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Automated analysis of vitamin E isomers in vegetable oils by continuous membrane extraction and liquid chromatography–electrochemical detection

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Abstract

A rapid and automated method for the analysis of α-, γ- and δ-tocopherols in vegetable oils is reported. Continuous extraction of vitamin E isomers from oil samples dissolved in Triton X-114 in the presence of methanol–hexane is achieved and coupled on-line with the chromatographic system. Using an acetic acid/sodium acetate buffer in a methanol–water (97:3) solution as the mobile phase, a C_{18} stationary phase and electrochemical detection in the coulometric mode, α-, γ- and δ-tocopherol isomers can be successfully analyzed within 17 min. Thirteen commercially available oils of olive, sunflower, corn and seed mixtures were analyzed using 2,2,5,7,8-pentamethyl-6-chromanol as internal standard. The results obtained using three methodologies, one of them including classical sample treatment for liposoluble vitamin analysis, were in good agreement. To validate the proposed method, analysis of the only BCR Reference Material available, with a certified content of α-tocopherol (margarine CRM 122), was carried out using the automated methodology, the results found being in agreement with the certified value.

Keywords: Automation; Vegetable oils; Extraction methods; Food analysis; Vitamins; Tocopherols

1. Introduction

Vitamin E consists of a group of eight vitamers, tocopherols and tocotrienols, all of which have biological activity. The biological activities of α-, β-, γ- and δ-tocopherols and α-tocotrienol have a ratio of 10:4:1:0,1:3. The various tocopherols also differ in their ability to protect fats and oils from oxidative rancidity [1].

Currently, vitamin E is considered to be one of the best biological antioxidants since it protects cytoplasmic membranes from oxidation and guards low-density plasma lipoproteins from dangerous lipid peroxidation processes; hence the coining of the term ''anti-ageing factor''. In addition, its antioxidative capacity and ability to act as a free radical scavenger can reduce the risk of cancer and delay the progression of precancerous lesions. Accordingly, control of vitamin E levels in foods is of great importance to ensure that daily ingestion will be optimal as an essential factor in human health.

The main sources of vitamin E are vegetable oils, fats, grains, nuts and seeds. Tocopherols are integral components of the unsaponifiable matter present in most vegetable oils and fats, while tocotrienols are present at significant levels in palm oil.

Since tocopherols are relatively non-polar mole-

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cules, their chromatographic determination can be accomplished in either normal-phase systems or reversed-phase systems. The latter are preferred over normal-phase systems due to the reproducibility of retention times, fast equilibration, and the greater robustness of reversed-phase columns over other stationary phases. Also, reversed-phase systems are compatible with electrochemical detection, which requires the presence of an electrolyte in the eluent solution to support the redox reactions that are the basis for detection. Only reversed-phase eluents – typically mixtures of short-chain alcohols or acetonitrile with water, buffers, or salt solutions – are sufficiently polar to carry electrolytes. Reversed-phase systems for vitamin E generally employ octadecylsilane (ODS, C_{18})-modified silica, although recently a C_{30} stationary reversed-phase has been developed [2]. The highly lipophilic tocopherols are well retained on reversed-phase columns and require strong eluents. The mobile phases most employed are pure methanol or methanol–water mixtures containing up to 10% water. Other eluents used are different mixtures of solvents such as acetonitrile, isopropanol, methanol, ethanol or water.

Normal-phase columns show certain unique advantages in the case of tocopherols and related substances because they provide greater selectivity and are generally better for separations involving numerous isomers or related compounds. Normal-phase systems are only suitable for the direct analysis of cooking oils and fats, since apolar normal-phase eluents are good solvents for these samples. Normal-phase eluents for the separation of the active compounds of vitamin E are composed of an alkane (hexane, heptane, isooctane) with a small amount of a polar modifier, which may be an alcohol (ethanol, methanol, butanol), an ether (tetrahydrofuran, methyl, *tert*.-butyl, isopropyl), or a chlorohydrocarbon (dichloromethane, chloroform). Cyano, amino and silica columns are those most used.

As a lipophilic substance, vitamin E is intimately associated with the lipid components of the sample matrix. The various sample preparation procedures serve to release vitamin E from the sample matrix and extract it into a solvent that is compatible with the chromatographic system. The determination of liposoluble vitamins in vegetable oils requires usually a sample preparation step to isolate the vitamins from other substances that might interfere.

The sample preparation step is generally an important part of chromatographic determinations, but is now one of the most tedious and time-consuming aspects of the overall analytical process. Another important feature in sample preparation, which is not always taken into account, is its effect on error propagation. Automatization of this stage may reduce analysis time to an important extent, considerably simplify the analytical process, and decrease its cost as well as reducing the errors inherent to the analytical method by as much as 50%.

The methods of sample preparation with this type of analyte most used include sample saponification or solvent extraction [3]. Saponification is carried out by treatment of the sample in a strong alkali environment; this decreases the load of material that is extracted together with tocopherol into the organic phase. Saponification procedures require prolonged heating of the sample in alcoholic solutions of potassium hydroxide, usually at elevated temperature, in the presence of an antioxidant, generally ascorbic acid. Saponification is always followed by extraction with an organic solvent, such as diethyl ether [1,4], diisopropyl ether [5,6], chloroform [7], hexane [8], or hexane–diisopropyl ether mixtures [9] to rid the sample of saponified material. Before injection into the chromatographic system, the extracts are purified, in some cases using alumina or Florisil columns [10] or, in others, by evaporating the organic phase and dissolving the residue in another solvent compatible with the mobile phase.

