of the samples. Accordingly, it is the method proposed.

Keywords: Food analysis; Membrane extraction; Extraction methods; Tocopherols; Vitamins

1. Introduction

The analysis of micronutrients in foods is of great interest both as regards nutrition and commercial aspects. A prominent area in this is the determination of liposoluble vitamins in massively consumed foods in which they are present.

The main problems involved in the determination of this type of analyte in samples as complex as foods derive from the low concentration of these components in foods and the need to isolate them beforehand. The isolation of liposoluble vitamins in this type of sample generally involves alkaline hydrolysis of the fatty material followed by extraction of the vitamins from the unsaponifiable material using a suitable organic solvent. This extract is then injected into a chromatographic system for the separation and determination of the analytes.

The term *oleaginous seed* is used to refer to the lipid-rich seeds and nuts from which oil is extracted. However, these seeds are also consumed as such (nuts) or in cooked foods or pastries. The main nutritional characteristic of oleaginous seeds is their high energy contribution, coming from their low water content (lower than 5%) and their high lipid content. The energy contribution of oleaginous seeds has one very important property: it is accompanied

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by a very high concentration of minerals and vitamins and such seeds are therefore of high nutritional value. These seeds are rich in proteins, whose nutritional value is somewhat mediocre since they are deficient in several essential aminoacids. Accordingly, a normal healthy diet must be completed with other protein sources. Glucids are present in small amounts in oleaginous seeds as a mixture of complex and simple glucids, which are assimilated progressively owing to the presence of lipids and fibre. Oleaginous seeds are the foods with the highest mineral contents. They contain important amounts of calcium, magnesium, potassium, iron, etc. The density of such minerals is so high, in fact, that they are counter-indicated in patients with renal insufficiency and in some cardiac insufficiencies.

The vitamin concentration in oleaginous seeds is high and indeed is one of their most important nutritional characteristics. Vitamin E is abundant, reaching 25 mg/100 g in hazelnuts and 46 mg/100 g in sunflower seeds. The recommended daily intake is 12 mg. This vitamin is important for the fruit itself since it protects unsaturated lipids from oxidation and allows their conservation. Vitamin E plays the same role in the human body but in circulating lipids and those present in cell membranes. Vitamins of the B group are present in large amounts: vitamin B_9 (folic acid), which is necessary for the synthesis of red blood cells, and vitamins B_1, essential for glucid metabolism, and B_6, involved in neuropsychic equilibrium.

The literature contains few references about the determination of vitamin E in seeds and nuts. Parcerisa et al. [1] analysed the fatty acid, tocopherol and sterol contents of hazel nuts using gas–liquid chromatography with mass spectrometric (GC–MS) or flame ionization detection (GC–FID) after saponification of the oil from the nuts. The same authors [2] also studied the influence of variety and geographic origin in the lipid fraction of hazelnuts from Spain. Lavedrine et al. [3] quantified tocopherols (α, γ and δ) and carotenes (α and β) in walnuts to determine the effect of geographical origin, variety and storage on their concentrations. These authors compared three methods, two of which included saponification. Demo et al. [4] detected the presence of nutrient antioxidants, such as tocopherols, in herbs and spices using thin layer chromatography (TLC), GC and GC–MS, while quantification was accomplished by reversed-phase high-performance liquid chromatography (HPLC), after saponification and extraction of unsaponifiable materials. Dolde et al. [5] quantified tocopherols from the seeds of wheat, sunflower, canola and soybean using normal-phase HPLC with UV detection. Lee et al. [6] used direct extraction to determine vitamin E in peanuts, peanut butter and nuts using LC. Hogarty et al. [7] determined the tocopherol content of several foods (breakfast cereals, cheeses, chips, fish, fruits, nuts, oils, salad dressings and vegetables) using reversed-phase HPLC with fluorescence detection after saponification of the samples with KOH. Fukuba et al. [8] determined tocopherols in nuts and spices by normal-phase HPLC with fluorimetric detection.

