Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 105 (2007) 1583–1590
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Chemometrics for the resolution of co-eluting peaks of β- and γ-tocopherols in RP-HPLC: Determination in edible oils and dietary supplements

T. Galeano Díaz *, M.I. Rodríguez Cáceres, B. Roldán Murillo *

Department of Analytical Chemistry, University of Extremadura, 06071 Badajoz, Spain

Received 18 October 2006; received in revised form 14 February 2007; accepted 10 March 2007

Abstract

This paper deals with the quantification of β- and γ-tocopherol by RP-HPLC, using a C18 column as stationary phase, and MeOH:H_2O (90:10, v/v) as mobile phase (flow rate, 2 mL min^{-1}). Both analytes appear together in the same chromatographic peak and, as consequence, a partial least squares (type PLS-1) chemometric method has been developed to analyze them separately. Different calibration matrices have been done with the fluorescence spectra, excitation and emission, absorbance and derivative absorbance spectra, obtained across the peak or in the peak apex. The matrix of data constructed with the derivative absorbance spectra obtained in the peak apex is the one that gives best results. The method has been successfully applied to edible oils and dietary supplements. No pretreatment of the samples was needed, only a dilution in 2-propanol was effected.

Keywords: Tocopherols; RP-HPLC; PLS; Edible oil; Dietary supplements

1. Introduction

Fat-soluble vitamins comprise vitamins A, D, E and K. Recommended dietary allowances (RDA) have been published all around the world. Some foods such as margarine, milk products, dietetic foods and infant formulas are commonly fortified with vitamins A, D and E. The addition of vitamins to a particular processed food is intended to provide a specific proportion of the RDA (Handbook of Food Analysis, 1996). General principles for the addition of nutrients to foods have been established by the Codex Alimentarius Commision (Codex Alimentarius Commission, 1987) and the US Food and Drug Administration (FDA, 1987).

Vitamin assays in foods are carried out for several purposes, among them, to provide data for food composition and for quality assurance. Eight vitamers of vitamin E occur in nature: four tocopherols and four tocotrienols. Tocopherols are methyl-substituted derivatives. Tocopherols and tocotrienols are designated as α-, β-, γ- and δ- according to the number and position of the methyl groups in the chromanol ring. The role of vitamin E in the body can be explained in general as a lipid antioxidant in stabilizing subcellular membranes, but, it is worth noting that every vitamer varies widely in biological activity.

The problem of the separation of α-, β-, γ- and δ-T has been the subject of numerous papers (Abidi, 2000; Pyka & Sliwiok, 2001; Rozzolo & Polesello, 1992). Abidi (2000) reviewed exhaustively the chromatographic analysis of tocol-derived lipid antioxidants performed until the date. The paper compiles published NP- and RP-HPLC and concluded that NP-HPLC have been used for the majority of investigators. It is worth noting that tocotrienols does not interfere in the analysis of tocopherols (Abidi & Rennick, 2001; Chen & Bergman, 2005).

* Corresponding authors. Tel./fax: +34 924289375 (B. Roldán Murillo).
E-mail addresses: tgaleano@unex.es (T. Galeano Díaz), broldan@unex.es (B. Roldán Murillo).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.03.032

When NP-HPLC is used, all the tocopherols can be quantified. Vegetable oils may be simply dissolved in hexane and analyzed directly by normal phase HPLC (Beltrán, Aguilera, Del Río, Sánchez, & Martínez, 2005; Cunha, Amaral, Fernandes, & Oliveira, 2006; Demir & Cetin, 1999; Gama, Casal, Oliveira, & Ferreira, 2000; Kornsteiner, Wagner, & Elmadfa, 2006; Marsin Sanagi, See, Wan Ibrahim, & Abu Naim, 2005). In these, the vitamin E is mainly present as α-tocopherol and γ-tocopherol (Pyka & Sliwiok, 2001). Tocotrienols are present in small amounts, except for palm oil, grape seed oil and the annatto lipid fraction, which have a relatively high content of these active components (Lercker & Rodriguez-Estrada, 2000). Either normal-phase (NP) or reversed-phase (RP) HPLC can be employed. Different mobile phases have been used, such us hexane–isopropyl alcohol (99:1, v/v) (Tan & Brzuskiewicz, 1989), *iso*-octane/*t*-butyl methyl ether (47:3, v/v) (Goffmann, Velasco, & Thies, 1999), hexane:isopropyl ether (93:7, v/v) (Hakansson, Jagerstad, & Oste, 1987) or hexane/propan-2-ol (Demir & Cetin, 1999; Gama et al., 2000).