Sample preparation can also be achieved by direct extraction, without saponification, of the liposoluble fraction with an organic solvent such as hexane [11–15] or hexane–2-propanol mixtures [16–18], for normal-phase systems, or anhydrous ethanol [19], acetonitrile–methanol–2-propanol mixtures [20], 2-propanol or tetrahydrofuran for reversed-phase systems, pentafluorophenyl [21] and ODS [22] phases. In some cases, after the oil extraction with the organic solvent, this is evaporated off and the residue is purified by freezing [23,24]. Determinations can also be carried out by direct injection of the oil, after dilution of an aliquot in a solvent, either in normal-phase [25,26] or in reversed-phase systems previous to a gas chromatographic determination [27].

Here we have developed an automated method for the determination of α-, γ- and δ-tocopherols in vegetable oils by reversed-phase high-performance

liquid chromatography (RP-HPLC) using electrochemical detection in the coulometric mode. For this, we propose a continuous extraction system employing non-porous membranes coupled to the chromatographic separation system without prior hydrolysis of the fat material.

2. Experimental

2.1. Apparatus

The set-up consists of two parts: one carries out sample treatment and the other performs separation and detection of the vitamin isomers. The sample preparation system comprises a Gilson (Villiers le Bel, France) Minipuls-3 peristaltic pump with Isoversinic 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 which is used when saponification is 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, 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.

The chromatographic system comprises a Spectra-Physics (San Jose, CA, USA) SP8800 ternary pump, an ESA (Chelmsford, MA, USA) Model 5020 ''Guard Cell'' (consisting of a porous graphite working electrode and a reference electrode and a counterelectrode, the latter two made of stainless steel) connected to the system to treat the mobile phase before it enters the column; an ESA Model 5010 ''Analytical cell'' (comprising two chambers in each of which there is a porous graphite working electrode, a double reference electrode and a double counterelectrode, both of stainless steel); an ESA coulometric detector and an integration system, consisting of the following: a Shimadzu (Duisburg, Germany) CBM-10A communication module, Class-LC10 Software, a Venturis 466 Digital (Madrid, Spain) Computer and a Hewlett-Packard (Madrid, Spain) 520 Deskjet printer. The chromatographic columns are: a Brownlee Labs. (Santa Clara, CA, USA) RP-18 pre-column (15×3.2 mm I.D., 7 μm) and a Brownlee Labs. OD-224 RP-18 column (220×4.6 mm I.D., 5 μm). The whole system, except the chromatographic pump, is controlled by the microprocessor.

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

2.2. Reagents

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 (BDH, Poole, UK), special HPLC quality *n*-hexane (Merck), R.A. grade glacial acetic acid (Scharlau, Barcelona, Spain), R.A. grade sodium acetate (Panreac, Barcelona, Spain), pure Triton X-114 (Fluka, Alcobendas, Madrid, Spain), R.A grade potassium hydroxide (Scharlau), R.A. grade ascorbic acid (Panreac), R.A. grade ethanol (Scharlau).

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

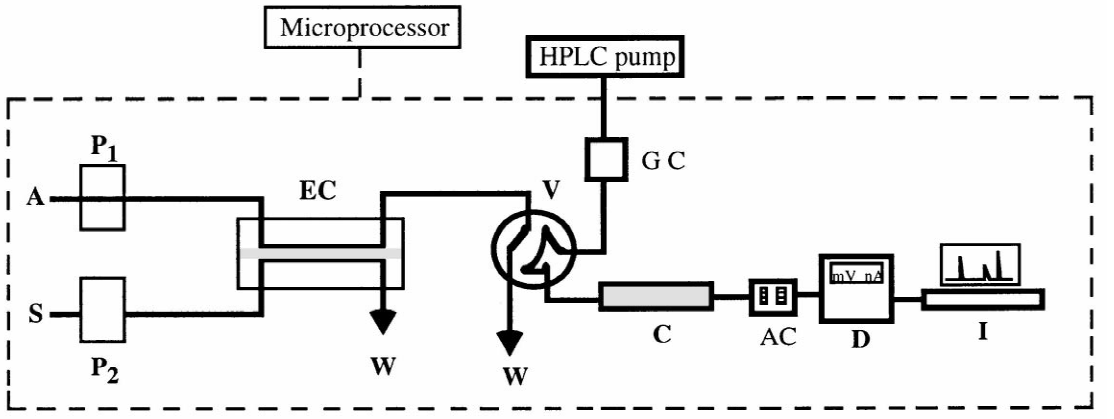
2.3. Samples

Certified sample CRM 122 supplied by the Institute for Reference Materials and Measurements (BCR Reference Materials, 98, Belgium). Oil samples were commercially available.

3. Procedure

The determination of different vitamin E isomers in vegetable oils is performed after continuous extraction of the analytes from the matrix using a silicone non-porous membrane coupled on-line to the chromatographic system.