Owing to the richness in vitamins of oleaginous seeds it is important to monitor the contents of such nutrients in these foods. This monitoring is even more important in the case of vegetarian diets since nuts are an important component of these. In the present work, a method is proposed for the determination of vitamin E isomers [α-, (β+γ)- and δ-tocopherols] in seeds and nuts by reversed-phase HPLC using electrochemical detection in the coulometric mode. Sample preparation was accomplished in the continuous mode, involving direct extraction of the analytes through a silicone membrane coupled on-line with the chromatographic system. This method, without alkaline hydrolysis, is compared with other two methods with alkaline hydrolysis coupled and not coupled to the HPLC system.

2. Experimental

2.1. Apparatus

2.1.1. Liquid chromatography

Isocratic HPLC determination was carried out with a Spectra-Physics (San José, CA, USA) SP8800 ternary pump. An ESA (Chelmsford, MA, USA) 5200 A coulochem detector with an ESA 5020 ''Guard cell'' connected to the system to treat the mobile phase before injection and an ESA 5010 ''Analytical cell'' containing dual porous graphite working electrodes was used. Data were processed

by a Shimadzu (Duisburg, Germany) CBM 10-A communication module and a computer using Class-LC10 Software (Shimadzu). Separation was achieved in an RP-18 precolumn (15×3.2 mm I.D., 7 μm) and an OD-224 RP-18 column (220×4.6 mm I.D., 5 μm) Brownlee Labs (Santa Clara, CA, USA).

2.1.2. Sample treatment

A Büchi (Flawil, Switzerland) RE 121 rotavapor with a Büchi 461 water-bath were used. Water was purified on an ElgaStat water purification system (Elga, High Wycombe, UK).

A Gilson (Villiers le Bel, France) Minipuls-3 peristaltic pump with Isoverinic and Silicone pump tubes (0.6 mm I.D.) and PTFE tubes (0.5 mm I.D.) for the rest of the channels, including the 5 m long coil reactor, used when saponification was carried out; a ''Global FIA'' (Seattle, WA, USA) extraction cell comprising two plastic blocks (11×2×3.2 cm) each containing an undulated slit through which both the donor and acceptor solutions flow, separated by a Perthese (Perouse Implant Labs., Bornel, France) non-reinforced silicone extraction membrane This system was controlled by a Gilson 231-401 microprocessor with a Rheodyne (Berkeley, CA, USA) six-port injection valve with a 20-μl loop and a Gilson 401 dilutor used as a piston pump.

2.2. Reagents

2.2.1. Chemicals

The following compounds and chemicals were used: α-tocopherol (98% Sigma, Alcobendas, Madrid, Spain), γ-tocopherol (Sigma), δ-tocopherol (90% Sigma), 2,2,5,7,8-pentamethyl-6-chromanol (97% Aldrich, Alcobendas, Madrid, Spain), special HPLC-quality acetonitrile (Merck, Darmstadt, Germany), special HPLC-quality methanol (Merck), special HPLC-quality *n*-hexane (Merck), reagent-grade glacial acetic acid (Scharlau, Barcelona, Spain), reagent-grade sodium acetate (Pancreac, Barcelona, Spain), pure Triton X-114 (Fluka, Alcobendas, Madrid, Spain), reagent-grade potassium hydroxide (Scharlau), reagent-grade ascorbic acid (Panreac) and reagent-grade ethanol (Scharlau). Nuts and seed samples were from commercial sources.

The mobile phase used was a solution of 2.5 m*M* acetic acid–sodium acetate in MeOH–water (97:3, v/v). This solution was filtered through Millipore (Madrid, Spain) nylon membranes with a pore size of 0.22 μm and a diameter of 45 mm and degassed with helium.

2.3. Procedure

The determination of liposoluble vitamins in foods usually requires saponification of the fatty material and extraction of the vitamins from the unsaponifiable residue prior to chromatographic separation and determination.

Here we used two methods for the quantification of the analytes of interest in the samples studied. In one of these, alkaline hydrolysis of the fatty material and analyte extraction were accomplished in a previous step and, in the other, these steps were coupled with chromatographic separation. Another procedure was also used; this included direct extraction of the analytes coupled in the continuous mode with chromatographic determination, with no previous hydrolysis.