On the other hand, when reversed-phase HPLC is used, the quantification of the individual β-T and γ-T is usually not possible. Tan and Brzuskiewicz (1989) carried out the separation of tocopherol and tocotrienol isomers using normal and reversed-HPLC. They preferred normal phase since the ODS column used for the reversed-phase mode is not able to separate β- from γ-T. Also, Gimeno, Castellote, Lamuela-Raventos, de la Torre, and Lopez-Sabater (2000) worked with an ODS-2 column and they expressed the content of Vitamin E as α-, (β + γ)- and δ-tocopherol. Similar results were obtained when a Nova-pack C18 column (Chen & Bergman, 2005) was used. However, resolution between β-T and γ-T has been possible using other less usual columns, such as polymeric C30 (Strohschein, Pursch, Lubda, & Albert, 1998), pentafluorophenylsilica (Richheimer, Kent, & Bernart, 1994) and non-silica based octadecyl polyvinyl alcohol (Abidi & Mounts, 1997; Abidi, 1999).

C30 phases exhibit superior shape selectivity in comparation of C18 for the separation of isomers of carotenoids and vitamins (Albert, 1998). The introduction of the C30 alkyl chain should result in increased hydrophobic interaction between the solutes and the alkyl chain. Carotenes and tocopherols have been simultaneously determinate using C30 reversed-phase column (Schieber, Marx, & Carle, 2002). In this case, the four tocopherols could be separated. The applicability of C30 stationary phases to food analysis has recently been reviewed by Sander, Sharpless, and Pursch (2000).

A survey of the literature shows that chemometric methods have been applied to the simultaneous determination of several analytes that co-eluted when the separation was performed by HPLC. Partial least squares (PLS-1 and PLS-2) and principal component regression (PCR) (Garrido Frenich, Martínez Galera, Martínez Vidal, & Gil García, 1996; Martínez Galera, Martínez Vidal, Garrido Frenich, & Gil García, 1996; Gil García et al., 1997) have been used. When the peaks are overlapped, the chemometric method can be applied to a chromatographic region (Garrido Frenich et al., 1996; Martínez Galera et al., 1996) or to the spectra of the analytes obtained in the peak (Gil García et al., 1997).

Other chemometric tools, such as, augmented iterative target transformation factor analysis (Van Zomeren, Metting, Coenegracht, & de Jong, 2005) and second-order calibration methods, such as generalized rank annihilation method (GRAM), parallel factor analysis (PARAFAC) and multivariate curve resolution-alternating least squares (MCR-PLS) have been used (Comas et al., 2004) for the resolution and quantification of overlapped chromatographic peaks. Fraga and Corley (2005) applied GRAM and PARAFAC to the resolution and quantification of overlapped peaks from comprehensive two-dimensional liquid chromatography (LC × LC).

The goal of this work is the resolution and quantification of the mixture of β-T and γ-T using a simple RP-HPLC method with a C18 column, which is the type more extensively used. The C18 columns of reverse phase are the most used since they present advantages such as the use of mobile phases with high percentage of water, therefore, compatible with aqueous matrices, or its fast equilibration when changes of composition in the mobile phase take place. Therefore, the chromatographic system could be used, not only for alpha and beta Tocopherols but for other analyses.The greater number of chromatographic analyses are carried out with this type of columns. Obviously systems in normal phase can be used, but the change of chromatographic modality if required, takes long time. Due to the total coincidence of the peaks, a chemometric tool (PLS-1) has been used with the fluorescence, absorbance or derivative absorbance spectra obtained in the peak and the results have been compared. The developed method here, that uses the derivative absorbance spectra obtained in the peak apex, has been successfully applied to different edible oils and dietary supplements.

2. Experimental procedures

2.1. Apparatus

The HPLC system used was an Agilent 1100 Series (Agilent Technologies Inc., Palo Alto, CA, USA) composed by a G1311A Agilent quaternary pump, a G1322A Agilent vacuum solvent delivery degasser, a G1315B Agilent UV–vis photodiode array detector, and a G1321A Agilent fluorescence detector. The liquid chromatographic system was controlled and the data collected and processed by the HP ChemStation for LC 3D software (Agilent Technologies Inc.).

2.2. Chromatographic system

The simultaneous analysis of β- and γ-tocopherols in edible oils and dietary supplements was performed using

a reversed-phase C18 column (Nova-Pack C18, 150 mm × 3.9 mm, Waters, Millipore). Two mobile phases, MeOH:H_2O (Gimeno et al., 2000) and ACN:MeOH (Gliszczynska & Sikorska, 2004), were assayed, based in the results from the literature in order to obtain the better resolution between the peaks of tocopherols. MeOH:H_2O, (90:10, v/v) with a flow rate of 2 mL min^{-1}, was selected. The absorbance spectra were obtained between 230 and 400 nm (maximum at 290 nm), and the emission fluorescence spectra were registered between 310 and 360 nm using an excitation wavelength of 290 nm.

The validation of the proposed method was carried out by normal-phase HPLC (IUPAC, 1988) using hexane:isopropanol (99.4:0.6, v/v) as mobile phase, a silica column and a flow rate of 1 mL min^{-1}.