The set-up is depicted in Fig. 1. The functioning of the system is as follows: the donor solution, obtained by dissolving the sample with a non-ionic



A: Acceptor. **S**: Sample. $\mathbf{P_1}$: Piston pump. $\mathbf{P_2}$: Peristaltic pump. **EC**: Extraction Cell.

GC: Guard Cell. **V**: Injection valve (20 μl loop). **C**: HPLC column.

A C: Analytical Cell. **D**: Coulometric Detector. **I**: Integrator. **W**: Waste.

Fig. 1. Diagram of coupled on-line sample treatment–LC for the determination of vitamin E isomers. LC conditions: mobile phase: methanol–water (97:3), 2.5 mmol l^{-1} HAcO/NaAcO; flow-rate: 1.0 ml min^{-1}. Electrochemical detection at +500 mV.

surfactant (Triton X-114) in the presence of methanol and hexane, passes along one side of the membrane in a time referred to as the enrichment time. 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 column 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.

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

4. Results and discussion

4.1. Optimization of variables in the extraction process

4.1.1. Displacement volume

In the methodology applied here, involving the extraction of Vitamin E isomers through a silicone membrane, the acceptor solution is enriched in analytes over a pre-set time, which we refer to as the enrichment time. After this, the enriched solution must be displaced by the acceptor phase pump, via a dilutor, to introduce the portion of acceptor solution containing the extracted isomers into the loop of the injection valve. The displacement volume necessary for the fraction of acceptor phase most enriched in analytes to be in the 20 μl of the loop depends on the

distance between the extraction cell and the loop of the valve. The acceptor solution was displaced with volumes ranging between 60 and 160 μl, obtaining the highest signal for 120 μl.

4.1.2. Acceptor phase

Owing to the lipophilic nature of vitamin E, the acceptor phase must be formed of a low-polarity solvent and must also be compatible with the stationary and mobile phases of the chromatographic system. Different acceptor phases were studied (acetone, methanol and acetonitrile) to check which one would afford the best analytical signals; these were obtained when acetonitrile was used as the acceptor phase.

4.1.3. Donor phase

Due to the high fat content of the samples analyzed, their solubilization requires the use of non-polar organic solvents or surfactants to form micellar solutions with polar solvents. In previous works [28,29], the advantage of using surfactants instead of organic solvents for this type of sample were acknowledged. Triton X-100 was used for the solubilization of butter and margarine. On using this surfactant for oil samples, the appearance of two phases was observed – i.e., the emulsion was not homogeneous – and the analytical signals decreased with time. However, on using another non-ionic surfactant – Triton X-114 – the samples were better solubilized and, further, the signals obtained over time could be reproduced.

To obtain the most suitable percentage of Triton X-114, samples of olive and sunflower oil were dissolved in this surfactant at concentrations between 1 and 10%. As may be seen in Fig. 2, the best signals were obtained with Triton X-114 percentages of around 6%.

In order to improve the extraction of vitamin E isomers, mainly γ- and δ-tocopherols, different assays were performed, adding different organic solvents such as methanol, hexane or acetonitrile to the solution of Triton X-114. Under these conditions a better extraction performance was observed.

First, a study was conducted to determine the most suitable percentage of methanol in the samples. This was performed for both olive and sunflower oil. The results obtained shown that, with methanol percentages around 20% or higher, the micelles were broken down and, experimentally, the appearance of two phases was observed. Because of this and in view of the results obtained, it was decided to work with 10% methanol for all types of oil studied.

Following this, we performed the study adding different percentages of acetonitrile to a sample of olive oil dissolved in Triton X-114 and 10% metha-

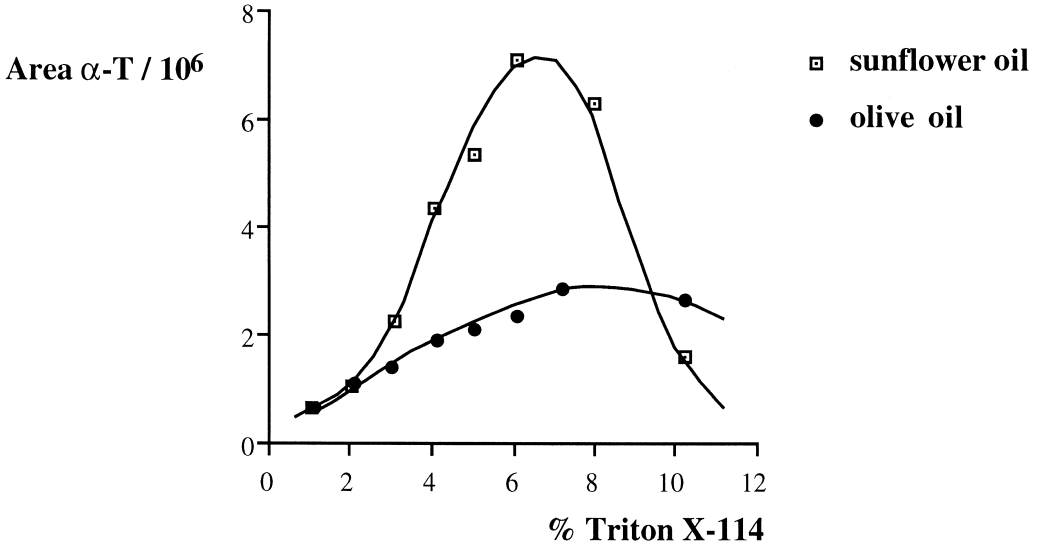


Fig. 2. Influence of Triton X-114 concentration in sample solubilization. LC conditions as in Fig. 1.