2.3.1. Discontinuous method

The samples, peeled and ground (approx. 2 g), were saponified and protected from light at room temperature over 2 h with stirring in an alcoholic solution of potassium hydroxide plus ascorbic acid to avoid oxidation of liposoluble vitamins. The composition of the hydrolysis reagent was as follows: 50 ml of ethanol, 5 ml of aqueous 10% ascorbic acid, 10 ml of aqueous 80% KOH and 25 ml of water. The analytes were then extracted with hexane (2×25 ml) and the extracts were washed with water (2×10 ml). The organic phase was removed by evaporation in a rotavapor under vacuum at 50°C and the residue was dissolved in methanol (50 ml) and filtered through Millipore (Madrid, Spain) nylon membranes with a pore size of 0.45 μm to clean the extracts before their injection into the chromatographic system.

2.3.2. Continuous method

The samples, peeled and ground (approx. 1 g), were mixed with Triton X-114 in the presence of methanol and acetonitrile. Water was added and the mixture was stirred for about 30 min to dissolve the analytes, after which the solution was brought up to a volume of 50 ml. Before the samples were injected

into the chromatographic system, the solutions obtained were centrifuged at 3000 rpm for 10 min and filtered through Osmonics (MN, USA) nylon filters with a pore size of 5.0 μm. In this method, the hydrolysis and extraction steps were coupled to the chromatographic system by a membrane extraction system. Coupling of the extraction step to the chromatographic separation was achieved using the system depicted in Fig. 1.

The functioning of the system is as follows: the sample, either alone or mixed with the hydrolysis reagent, circulates through a reactor in which saponification is accomplished, forming the donor solution, which passes across one side of the extraction membrane for a time referred to as the enrichment time (6 min). During this time, the acceptor solution (acetonitrile) is stopped and it extracts the analytes that have previously diffused across the membrane. After the enrichment time, the microprocessor forces the acceptor solution to move on via a dilutor, displacing a given volume of this solution (displacement volume) to fill the injection loop. Then, also controlled by the microprocessor, the valve turns, injecting 20 μl into the chromatographic system,

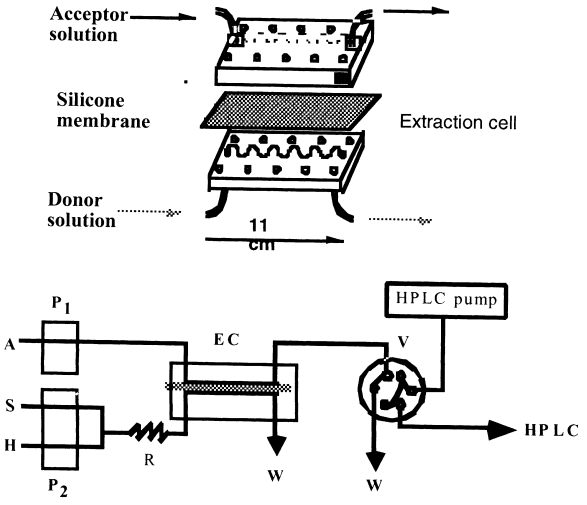

Fig. 1. Diagram of coupled on-line sample treatment-HPLC for the determination of vitamin E isomers. HPLC conditions: mobile phase=methanol–water (97:3), 2.5 mmol l^{-1} HAcO–NaAcO; flow-rate=1.0 ml min^{-1}. Electrochemical detection was at +500 mV. A: Acceptor solution; S: sample; H: hydrolysis reagent; P_1: piston pump; P_2: peristaltic pump; R: reactor; EC: extraction cell; V: injection valve (20-μl loop); W: waste. R and H are only used when alkaling hydrolysis is desired.

where separation and later detection of analytes are accomplished. Between each successive determination, it is necessary to perform a washing step by passing 4 ml of acceptor phase, thus avoiding the memory effect of the membrane.