2.3. Software

The UNSCRAMBLER (Unscrambler software, version 6.11 of CAMO, Trondheim, Norway) software package was used for statistical treatment of the data. The digitalized spectra, acquired with the Hewlett–Packard HPLC instrument, were converter in the same instrument to ASCII format and imported in this extension in Unscrambler. The derivation of the absorbance spectra was made also with Unscrambler software package through the use of the Savitzky and Golay algorithm (using 5 data points for the averaging and 2 as polynomial order).

2.4. Chemicals and reagents

A tocopherol set (α-, β-, γ- and δ-T, 95%) was purchased from Calbiochem (an affiliate of Merck, Darmstadt, Germany). Stock solutions of tocopherols (α-, β-, γ- and δ-T) of 500 μg mL^{-1} were prepared in hexane (Merck, HPLC grade, Darmstadt, Germany). These solutions were stored at 4 °C for several months in dark bottles. Working standard solutions consisted in mixtures of different concentrations of the four tocopherols in 2-propanol were prepared in 10-mL volumetric flasks.

For all experiments analytical reagent grade or better chemical and solvents were used. HPLC grade water was produced from a Milli-Q system (Millipore, USA).

2.5. Chemometric methods

In this work, the experimental data (absorbance and fluorescence spectra) obtained before, after and in the peak apex of the chromatogram were treated by the multivariate calibration method known as PLS-1. The theory and application of this method can be found extensively in the literature (Escandar et al., 2006; Helland, 2001; Wold, 2001). For that reason, only the outlines will be provided in this paper.

Partial least squares (PLS) uses the full-spectrum information and has the advantage that it is not necessary to explain the variations on the concentration of the analytes. It is a multivariate calibration procedure based on a principal components or factor analysis method (Geladi & Kowalski, 1986). The optimum number of PCs is linked to the complexity of the spectra and can be determined by using cross-validation (internal validation) method leaving out one sample each time (Eastment & Krzanowski, 1982). The predicted concentrations are compared with the known concentrations and the prediction error sum of squares (PRESS) is calculated as follows:

$$\mathrm{PRESS} = \sum_{i=1}^{N} (\hat{c}_i - c_i)^2$$

where N is the total number of calibration samples, $\hat{c}_i$ represents the estimated concentration and c_i is the reference concentration for the ith sample.

The number of factors was chosen in order to minimize the PRESS. The smaller the PRESS value, the better the model predicts the concentrations of the constituents in the samples. This parameter is a measure of how well a particular model fits the concentration data. One reasonable choice for the optimum number of factors would be that number which yielded the minimum PRESS. Thomas and Haaland (1988) have determined that the number of factors for the first PRESS value that had an F-ratio probability failing below 0.75 can be selected as the optimum.

To quantify the prediction ability of the models, the root mean standard error of prediction (RMSEP) was used

$$\mathrm{RMSEP} = \left(\frac{\mathrm{PRESS}}{N-1}\right)^{0.5}$$

and the square of the correlation coefficient (R^2), which is an indication of the quality of fit of all the data to a straight line, that is calculated with the following formulae,

$$R^2 = \frac{\sum_{i=1}^{N} (\hat{c}_i - c_i)^2}{\sum_{i=1}^{N} (c_i - \bar{c}_i)^2}$$

where $\bar{c}_i$ represents the mean of the true concentration in the prediction set.

2.6. Recommended procedure for tocopherols determination

Different real samples (oil and dietary supplements) were analysed. When oil samples were analysed, appropriate amounts were weighed and placed in 10-mL volumetric flasks filled until the mark with 2-propanol.

All the dietary supplements were present as capsules. For all of them, the content of some capsules was emptied, and once the appropriate amount was weighed, 2-propanol was added and the solution was placed in the ultrasound for 15 min. After that, it were filtrated and the solution were placed in 10-mL volumetric flask and filled until the mark with 2-propanol. Once the solutions were prepared, they were injected in the chromatographic system.

2.7. Real samples analysed

Once the method was developed, the following real samples were analysed:

- Oil samples:
  - (S1) Olive oil [Spain]
  - (S2) Corn oil [Spain]
  - (S3) Sojasol [Koipesol, Spain] → commercial mixture of soybean and sunflower
  - (S4) Soyplus [La Española, Spain] → commercial mixture of soybean and sunflower

- Dietary Supplements:
  - (S5) Vitamin E [Lab. Diafarm, S.A., France] → Labelled content 10 mg of Vit E./tablet
  - (S6) Mensoy [Dr. Düneer, Switzerland] → Labelled content 360 mg of Vit E/100 g
  - (S7) Polen y Jalea Real [Santiveri, Spain] → Labelled content 1.270 mg of Vit. E/100 g

3. Results and discussion

3.1. Optimization of the mobile phase for the analysis

In order to select the optimum wavelengths, absorbance and fluorescence spectra were recorded previously. The maximum for the absorbance spectra is located at 290 nm. For the fluorescence spectra, the excitation and emission maxima are located at 290 and 330 nm, respectively. Thus, those wavelengths were selected for obtaining chromatograms.