nol. It was observed that upon adding acetonitrile to the donor phase the signals increased with increasing acetonitrile concentrations. This increase in the signals was greater for the most abundant isomer – α-tocopherol, about 9 times – and was four-fold increased for γ-tocopherol. In most oil samples, the γ and δ isomers are present in much lower amounts than α-tocopherol; using a suitable amount of sample to be able to quantify the former two isomers, the working electrode would become saturated due to the presence of α-tocopherol.

The addition of hexane to the solution of samples in Triton X-114 and methanol was studied to determine the most appropriate percentage of this solvent as regards the response of the analytes. After applying the procedure, the signals shown in Fig. 3 were obtained. For percentages of hexane above 8%, the analytical responses remained constant after an approximately three-fold relative increase with respect to the signal in the absence of hexane. This would permit the use of larger sample amounts for the determination of all the isomers without the appearance of possible saturations in the signal due to the presence of α-tocopherol.

It was decided to work dissolving the samples in 6% Triton X-114 with 10% MeOH and 10% hexane. To check that the emulsions obtained were homogeneous, and hence that the signals obtained would be reproducible, the relative standard deviation (RSD) was determined after applying the procedure 12 times to a sample of olive oil. Values of 8.87% and 5.84% were obtained for γ- and α-tocopherols, respectively.

4.1.4. Amount of sample

In order to determine the most suitable amount of sample to be used, the procedure was applied, weighing different amounts of olive and sunflower oils, and it afforded the signals shown in Fig. 4. A gradual and linear increase occurred in the signals as the amount of sample was raised. For amounts above 2 g, the signals tended towards a constant value asymptotically. Because the relationship between the amount of sample used and the analytical signal must be linear, it was necessary to work in the increasing zone of the curve, such that amounts of less than 1 g were used for all types of oil.

4.1.5. Enrichment time

The time during which the donor solution passes along one side of the silicone membrane while the acceptor solution remains on the other side, becoming enriched in analytes, is called the enrichment time. To optimize this parameter, the general procedure was applied to a sample of olive oil and another of sunflower oil, varying the enrichment time between 1 and 12 min; the signals shown in Fig. 5 were obtained. The signal was seen to increase

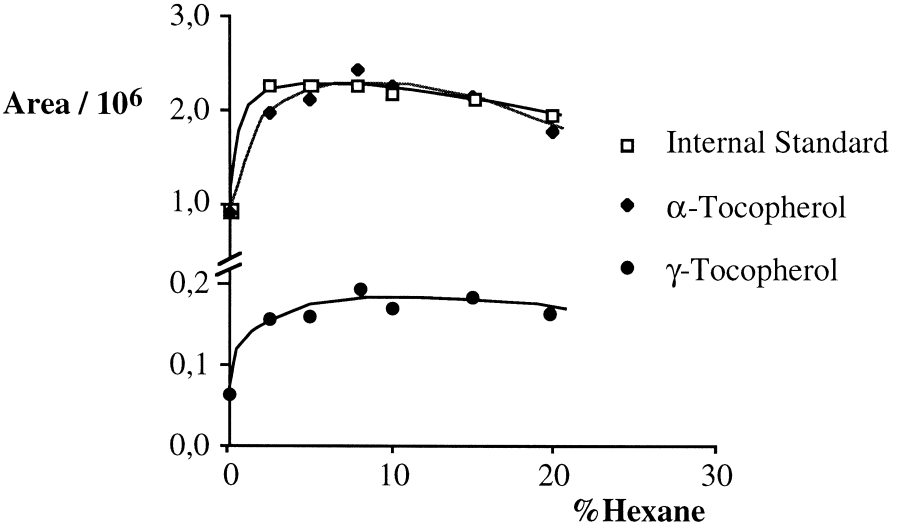


Fig. 3. Influence of hexane concentration on the chromatographic signal. LC conditions as in Fig. 1.