3. Results and discussion

3.1. Chromatographic conditions

The variables affecting the chromatographic separation and detection were optimised in earlier works [9]. The mobile phase used was a 2.5 m*M* acetic acid–sodium acetate buffer as the supporting electrolyte in a methanol–water (97:3, v/v) solution. The flow-rate was set at 1.0 ml/min.

Detection was carried out by the coulometric detector. A potential of +1000 mV was applied to the guard cell with a view to oxidising possible impurities entering with the mobile phase, hence reducing background noise. The first graphite electrode of the analytical cell was subjected to reduction potentials in order to obtain ''cleaner'' measurement signals and less background noise, as well as to reduce any vitamin E that might have been oxidised during sample preparation. This potential was set at –1000 mV. An oxidation potential was applied to the second working electrode of the analytical cell to measure the vitamin E was being eluted. This working potential was set at +500 mV. Current intensity was measured with this second electrode.

It is necessary to note that the chromatographic peak at 12 min, when seeds and nuts had been analysed, could correspond to β- and γ-tocopherol. It is known that the positional β-and γ-tocopherol isomers cannot be completely resolved by reversed-phase HPLC. Normally, β-tocopherol levels in these samples are lower than those of γ-tocopherol. Only in a few cases is the β-isomer concentration important with respect to that of the γ-isomer.

3.2. Quantification

Analyte quantification was carried out using the internal standard method, employing 2,2,5,7,8-pentamethyl-6-chromanol (PMC) in all methodologies used. This compound is electroactive at the working

Table 1
Calibration graphs for α-, γ- and δ-tocopherol isomers using 2,2,5,7,8-pentamethyl-6-chromanol as internal standard

Tocopherol	Quantification equation: y(ratio of analyte/internal standard areas) = $a + bx$ (mol l^{-1})			
	Concentration range (M)	a	b	r^2 ($N=9$)
α	$(4.16\text{-}208)\cdot10^{-7}$	0.02±0.07	$(1.98\pm0.06)\cdot10^{5}$	0.992
γ	$(4.34\text{-}217)\cdot10^{-7}$	0.06±0.05	$(1.61\pm0.05)\cdot10^{5}$	0.994
δ	$(4.15\text{-}208)\cdot10^{-7}$	0.05±0.03	$(1.26\pm0.03)\cdot10^{5}$	0.995

potentials and its signal does not interfere with those of the analytes. In addition, it was observed that the relationship between the signals of the analytes and that of the internal standard and the analyte concentration was linear (Table 1). The concentration of the internal standard was $1.241\cdot10^{-3}$ M. The addition was carried out before sample treatment.

3.3. Analysis of nut and seed samples

3.3.1. Discontinuous method

Using the procedure described in Section 2.3.1 for analysis in the discontinuous mode, the following samples were analysed: raw walnuts, almonds (both raw and roasted), raw and roasted hazelnuts, raw and fried sunflower seeds, and raw and fried peanuts. The results obtained following this methodology, in which the hydrolysis and extraction steps took place before separation and chromatographic determination are shown in Table 2.

3.3.2. Continuous method

Use of the continuous method requires optimisation of a set of variables – among which are the donor and acceptor phases, the enrichment time and the amount of sample – in order to obtain the best analytical signal possible. The general procedure was applied to samples of walnuts, since these contain the analytes of interest, to optimise these variables before carrying out analysis of vitamin E isomers in the different types of nuts and seeds.

In a previous work [10], it was seen that the best acceptor phase when attempting to extract vitamin E isomers through a non-porous silicone membrane was acetonitrile. With the geometry of the continuous mode system available for this work, the optimum displacement volume was 120 μl – for the fraction of acceptor phase most enriched in analytes to be in the 20 μl loop injection. Accordingly, acetonitrile was used as the acceptor phase and that volume was employed for injection into the chromatographic system.