In the first case a proportion MeOH:H_2O (96:4, v/v) was selected at a 2 mL min^{-1} flow rate, only 6 min were required for the analysis of tocopherols in these conditions ($t_R = 4.8$ min for β- + γ-ocopherol).

On the other hand, the proportion ACN:MeOH selected to achieve better resolutions values on the chromatogram was 65:35, also with a flow rate of 2 mL min^{-1}, being the analysis time, in this case, lower than 3 min ($t_R = 2.7$ min for β- + γ-Tocopherol).

At the same time that the chromatograms were registered, absorbance and fluorescence spectra were recorder before, after and in the peak apex.

3.2. Simultaneous determination of β- and γ-tocopherol

Initially, the studies were performed using MeOH:H_2O (90:10, v/v) as mobile phase and a flow rate of 2 mL min^{-1}. Firstly, different calibration matrices were constructed, as multivariate data different signals corresponding to 19 samples prepared according a central composite design, with a concentrations range between 0 and 10 μg mL^{-1} for both β- and γ-Tocopherols. The chromatograms of these samples were run and the different signals were taken in each peak, one before the peak apex, another in the maximum or apex and the third one after the peak apex. The different signals used were absorbance, excitation and emission spectra, giving rise to different matrices, absorbance spectra matrix (ASM), excitation spectra matrix (ExSM) and emission spectra matrix (EmSM). Once all the chromatograms were run, PLS-1 was applied to the different matrices constructed with the different spectra obtained at the different moments.

Thus, nine matrices were constructed, three with each type of spectra. The analysis of the statistical parameters (data not shown) obtained for each one of them, reveals that the better results were obtained when the spectra taken in the peak apex were used. In Table 1, the statistical parameters corresponding to the matrices constructed using the different kinds of spectra, taken in the peak apex, are summarized. Taking into account that these statistical parameters show similar and acceptable values for all the three models, the number of factors was the variable considered. As can be seen, when MeOH:H_2O is used as mobile phase, the lowest values for the number of factors (PCs) are given by ASM, followed by ExSM. The worst values are for EmSM. On the basis of that, EmSM was discarded and the two matrices remaining were assayed with the second mobile phase. Thus, when ACN:MeOH was used as mobile phase, the spectra were recorded only in the peak apex, and only two matrices (ASM and ExSM) were constructed. A comparative analysis between the different

Table 1
Statistical parameters for the different matrices assayed using the spectra taken in the peak apex

Mobile phase	Matrix	Analyte	PCs	R^2	RMSEP	Var. explic (%)
MeOH:H_2O	ASM	β-T	3	0.9970	0.2608	98.6
		γ-T	2	0.9899	0.4628	98.0
	ExSM	β-T	3	0.9396	1.1290	86.2
		γ-T	3	0.9622	0.8988	91.4
	EmSM	β-T	4	0.9808	0.6413	96.6
		γ-T	4	0.9666	0.8086	94.1
	DASM	β-T	2	0.9957	0.3066	99.2
		γ-T	2	0.9938	0.3148	98.9
ACN:MeOH	ASM	β-T	5	0.9985	0.2842	99.2
		γ-T	4	0.9962	0.3238	99.0
	ExSM	β-T	3	0.9749	0.7121	95.0
		γ-T	3	0.9861	0.5592	97.2

Table 2
Recoveries obtained for synthetic samples using PLS-1

Mobile phase	Matrix	β-T		γ-T	
		Added (μg mL^{-1})	% Rec ± SD	Added (μg mL^{-1})	% Rec ± SD
MeOH:H_2O	ASM	3.0–8.0	105 ± 17	2.0–8.0	98 ± 20
	ExSM		96 ± 22		102 ± 60
ACN:MeOH	ASM	3.0–8.0	123 ± 19	2.0–8.0	116 ± 33
	ExSM		124 ± 44		149 ± 42

matrices, on the basis of the number of factors, reveals that when the absorbance spectra matrices are compared, better results were obtained with the MeOH:H_2O mobile phase, however, no difference exist between ExSM.

Once the emission spectra have been discarded, synthetic samples were prepared and were predicted with the other four matrices. Table 2 summarizes the recovery values obtained for the synthetic samples. As can be observed in the table, worst recoveries were obtained when the mobile phase was ACN:MeOH. In conclusion, MeOH:H_2O was selected, as mobile phase, for further experiments. Also, a comparison between the recoveries obtained with the excitation spectra and the absorbance spectra when the mobile phase was MeOH:H_2O reveals that the best results were obtained with the absorbance data.