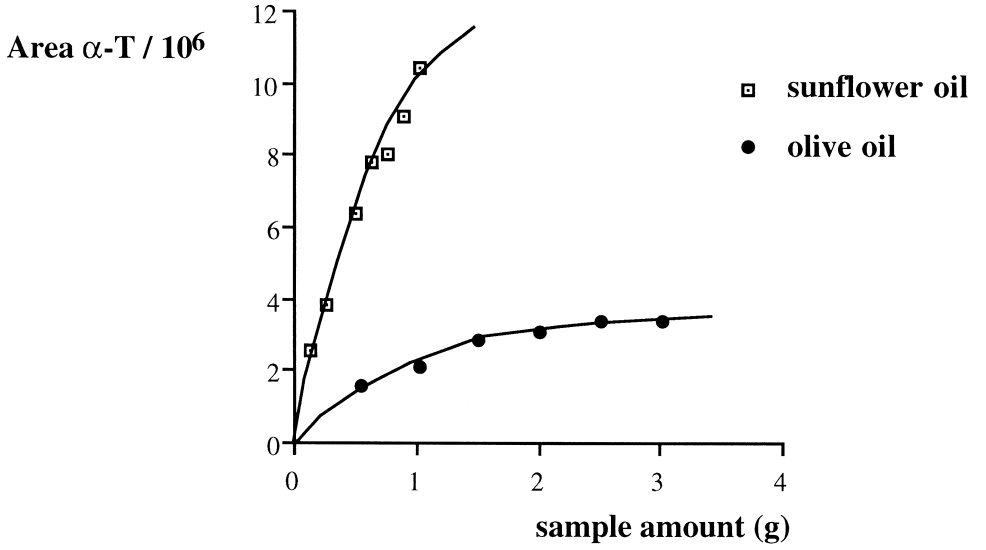


Fig. 4. Influence of sample amount on the chromatographic signal. LC conditions as in Fig. 1.

linearly up to about 6–8 min. In order not to lengthen the analysis time excessively and to avoid the memory effect that arises when the signals are very strong and the acceptor solution does not completely remove the analytes in an experiment (the analytes accumulating on the membrane and being removed in greater proportions during the next experiment), it was decided to work with enrichment times of 6 min, thus permitting complete determination in some 17 min.

4.2. Optimization of variables in the separation and detection processes

In previous works [28,30], a systematic study was made of the variables affecting the chromatographic

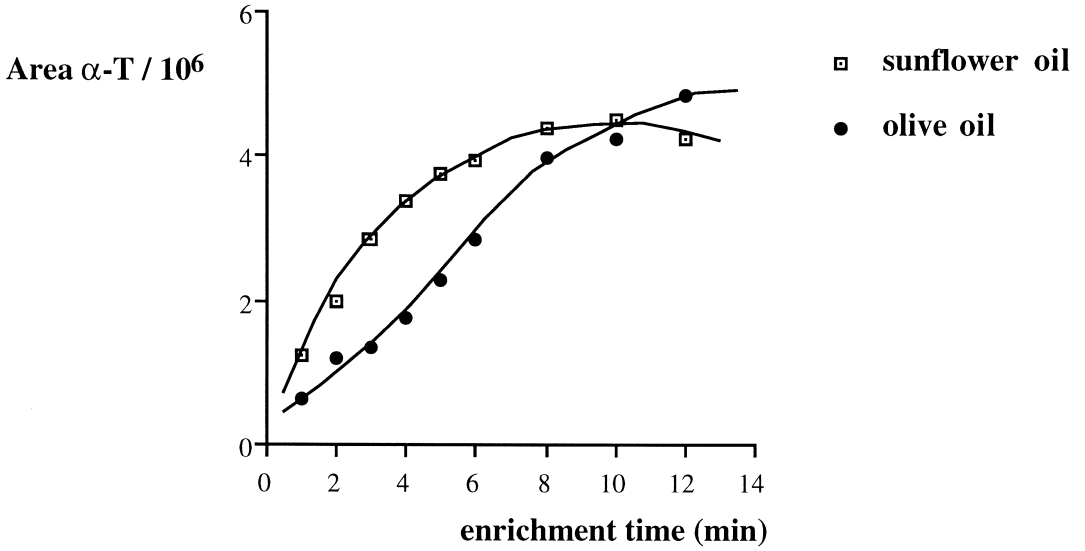


Fig. 5. Influence of enrichment time on the chromatographic signal. LC conditions as in Fig. 1.

system. The mobile phase used was methanol–water (97:3, v/v), with 2.5 m*M* acetic acid/sodium acetate, the flow-rate of the mobile phase being 1.0 ml min^{-1}.

To obtain the best analytical signal possible, the potentials applied to the two graphite electrodes of the analytical cell were optimized. First, the effect of the potential applied to the first electrode on the analytical signal was studied. The potential of the first electrode was varied, keeping the measurement potential constant ($E_2 = +650$ mV). In the case of standard samples, no variations in the signal were observed on modifying the potential applied to the first electrode. However, in the case of real samples this potential affected the signal. It was thus decided to work with a E_1 potential of -1000 mV applied to this electrode.

Secondly, the effect of the potential of the measurement electrode on the analytical signal was studied, keeping the potential of the first electrode constant ($E_1 = -1000$ mV). As in the previous case, the procedure was applied to both a standard sample and a real sample. It was decided to use a working potential of $+500$ mV since at this value the maximum intensity of the intensity-potential curve was reached.

4.3. Analytical characteristics

Using the optimum conditions deduced from the study of the different variables, calibration straight lines corresponding to the different isomers were obtained.