One of the best ways of solubilizing samples with high fat contents is to treat them with surfactants. The use of Triton X-114, in the presence of a polar organic solvent had already been seen to be very useful for the solubilization of oils and margarines [9,10]. In order to find the best concentration of

Table 2
Determination of vitamin E isomer contents in various nut and seed samples after alkaline hydrolysis in discontinuous mode (results from three replicate analyses)

	Vitamin E isomer contents (mg/100 g)		
	α-Tocopherol	(β+γ)-Tocopherol	δ-Tocopherol
Walnuts	0.61±0.13	12.9±1.2	1.00±0.30
Raw almonds	16.5±1.4	1.78±0.25	–
Roasted almonds	20.9±2.0	1.36±0.19	–
Raw hazelnuts	17.6±0.9	0.80±0.51	–
Roasted hazelnuts	17.8±0.1	1.14±0.27	–
Raw peanuts	5.68±0.20	3.37±0.15	0.24±0.01
Fried peanuts	0.68±0.11	1.29±0.11	0.14±0.02
Raw sunflower seeds	22.7±0.6	0.71±0.11	–
Fried sunflower seeds	12.8±0.2	1.39±0.28	–

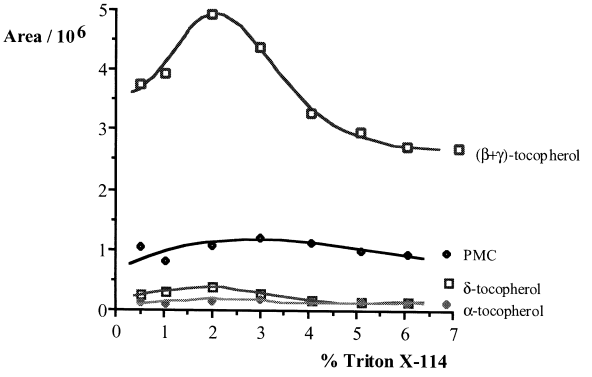

Fig. 2. Influence of Triton X-114 concentration in the chromatographic signal. The same sample amount of walnut in the presence of the internal standard (PMC) has been taken for solubilization in all experiences. HPLC conditions as in Fig. 1.

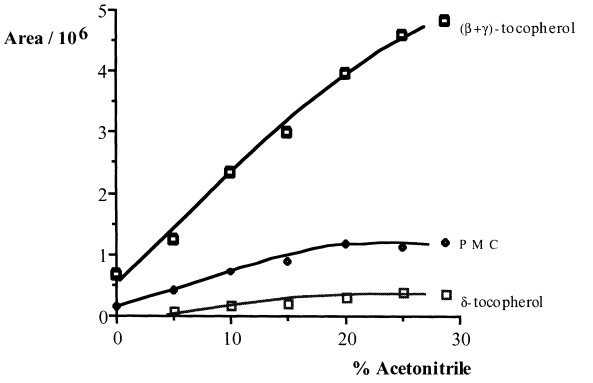

Fig. 3. Influence of acetonitrile concentration of donor solution in the chromatographic signal. The same sample amount of walnut in the presence of the internal standard (PMC) has been taken in these experiences. HPLC conditions as in Fig. 1.

Triton X-114 for the solubilization of nuts, samples of walnut were treated with this surfactant at different concentrations. The results are shown in Fig. 2. The strongest analytical signal was obtained when 2% Triton X-114 was employed and therefore the nut samples were treated with Triton X-114 at 2%.

The presence of polar solvents, such as methanol or acetonitrile, in solutions of surfactants improves the extraction of analytes through the membrane, possibly because contact between the solution and the membrane is increased. Thus, after the different assays, it was seen that the presence of methanol and acetonitrile afforded higher analytical signals. A solvent concentration of 10% was considered to be suitable for work with these samples. Higher concentrations than 20% could lead to phase separation owing to micelle breakage. Fig. 3 shows the effect of the acetonitrile concentration on the chromatographic signal.

The enrichment time, i.e., the time the acceptor solution must remain halted while the donor solution circulates across the other side of the membrane, was also optimised. The enrichment time was varied between 1 and 10 min and it was observed that the signal increased in a linear fashion up to an enrichment time of about 6 min, after which it remained constant. An enrichment time of 6 min was therefore chosen, allowing analysis to be accomplished in some 20 min.