Taking into account the conclusions obtained above, real samples were analyzed using MeOH:H_2O (90:10, v/v) as mobile phase (flow rate, 2 mL min^{-1}), and recording only the absorbance spectra at the peak apex. Samples are registered in triplicate. The recoveries obtained for the different real samples are summarized in Table 3. Several samples were not spiked with β- and γ-T, but the model predicted the values that appear in the table denoted with (∗). As can be seen in the table, the results are not satisfactory at all.

It was observed that baseline of spectra changed between successive runs. Because of this, trying to improve the method, a new matrix was constructed in the same con-

Table 3
Recoveries obtained for real samples using ASM matrix

Sample	β-T		γ-T	
	Added (mg g^{-1})	% Rec. ± SD (*Found, mg g^{-1})	Added (mg g^{-1})	% Rec. ± SD (*Found, mg g^{-1})
S1	0.0	106 ± 16*	0.0	–
	0.059	114.0 ± 3.7	0.059	19 ± 12
	0.098	82 ± 20	0.138	43 ± 12
S2	0.0	62 ± 11*	0.0	56 ± 39*
	0.254	81.5 ± 0.8	0.254	92 ± 17
	0.423	108.5 ± 2.5	0.593	81.9 ± 2.4
S3	0.0	74 ± 19*	0.0	–
	0.094	180 ± 101	0.094	–
	0.156	126 ± 53	0.219	–
S4	0.0	60.90 ± 0.01*	0.0	72.4 ± 2.4*
	0.099	132 ± 42	0.099	102 ± 34
	0.165	116 ± 33	0.231	35.6 ± 6.6
S5	0.0	1072 ± 162*	0.0	–
	0.275	64 ± 80	0.275	–
	0.459	100 ± 13	0.642	–
S6	0.0	113 ± 42*	0.0	–
	0.192	0	0.192	108 ± 17
	0.321	83.7 ± 2.1	0.448	109 ± 12
S7	0.0	–	0.0	195 ± 95*
	0.268	44 ± 22	0.268	108 ± 17
	0.446	77 ± 18	0.625	109 ± 12

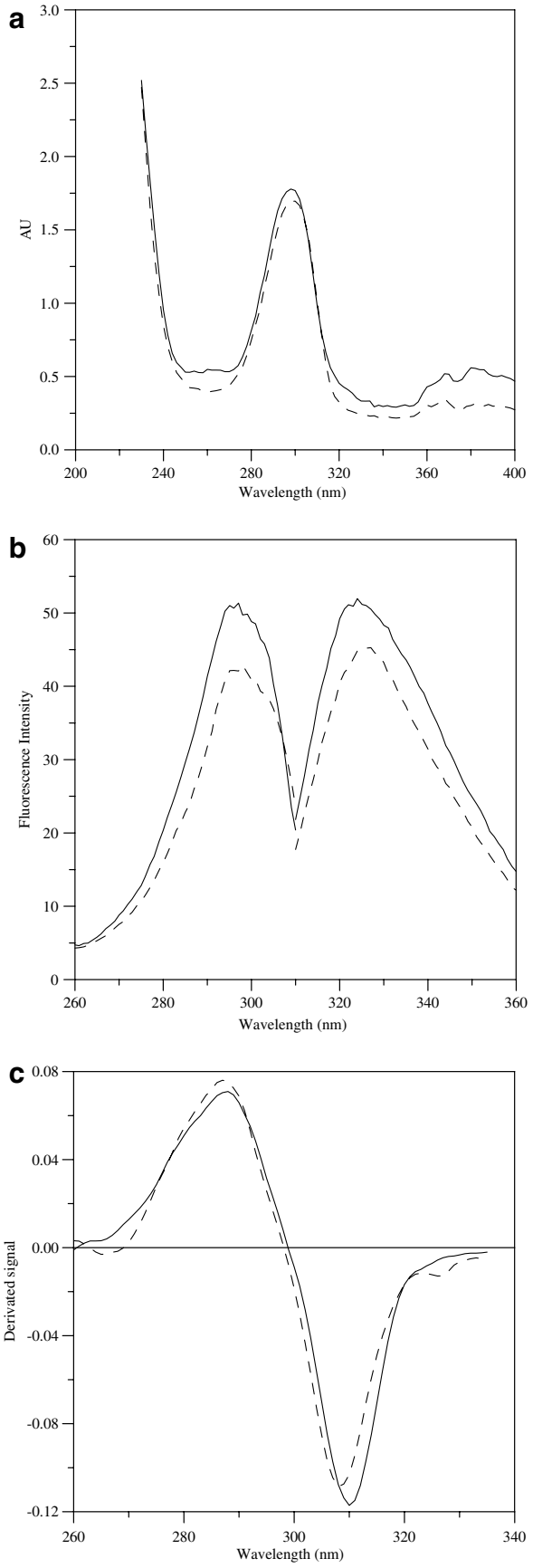

Fig. 1. (a) Absorbance; (b) excitation and emission spectra and (c) derivate absorbance of β-T (solid line) and γ-T (dashed line).

ditions mentioned above, but using this time the derivative absorbance spectra as multivariate signal. Thus, the absorbance spectra taken in the peak apex were derived with the Unscrambler software package. Fig. 1c shows the derivative absorbance spectra of β- and γ-tocopherol, in which it can be noted the little difference that exists between the spectra of both tocopherols. Table 2 summarizes the statistical parameters obtained for the new matrix constructed in this way, named DASM. If these parameters are compared with the ones for the ASM, it can be observed an improvement.