On plotting the areas of the chromatographic peaks (quantitative variable obtained with tree replicates) against the concentrations of the different isomers, straight lines were obtained with the following equations and linear correlation coefficients:

y (units of area)$=a+bx$ (mol l^{-1})				
	Concentration range	a	b	r^2 ($n=9$)
α-Tocopherol	$(2.10–210)\cdot10^{-7}$	$(1.8\pm1.3)\cdot10^{5}$	$(1.55\pm0.01)\cdot10^{12}$	0.999
γ-Tocopherol	$(2.11–211)\cdot10^{-7}$	$(1.8\pm1.3)\cdot10^{5}$	$(9.93\pm0.14)\cdot10^{11}$	0.999
δ-Tocopherol	$(2.21–221)\cdot10^{-7}$	$(1.9\pm0.4)\cdot10^{5}$	$(3.92\pm0.04)\cdot10^{11}$	0.999

The detection limits, evaluated by the term L.D.$= 3S/b$, obtained under the same working conditions were $3.75\cdot10^{-8}$ mol l^{-1}, $5.85\cdot10^{-8}$ mol l^{-1} and $1.48\cdot10^{-7}$ mol l^{-1} for α-, γ- and δ-tocopherol, respectively.

The RSDs obtained on carrying out a study on inter-day precision ($n=10$) for a standard sample containing α-, γ- and δ-tocopherols at concentrations of $4.10\cdot10^{-6}$ mol l^{-1}, $4.46\cdot10^{-6}$ mol l^{-1} and $4.27\cdot10^{-6}$ mol l^{-1}, respectively, were 6.65% for α-tocopherol, 6.47% for γ-tocopherol and 6.35% for δ-tocopherol.

4.4. Quantification

Quantification was performed using the internal standard method, which corrects the matrix effect and also the losses of analyte that may occur during sample preparation. In a previous work [29], we confirmed the possibility of using 2,2,5,7,8-pentamethyl-6-chromanol (PMC) as internal standard since this compound is electroactive at the working potentials and, also, it elutes at a sufficient distance from the analytes to be determined separately. Accordingly, its signal does not interfere with those of the analytes.

It was further observed that the relationship between the signals of the analytes and of the internal standard (used as an analytical variable in this type of methodology) and the analyte concentration was linear. All values were obtained with three replicates.

y (ratio of analyte/internal standard areas)$=a+bx$ (mol l^{-1})			
	a	b	r^2 ($n=9$)
α-Tocopherol	$(2.7\pm7.1)\cdot10^{-3}$	$(1.706\pm0.008)\cdot10^{5}$	1
γ-Tocopherol	$(0.9\pm1.2)\cdot10^{-2}$	$(1.093\pm0.014)\cdot10^{5}$	0.999
δ-Tocopherol	$(1.6\pm0.27)\cdot10^{-2}$	$(4.320\pm0.029)\cdot10^{4}$	1

4.5. Proposed procedure for the determination of α-, γ- and δ-tocopherols in vegetable oils

The general procedure proposed for the automatized analysis of α-, γ- and δ-tocopherols in vegetable oils using the described methodology is as follows:

Table 1
Determination of different vitamin E isomer contents (mg/100 g) in different oil samples by different analytical methods: (a) automatized method without prior hydrolysis, (b) automatized method with prior hydrolysis, (c) discontinuous method with hydrolysis

		Olive oils					Sunflower oils			Corn oils		Mixed seed oils		
		O-1	O-2	O-3	O-4	O-5	SF-1	SF-2	SF-3	C-1	C-2	S-1	S-2	S-3
α-Tocopherol	a	12.9±1.0	11.5±0.6	14.7±0.3	19.1±1.0	18.0±0.4	62.5±2.7	72.8±0.6	71.0±0.5	25.4±2.4	15.5±0.6	73.5±0.6	52.6±0.5	33.4±1.2
	b	15.5±0.8	11.5±0.1	15.8±0.6	20.2±0.5	20.5±0.8	69.5±1.0	64.8±3.0	63.5±0.4	23.4±0.4	15.9±0.5	62.0±1.3	57.0±1.2	36.5±0.8
	c	12.4±0.4	10.3±1.4	18.1±0.5	18.0±0.2	19.2±0.7	65.1±3.9	64.0±3.8	69.2±2.3	26.2±2.1	18.2±1.3	69.5±0.1	57.6±3.4	37.1±2.6
γ-Tocopherol	a	1.04±0.10	0.96±0.05	1.49±0.06	1.96±0.03	1.46±0.14	4.58±0.07	4.83±0.09	5.41±0.24	48.8±1.3	24.2±1.1	9.36±0.35	11.4±0.8	26.1±2.0
	b									61.6±2.0	31.9±1.4	12.1±0.8	18.6±0.9	37.1±2.7
	c	1.20±0.12	1.23±0.06	1.45±0.10	1.49±0.03	1.28±0.12	4.82±0.18	3.86±0.55	4.69±0.14	50.3±2.9	28.5±2.2	8.04±0.29	13.0±0.7	29.6±2.3
δ-Tocopherol	a									2.84±0.32	2.06±0.18		3.44±0.22	9.75±1.31
	c									1.67±0.38	1.07±0.18		2.69±0.26	8.52±1.48