To find the most suitable amount of sample for the analysis, different amounts of sample were weighed, obtaining the results shown in Fig. 4. It may be seen that the signal increases up to sample amounts of about 2 g, after which it remains constant. An optimum sample amount of 1 g was chosen since it is necessary to choose a value from the zone in which the signal increases with the increase in sample mass.

Once the best conditions for the extraction of analytes through the silicone membrane had been determined, application of the method to samples of nuts and seeds was studied in two different ways. One of them involved performing alkaline hydrolysis

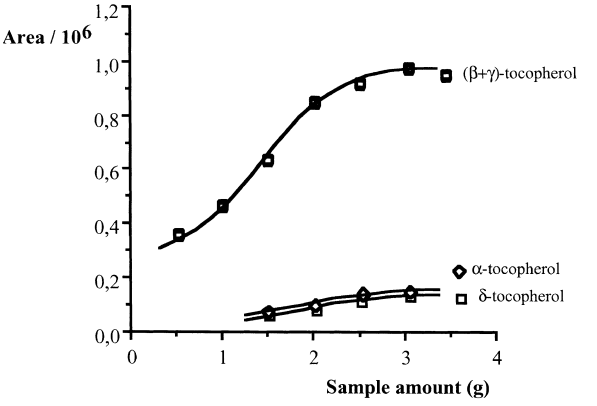

Fig. 4. Influence of walnut sample amount in the chromatographic signal. HPLC conditions as in Fig. 1.

Table 3
Determination of vitamin E isomer contents in various nut and seed samples using alkaline hydrolysis in continuous mode (results from three replicate analyses)

	Vitamin E isomer contents (mg/100 g)		
	α-Tocopherol	(β+γ)-Tocopherol	δ-Tocopherol
Walnuts	0.76±0.08	20.2±0.9	3.74±0.39
Raw almonds	15.7±0.4	0.84±0.06	–
Roasted almonds	19.0±1.1	2.01±0.07	–
Raw hazelnuts	15.1±0.8	1.01±0.15	–
Roasted hazelnuts	17.9±0.6	5.15±0.38	–
Raw peanuts	6.49±0.39	6.33±0.53	0.41±0.06
Fried peanuts	1.01±0.07	2.98±0.05	1.91±0.29
Raw sunflower seeds	25.8±1.4	1.62±0.02	–
Fried sunflower seeds	17.1±0.4	1.79±0.04	–

of the fatty material prior to analyte extraction and the other involved direct extraction. In both cases, the contents in α-, (β+γ)- and δ-tocopherols were determined in samples of raw walnut, raw and roasted almonds, raw and roasted hazelnuts, raw and fried sunflower seeds, and raw and fried peanuts.

3.3.2.1. With hydrolysis

Hydrolysis was achieved by using the system described in Section 2.3.2. The sample, dissolved in Triton X-114 in the presence of methanol and acetonitrile, was made to merge with the hydrolysis reagent in a 5 m long reactor where alkaline hydrolysis of the fatty material took place. This hydrolysed material was the donor phase that provided the analytes, which were extracted by the acceptor phase through the silicone membrane. The results obtained on applying this procedure are shown in Table 3.

3.3.2.2. Without hydrolysis

The sample, dissolved in the presence of Triton X-114, methanol and acetonitrile (donor phase), was passed directly across the donor side of the extraction membrane during the enrichment step. The analytes diffused through the membrane to the acceptor phase, which was injected into the chromatographic system where separation and later determination were accomplished. The results obtained on using this method are shown in Table 4.

The chromatograms obtained on applying each of the three methods used for the analysis of walnut samples are shown in Fig. 5. As may be seen, in all cases the chromatographic peaks corresponding to the three analytes and the internal standard are perfectly resolved and readily quantifiable.