The detection limits were estimated evaluating the repetivity of the samples without one tocopherol and variable concentration of the other. A good measure of the detection limit can be given by 3 S_D, being S_D the standard deviation obtained for the concentration of the tocopherol examined. This procedure could be as an extension of the IUPAC method. The detection limits calculated were: 0.22 μg mL^{-1} and 0.37 μg mL^{-1} for β- and γ-T, respectively.

Once the model has been optimized, the next step was the prediction of the synthetic and real samples. The recoveries obtained for the synthetic and real samples are compiled in Table 4 and 5, respectively. Again, several samples were not spiked with β- and γ-T. The values predicted by the model in these cases appear in the table denoted with (∗). It is worth noting that recoveries for synthetic samples around 100% are obtained. Again, in the case of spiked real samples, good recoveries were obtained.

3.3. Validation of the method

The procedure was the following: the appropriate amounts of the samples were weighed, dissolved in hexane, filtered and injected (20 μL) into the chromatographic system. Samples were registered in triplicate. Later, the standard addition method was applied. Both detectors (DAD and fluorescence) were used in the same conditions mentioned above.

The results obtained for the real samples analysed are summarized in Table 5 and compared with the ones found with the proposed RP-HPLC method. A good agreement can be found.

Table 4
Recoveries obtained for the synthetic samples using DASM matrix

Matrix	Sample	β-T		γ-T	
		Added (μg mL^{-1})	% Rec ± SD	Added (μg mL^{-1})	% Rec ± SD
DASM	1	3.0	106.6 ± 3.5	3.0	99.7 ± 3.8
	2	5.0	104.2 ± 0.5	5.0	96.3 ± 1.7
	3	3.0	104.7 ± 5.6	5.0	99.4 ± 1.6
	4	5.0	98.9 ± 0.4	7.0	99.9 ± 1.0
	5	7.0	101.2 ± 0.6	7.0	99.7 ± 1.0

Table 5
Results obtained for the analysis of real samples using DASM matrix

Sample	β-T			γ-T		
	Added (mg g^{-1})	% Rec. ± SD (*Found, mg g^{-1})	Results of validation by NP_HPLC (mg g^{-1})	Added (mg g^{-1})	% Rec. ± SD (*Found, mg g^{-1})	Results of validation by NP_HPLC (mg g^{-1})
S1	0.0	–	3.0 ± 0.1	0.0	–	9.4 ± 0.9
	0.059	105.7 ± 4.2	–	0.059	92.0 ± 8.0	–
	0.098	102.4 ± 9.4	–	0.138	87 ± 15	–
S2	0.0	19.9 ± 3.5*	14.5 ± 0.9	0.0	1051 ± 28*	1051 ± 102
	0.254	105.7 ± 4.7	–	0.254	72.5 ± 2.7	–
	0.423	107.1 ± 9.9	–	0.593	78.1 ± 1.6	–
S3	0.0	29.5 ± 4.5*	33.7 ± 1.8	0.0	207.0 ± 9.0*	232 ± 13
	0.094	104 ± 14	–	0.094	100 ± 24	–
	0.156	112.0 ± 2.0	–	0.219	87 ± 18	–
S4	0.0	27.0 ± 5.0*	24.8 ± 0.2	0.0	18.0 ± 6.7*	18.5 ± 0.4
	0.099	114.0 ± 9.0	–	0.099	109 ± 11	–
	0.165	99 ± 22	–	0.231	80.5 ± 2.1	–
S5	0.0	848 ± 47*	928 ± 82	0.0	424 ± 24*	545 ± 86
	0.275	116 ± 12	–	0.275	96.1 ± 4.5	–
	0.459	152 ± 38	–	0.642	108.9 ± 3.1	–
S6	0.0	6.4 ± 2.3*	7.2 ± 0.1	0.0	114.4 ± 9.8*	121.7 ± 1.7
	0.192	97.1 ± 7.6	–	0.192	82 ± 19	–
	0.321	113.7 ± 2.3	–	0.448	98.0 ± 0.9	–
S7	0.0	3.1 ± 0.3*	4.0 ± 0.1	0.0	7.4 ± 0.2*	5.8 ± 0.1
	0.268	101.9 ± 4.4	–	0.268	99.1 ± 1.1	–
	0.446	111.8 ± 5.3	–	0.625	96.3 ± 9.6	–

4. Conclusions

A simple RP-HPLC method has been developed for the analysis of β-T and γ-T using a column of the most widely type used, C18. Due to the total overlapping of their chromatographic peaks, a PLS multivariate method has been used for the resolution. Different calibration matrices have been done and the best results have been obtained with the constructed with the derivative absorbance spectra obtained in the peak apex, as multivariate data. The method, once optimised, was applied to synthetic samples with good recoveries. Later, it was applied to different real samples. When the method was applied to the unspiked samples, the concentrations of β-T and γ-T found were in agreement with the ones found by a NP-HPLC validation method. In addition, for the spiked samples, the recoveries were, in general, between 97% and 116% for β-T and between 72% and 109% in the case of γ-T. In summary, the method allows the easy analysis of the tocopherols with the most commonly used HPLC technique.