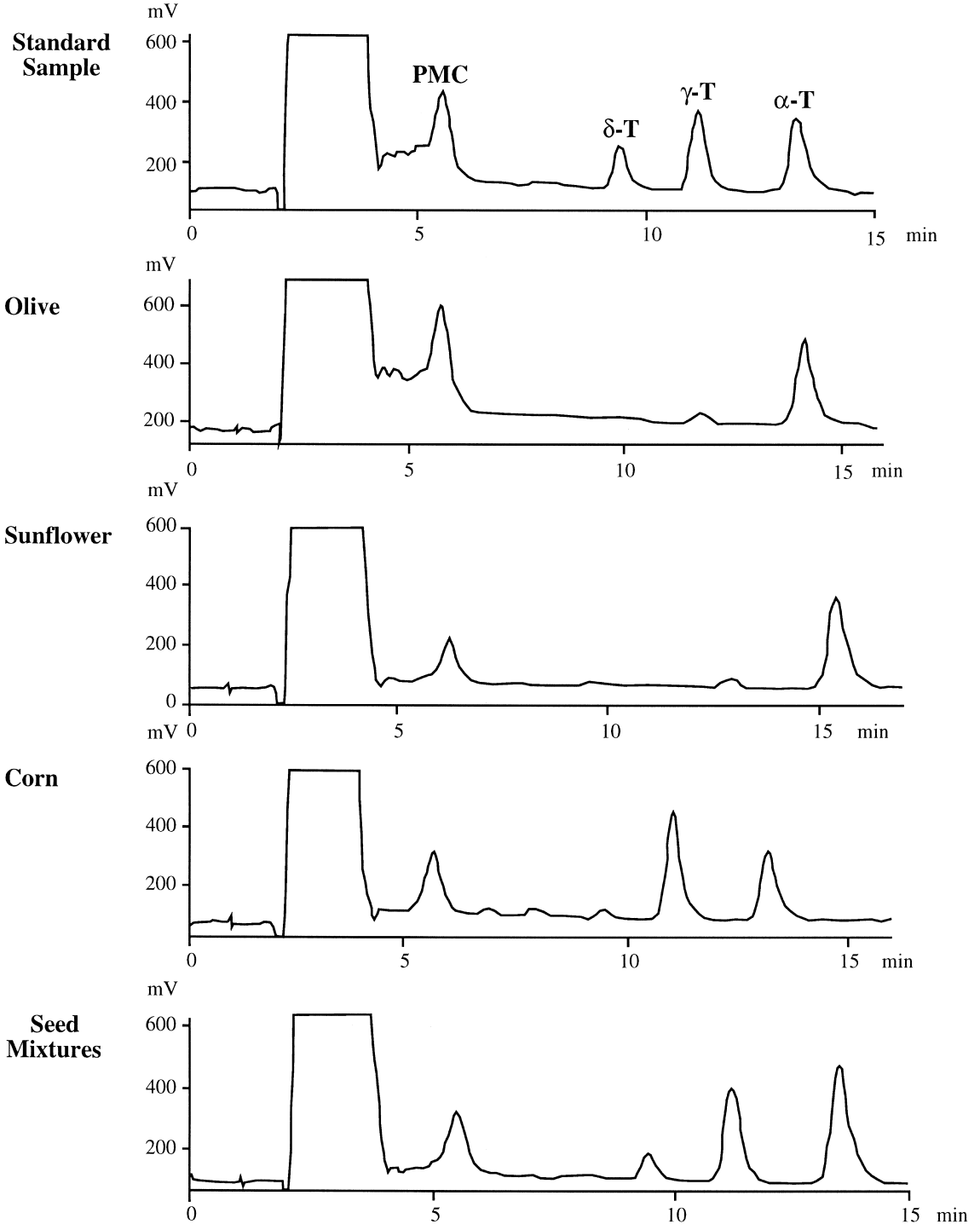


Fig. 6. Chromatograms obtained after application of the proposed method to the different types of oil. LC conditions as in Fig. 1.

The donor solution was obtained by weighing between 0.5 and 1.0 g of sample, depending on the type of oil, and adding about 3 g of Triton X-114, 5 ml of methanol, 5 ml of hexane and the internal standard (PMC). This solution is mixed well and brought up to volume of 50 ml with bidistilled water. This solution is passed along one side of the membrane while on the other side the acceptor solution (acetonitrile) is allowed to remain in place for 6 min. After this time, the acetonitrile enriched in the analytes is displaced to the loop of the injection valve, dispensing a volume of 120 μl via a dilutor. This is injected into the chromatographic system, where separation and later quantification of the analytes is achieved by the coulometric detector, applying a potential of +500 mV to the working electrode. Under these working conditions, the elution times are approximately 5, 9, 11 and 13 min for PMC and δ-, γ- and α-tocopherols, respectively.

4.6. Analysis of vegetable oils

Thirteen samples of different vegetable oils were chosen for analysis. These included olive oil, virgin olive oil, sunflower oil, corn oil and mixed seed oil. The samples all had different contents in α-, γ- and δ-tocopherol isomers. The samples were analyzed in triplicate and the results are shown in Table 1. Fig. 6 shows the chromatograms obtained on applying the method to the different types of vegetable oils. The RSDs lie in the 5–9% range, which are very acceptable values for the analysis of real samples.

5. Comparison of analytical methods

To validate the proposed method, the samples were analyzed using another two methods.

5.1. Sample analysis in continuous mode with alkaline hydrolysis

The aim underlying this type of determination was to include alkaline hydrolysis in the proposed methodology and to check whether any significant improvement could be obtained in the results obtained by using only isomer extraction.