These methodologies were compared for each isomer analysed using Student's *t*-test for paired

Table 4
Determination of vitamin E isomer contents in different nut and seed samples using the continuous method without previous hydrolysis (results from three replicate analyses)

	Vitamin E isomer contents (mg/100 g)		
	α-Tocopherol	(β+γ)-Tocopherol	δ-Tocopherol
Walnuts	0.79±0.05	20.7±0.5	3.01±0.05
Raw almonds	14.8±0.3	1.42±0.15	–
Roasted almonds	19.6±0.4	2.55±0.16	–
Raw hazelnuts	17.3±0.1	1.11±0.16	–
Roasted hazelnuts	17.5±0.2	5.17±0.30	0.45±0.07
Raw peanuts	5.68±0.37	7.74±0.22	0.56±0.05
Fried peanuts	0.76±0.03	3.09±0.15	0.90±0.14
Raw sunflower seeds	26.9±0.6	1.54±0.08	–
Fried sunflower seeds	14.1±0.5	5.56±0.33	–

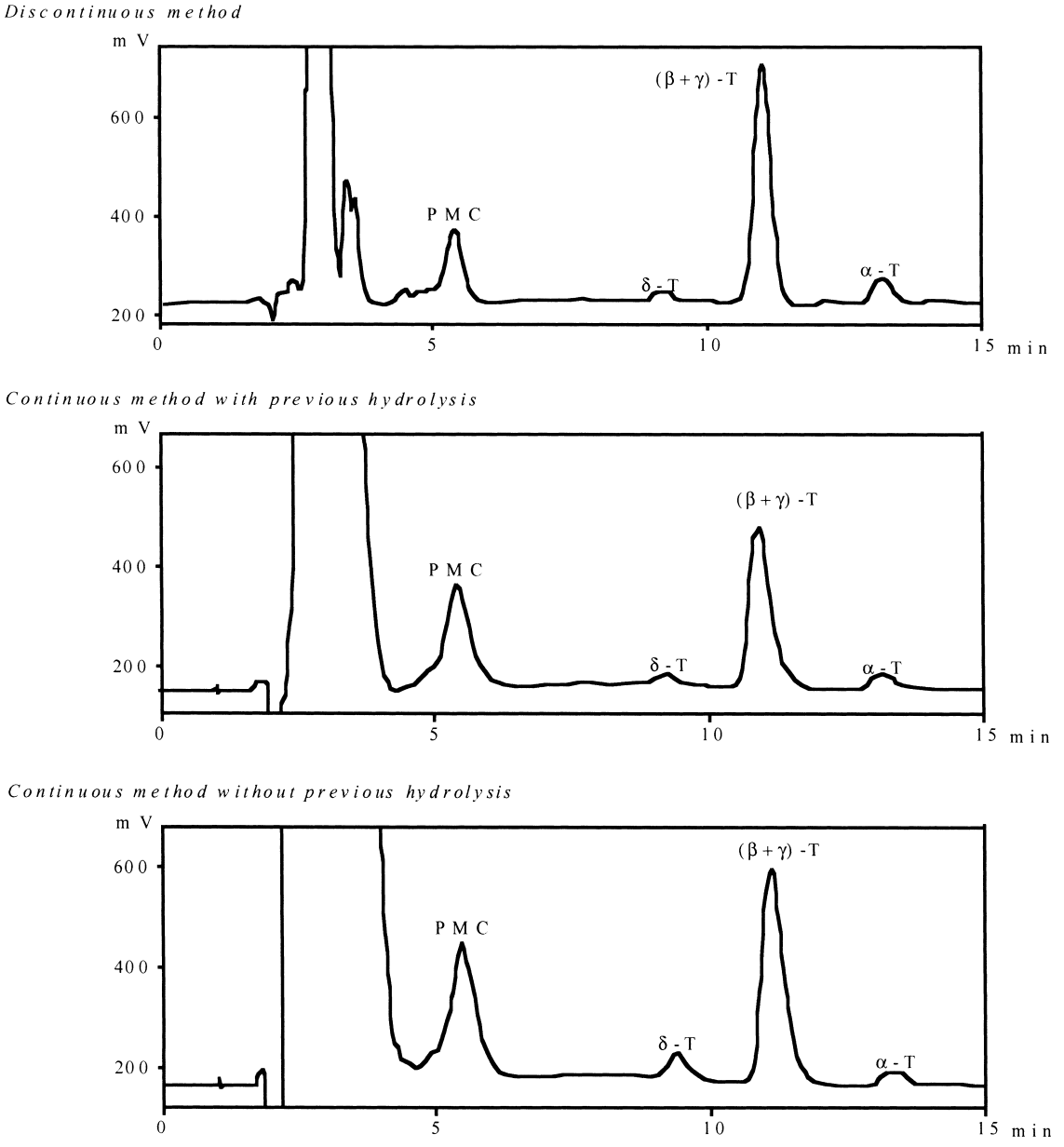

Fig. 5. Chromatograms obtained after application of the three proposed methodologies to a walnut sample. HPLC conditions as in Fig. 1. For details see Section 2.

values. The levels of significance obtained are shown in Table 5. In most cases, the level of significance was higher than 0.05 (chosen as the minimum level of significance), i.e., there were no differences among the results obtained. It may be concluded that the different analytical methods used afford significantly similar results, mainly in the α-tocopherol isomer. However, differences were found between

Table 5
Levels of significance obtained after application of Student's *t*-test for paired values to the three methodologies: (a) discontinuous method; (b) continuous method with previous hydrolysis; (c) continuous method without previous hydrolysis

	a–b	a–c	b–c
α-Tocopherol	0.5978	0.6862	0.7512
(β+γ)-Tocopherol	0.0520	0.0150	0.0922
δ-Tocopherol	0.1728	0.1789	0.2686

the quantity of (β+γ)- and δ-tocopherol obtained, depending on method used. This effect can be seen in Table 5 in the low value obtained for the levels of significance upon comparing the method of hydrolysis in discontinuous mode and the method without hydrolysis with direct extraction of the isomers. This effect can be accounted for because even in presence of antioxidants (ascorbic acid) alkaline hydrolysis leads to the partial oxidation of tocopherols, mainly the γ- and δ-isomers. The effect is more important when the hydrolysis is carried out in discontinuous mode than when it is coupled to chromatographic system.