Acknowledgements

The authors are grateful to the Ministerio de Ciencia y Tecnología (Proyect BQU 2005-02389) and Junta de Extremadura (Project 2PR03A073) for financial support.

References

Abidi, S. L. (1999). Reversed-phase retention characteristics of tocotrienols antioxidants. *Journal of Chromatography A, 844*(1–2), 67–75.

Abidi, S. L. (2000). Chromatographic analysis of tocol-derived lipid antioxidants. *Journal of Chromatography A, 881*(1–2), 197–216.

Abidi, S. L., & Mounts, T. L. (1997). Reversed-phase high-performance liquid chromatographic separations of tocopherols. *Journal of Chromatography A, 782*(1), 25–32.

Abidi, S. L., & Rennick, K. A. (2001). Capillary electrochromatographic evaluation of vitamin E-active oil constituents: tocopherols and tocotrienols. *Journal of Chromatography A, 913*, 379–386.

Albert, K. (1998). Correlation between chromatographic and physicochemical properties of stationary phases in HPLC: C30 bonded reversed-phase silica. *Trends in Analytical Chemistry, 17*(10), 648–658.

Beltrán, G., Aguilera, M. P., Del Río, C., Sánchez, S., & Martínez, L. (2005). Influence of fruit ripening process on the natural antioxidant content of Hojiblanca virgin olive oils. *Food Chemistry, 89*, 207–215.

Chen, M. H., & Bergman, C. J. (2005). A rapid procedure for analysing rice bran tocopherol, tocotrienols and gamma-oryzanol contents. *Journal of Food Composition and Analysis, 18*(2–3), 139–151.

Codex Alimentarius Commission. (1987). General principles for the addition of essential nutrients to foods, Alinorm 87/26, Appendix 5, Food and Agriculture Organization, Rome.

Comas, E., Gimeno, R. A., Ferré, J., Marcé, R. M., Borrull, F., & Rius, F. X. (2004). Quantification from highly drifted and overlapped chromatographic peaks usig second-order calibration methods. *Journal of Chromatography A, 1035*, 195–202.

Cunha, S. C., Amaral, J. S., Fernandes, J. O., & Oliveira, M. B. P. P. (2006). Quantification of tocopherols and tocotrienols in Portuguese olive oils using HPLC with three different detection systems. *Journal of Agricultural and Food Chemistry, 54*, 3351–3356.

Demir, C., & Cetin, M. (1999). Determination of tocopherols, fatty acids and oxidative stability of pecan, walnut and sunflower oils. *Deutsche Lebensmittel Rundschau, 95*(7), 278–282.

Eastment, H. T., & Krzanowski, W. J. (1982). Cross-validatory choice of the number of components from a principal component analysis. *Technometrics, 24*, 73–77.

Escandar, G. M., Damiani, P. C., Goicoechea, H. C., & Olivieri, A. C. (2006). A review of multivariate calibration methods applied to biomedical analysis. *Microchemical Journal, 82*, 29–42.

Unites States Food and Drug Administration, Code of Federal Regulations, Title 21, Part 104.5, Food and Drug Administration, Washington DC, 1987.

Fraga, C. G., & Corley, C. A. (2005). The chemometric resolution and quantification of overlapped peaks from comprehensive two-dimensional liquid chromatography. *Journal of Chromatography A, 1096*, 40–49.

Gama, P., Casal, S., Oliveira, B., & Ferreira, M. A. (2000). Development on an HPLC/diode-array/fluorimetric detector method for monitoring tocopherols and tocotrienols in edible oil. *Journal of Liquid Chromatography and Related Technology, 23*(19), 3011–3022.

Garrido Frenich, A., Martínez Galera, M., Martínez Vidal, J. L., & Gil García, M. D. (1996). Partial least-squares and principal component regression of multi-analyte high-performance liquid chromatography with diode-array detection. *Journal of Chromatography A, 727*, 27–38.

Geladi, P., & Kowalski, B. R. (1986). Partial-least squares regression: a tutorial. *Analytical Chimica Acta, 185*, 1–17.