Continuous mode determination was carried out, extracting the analytes in the same way – i.e., using the silicone membrane – but performing alkaline hydrolysis of the sample. The sample, mixed in Triton X-114, methanol and the internal standard at the same proportions listed above, was made to merge with the hydrolysis reagent. The latter comprised 20 ml of 60% KOH, 70 ml of ethanol, 10 ml of 5% ascorbic acid and 50 ml of bidistilled water. The presence of ascorbic acid was mandatory to avoid oxidation of the analytes in this strongly alkaline medium. The mixture was passed through a 5 m coiled reactor and then passed along one side of the membrane where the vitamin E isomers were extracted, as described for the proposed procedure above.

5.2. Sample analysis in discontinuous mode

The generally accepted procedure [31] to analyze liposoluble vitamins in food such as milk, butter and oil was applied. To do so, between 0.5 and 1.0 g of sample are mixed with the hydrolysis reagent, whose composition is 10 ml of 80% KOH, 50 ml of ethanol, 5 ml of 10% ascorbic acid and 25 ml of bidistilled water. The mixture is stirred for 2 h at room temperature protected from light. After this time, the analytes are extracted with three portions of 10 ml of hexane for 1 min, after which the extracts are washed with two 10 ml portions of bidistilled water for 30 s and the organic phase is evaporated to dryness in a rotavapor at 50°C. The residue is dissolved in methanol, filtered through 0.45 μm mesh nylon, and injected directly into the chromatographic system.

These methods were applied to 13 samples of commercial oils (five of olive, three of sunflower, two of corn and three of seed mixtures), obtaining the results shown in Table 1. Using all three methods, the results were similar. It is precise to indicate that, when the δ and γ isomers are present in lower concentrations, the method including continuous alkaline hydrolysis produces mistakes and the results were not included.

5.3. Student's t-test

The three methods were compared with each other for each type of oil using Student's *t*-test for paired

Table 2
Paired *t*-test for different quantification methods applied to oil samples: (a) without prior hydrolysis, (b) with prior hydrolysis and (c) discontinuous method – levels of significance obtained

Oil		a–b	a–c	b–c
Olive	α-Tocopherol	0.0404	0.7013	0.2965
	γ-Tocopherol		0.7104	
Sunflower	α-Tocopherol	0.6228	0.5061	0.9602
	γ-Tocopherol		0.3203	
	α-Tocopherol	0.6257	0.3166	0.0622
Corn	γ-Tocopherol	0.1552	0.2863	0.3139
	δ-Tocopherol		0.0529	
	α-Tocopherol	0.8181	0.6331	0.3345
Seed mixtures	γ-Tocopherol	0.0998	0.4636	0.0289
	δ-Tocopherol		0.1514	

values. In each case, the level of significance was obtained. When this value was greater than 0.05 (chosen as the minimum level of significance) the null hypothesis was accepted – there were no differences among the different results. Table 2 shows the levels of significance obtained on applying the *t*-test to the contents found in the different types of oil studied with the different methods. Stat View 4.01 Macintosh version program was employed for the statistical treatment of data.

In most cases, the level of significance obtained was greater than 0.05. Two values were lower than 0.05 but greater than 0.01, such that they were considered acceptable. In the light of the results, it may be concluded that the different analytical methods used afford significantly similar results.

For the analysis of the vitamin E isomer contents we propose the automatized procedure including direct extraction of the isomers through the silicon membrane since it permits quantification of the isomers found at lower concentrations and also simplifies sample treatment.

5.4. Analysis of a margarine sample with a certified content in α-tocopherol

We were unable to find samples of oils whose contents in micronutrients, such as vitamins, have been certified. However, The Institute for Reference Materials and Measurements (BCR Reference Materials, 98, Belgium) has available the CRM122 sample of margarine with a certified α-tocopherol content of 241 mg kg^{-1}. Although, this was not exactly the same matrix, it was the the most similar sample that we were able to find with a certified content in this type of analyte.

Table 3
Determination of the α-tocopherol content in a certified margarine sample using different methods (results from six replicate analyses): (a) automatized method without prior hydrolysis, (b) automatized method with prior hydrolysis, (c) discontinuous method with hydrolysis. α-tocopherol[a]

	a	b	c
Content (mg/100 g)	23.4±0.7	25.4±1.0	23.4±1.5
Relative error (%)	2.9	5.4	2.9
RSD (%)	3.0	3.9	6.5

[a] Certified content: 24.1±1.2 mg/100 g.

Six different portions of this margarine were analyzed, obtaining the results shown in Table 3. As may be seen, the proposed method did not produce any bias error in content assessment. Additionally, precision was highly acceptable, as was previously observed in the analysis of oil samples.

6. Conclusions

The proposed methodology, which includes direct extraction of analytes across the membrane coupled ''on-line'' with chromatographic determination, is successful. This is very important because automatization simplifies the analytical process, reduces analysis time (this is about 25 min from sample weighing to quantification) and decreases the errors in the determinations. This method is slower than methods with direct injection after sample dissolution, although in this case a normal-phase silica must be used and the disadvantages of the use of organic solvents are well known. We believe that the advantages of our method outweigh the disadvantages of faster methodologies. With these characteristics, the proposed methodology can be applied to routine analysis in the food industry for the control of these tocopherols in oil samples.

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