We propose use of the methodology that includes analyte extraction in the continuous mode by silicone membranes without performing prior alkaline hydrolysis. The detection limits evaluated, RSDs obtained carried out a study on intra-day and inter-day precision and recovery obtained for the α-, γ- and δ-isomers using the method without alkaline hydrolysis are showed in Table 6.

Using this proposed methodology, we analysed a sample of margarine with a certified content of α-tocopherol of 24.1 mg/100 g. This sample (CRM 122) was supplied by the Institute for Reference Materials and Measurements (BCR Reference Materials, Belgium). The results obtained after application of the proposed procedure (six replicates) were 23.4±0.7 mg/100 g, with an accuracy of 3.0%.

4. Conclusions

The three methodologies used for the determination of vitamin E isomers in nut and seed samples afford good results, mainly for α-tocopherol not for other isomers when alkaline hydrolysis, in discontinuous mode, was used. We propose use of the methodology that includes analyte extraction in the continuous mode by silicone membranes without performing prior alkaline hydrolysis. This method is the simplest one to carry out and analysis takes no longer than 40 min as from weighing of the samples. It was validated in the analysis of certified sample and, additionally, when used the results for (β+γ)- and δ-tocopherols were the best.

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Table 6
Detection limits, precision and recovery of proposed method: continuous mode without alkaline hydrolysis

	L.D.[a] (*M*)	Precision (RSD)[b], intra-day (%)	Precision (RSD)[b], inter-day (%)	Recovery $1\cdot10^{-6}$ *M* added (%)
α-Tocopherol	$8.08\cdot10^{-8}$	2.87	9.10	101
γ-Tocopherol	$9.93\cdot10^{-8}$	2.99	7.52	102
δ-Tocopherol	$1.26\cdot10^{-7}$	4.26	9.13	96.6

[a] Detection limits evaluated in term $3S/b$.
[b] $N=10$ Replicates.

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