Gil García, M. D., Garrido Frenich, A., Martínez Vidal, J. L., Martínez Galera, M., Muñoz de la Peña, A., & Salinas, F. (1997). Resolution of overlapping peaks in HPLC with diode array detection by application of partial least squares calibration to cross-sections of spectrochromatograms. *Analytical Chimica Acta, 348*, 177–185.

Gimeno, E., Castellote, A. I., Lamuela-Raventos, R. M., de la Torre, M. C., & Lopez-Sabater, M. C. (2000). Rapid determination of vitamin E in vegetable oils by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 881*(1–2), 251–254.

Gliszczynska, A., & Sikorska, E. (2004). Simple reversed-phase liquid chromatography method for determination of tocopherols in edible plants oils. *Journal of Chromatography A, 1048*(2), 195–198.

Goffmann, F. D., Velasco, L., & Thies, W. (1999). Quantitative determination of tocopherols in single seed of rapeseed (Brassica napus L.). *Fett/Lipid, 101*(4), 142–145.

Hakansson, B., Jagerstad, M., & Oste, R. (1987). Determination of vitamin E in wheat products by HPLC. *Journal of Micronutrients Analysis, 3*(4), 307–318.

Handbook of Food Analysis (1996). Nollet, Leo M.L. (Ed.) Vol. 1. New York, Basel: Marcel Dekker Inc.

Helland, I. S. (2001). Some theoretical aspects of partial least squares regression. *Chemometrics and Intelligent Laboratory Systems, 58*, 97–107.

International Union of Pure and Applied Chemistry (1988). Pure Applied Chemistry, 60, 877–892.

Kornsteiner, M., Wagner, K.-H., & Elmadfa, I. (2006). Tocopherols and total phenolics in 10 different nyt types. *Food Chemistry, 98*, 381–387.

Lercker, G., & Rodriguez-Estrada, M. T. (2000). Chromatographic analysis of unsaponifiable compounds of olive oils and fat-containing foods. *Journal of Chromatography A, 881*(1–2), 105–129.

Marsin Sanagi, M., See, H. H., Wan Ibrahim, W. A., & Abu Naim, A. (2005). Determination of carotene, tocopherols and tocotrienols in residue oil from palm pressed fiber using pressurized liquid extraction-normal phase liquid chromatography. *Analytica Chimica Acta, 538*, 71–76.

Martínez Galera, M., Martínez Vidal, J. L., Garrido Frenich, A., & Gil García, M. D. (1996). Determination of cypermethrin, fenvalerate and *cis*- and *trans*-permethrin in soil and groundwater by high-performance liquid chromatography using partial least-squares regression. *Journal of Chromatography A, 727*, 39–46.

Pyka, A., & Sliwiok, J. (2001). Chromatographic separation of tocopherols. *Journal of Chromatography A, 935*(1–2), 71–76.

Richheimer, S. L., Kent, M. C., & Bernart, M. W. (1994). Reversed-phase high-performance liquid chromatographic method using a pentafluorophenyl bonded phase for analysis of tocopherols. *Journal of Chromatography A, 677*(1), 75–80.

Rozzolo, A., & Polesello, S. (1992). Chromatographic determination of vitamins in foods. *Journal of Chromatography A, 624*(1–2), 103–152.

Sander, L. C., Sharpless, K. E., & Pursch, M. (2000). C30 stationary phases for the analysis of food by liquid chromatography. *Journal of Chromatography A, 880*, 189–202.

Schieber, A., Marx, M., & Carle, R. (2002). *Simultaneous determination of carotenes and tocopherols in ATBC drinks by high-performance liquid chromatography, 76*, 357–362.

Strohschein, S., Pursch, M., Lubda, D., & Albert, K. (1998). Shape selectivity of C30 phases for RP-HPLC separation of tocopherol isomers and correlation with MAS NMR data from suspended stationary phases. *Analytical Chemistry, 70*(1), 13–18.

Tan, B., & Brzuskiewicz, L. (1989). Separation of tocopherol and tocotrienol isomers using normal- and reversed-phase liquid chromatrography. *Analytical Biochemistry, 180*(2), 368–373.

Thomas, E. V., & Haaland, D. M. (1988). Partial least-squares methods for spectral analyses. I. Relation to other quantitative calibration methods and the extraction of qualitative information. *Analytical Chemistry, 60*(11), 1193–1202.

Van Zomeren, P. V., Metting, H. J., Coenegracht, P. M. J., & de Jong, G. J. (2005). Simultaneous resolution of overlapping peaks in high-performance liquid chromatography and micellar electrokinetic chromatographywith diode array detecting using augmented iterative target transformation factor analysis. *Journal of Chromatography A, 1096*, 165–176.

Wold, S., Sjöström, M., & Eriksson, L. (2001a). PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems, 58*, 109–130.

Wold, S., Trygg, J., Berglund, A., & Antti, H. (2001b). Some recent development in PLS modelling. *Chemometrics and Intelligent Laboratory Systems, 58*, 131–150.