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On-line HPLC Detection of Tocopherols and Other Antioxidants through the Formation of a Phosphomolybdenum Complex

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An on-line method to detect and quantify antioxidant species in complex extracts has been developed as a combination of conventional HPLC separation and a postcolumn reaction with phosphomolybdenum reagent at acidic pH. Sample analytes were chromatographed by HPLC, and the postcolumn formation of a phosphate/Mo(V) complex was detected at 598 nm with an on-line absorbance detector. An optimized instrumental system was set up using pure α-tocopherol, and it was successfully tested with complex food extracts including lettuce, tomato, red pepper, and soybean seed, where several tocopherols and carotenoids were identified. A potential application of this detection method to quantitatively determine different antioxidants was considered, and a specific application to the determination of tocopherols was developed. The new method was characterized with respect to linearity interval, repeatability, and reproducibility, the quantitative results obtained were validated by comparison with a conventional HPLC method with fluorometric detection, and it was applied to the determination of tocopherols in different foods. The results suggest that the proposed on-line HPLC method can be a powerful instrument for the detection, purification, and characterization of natural antioxidants.

KEYWORDS: Antioxidants; tocopherols; on-line detection; HPLC (high-performance liquid chrmatography); molybdenum; phosphate

INTRODUCTION

Numerous environmental factors can induce the production of free radicals, namely, radiations, smokes, and pesticides (*1*, *2*). When these compounds accumulate above a critical level, they may become dangerous and unhealthy for living organisms. Oxidative processes triggered by free radicals are also responsible for food deterioration, causing the loss of nutritional value and unwanted changes of odor, taste, texture, consistency, and appearance. Food technology uses two strategies to fight these problems, a passive one based on the avoidance of oxidation-causing factors and an active strategy based on the addition of antioxidants (*3*, *4*).

Tocopherols and other phytochemicals isolated from herbs, fruits, and vegetables are the major focus of the studies on natural antioxidants. Beginning with the early works of Chipault et al. (*5*−*7*), there are many studies on the antioxidant capacity of plant extracts with potential applications as preservatives in foods, cosmetics, and pharmaceuticals (*8*−*14*). A number of methods have been developed for the determination of the antioxidant capacity of pure compounds or complex mixtures to be used in the food, drug, and cosmetic industries (*15*−*21*). Most of them, however, were not designed for extensive and rapid screenings of potential sources of antioxidants, and only recently has a method been developed for the on-line detection of free radical scavenging compounds after HPLC (*22*).

A spectrophotometric method based on the formation of a blue-green complex of phosphate and Mo(V) was proposed by Fiske and Subbarrow (*23*) to determine inorganic phosphate. This method was modified later by Chen et al. (*24*). The requirement of a reducing agent such as ascorbic acid in the reagent mixture, to obtain Mo(V) from Mo(VI), suggested the modification of this method to determine any reducing species. We developed an easy and fast spectrophotometric method to estimate the total antioxidant capacity of aqueous and nonaqueous extracts, and we applied the method to the specific determination of different water-soluble and lipid-soluble antioxidants including vitamin C, reduced glutathione (GSH), butylated hydroxytoluene (BHT), and vitamin E (*21*). The problems related to the limited selectivity of spectrophotometric batch methods motivated us to try to adapt our phosphomolybdenum method to enable its application as an on-line method to monitor the antioxidant capacity of the eluant in a chromatographic system such as HPLC.

The present paper responds to our interest in the plant biosynthesis of tocopherols and in the identification of new sources of natural antioxidants. We propose a combination of HPLC with postcolumn formation of a phosphomolybdenum complex as a powerful method to identify new sources of known

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antioxidants as well as to detect and determine the concentration of putative antioxidants in complex mixtures from any source.

MATERIALS AND METHODS

Chemicals. Ammonium molybdate, butylated hydroxytoluene (BHT), α-, γ-, and δ-tocopherol, and α-tocopherol acetate were obtained from Sigma (St. Louis, MO). HPLC grade methanol and hexane and analytical grade sodium phosphate and sulfuric acid were obtained from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

Instrumentation. The molecular absorption spectra and absorbance at specific wavelengths were recorded with a DU-62 UV−visible spectrophotometer (Beckman, Palo Alto, CA) equipped with quartz cells of 1 cm light path. The HPLC equipment was a System Gold (Beckman, Palo Alto, CA) consisting of a 200 μL injection valve (model 7125 from Rheodyne, Cotati, CA), a 126 solvent delivery module, and a 168 diode array detector. Alternatively, a JASCO FP-920 fluorescence detector (Jasco, Tokyo, Japan) was used with excitation at 296 and emission at 340 nm. Reversed phase (RP) chromatography was run on a stainless steel analytical column packed with Spherisorb ODS2 (25 cm × 4.6 mm i.d.) from Teknocroma (Barcelona, Spain). For tocopherols determination, chromatography was developed with a linear gradient of 90−100% methanol in water (v/v) at a flow rate of 2 mL/min.

The postcolumn reaction system was set up and coupled to the HPLC system. A 110B reagent delivery module, a pulse damper, and a low dead volume tee were used for mixing the phosphomolybdenum reagent (0.6 M sulfuric acid, 32 mM sodium phosphate, and 4 mM ammonium molybdate in water) with the HPLC column eluant. A linear reactor was developed with a stainless steel tubing (30 cm × 0.01 mm i.d.), and the reactor temperature was controlled with a water bath thermostat. All of these components were acquired from Beckman.

Sample Preparation. Stock solutions of α-, γ-, and δ-tocopherol and α-tocopherol acetate were prepared in hexane, and exact concentrations were determined spectrophotometrically on the basis of their absorption coefficients.

Soybean seeds (*Glycine max* L. var. Williams) were kindly provided by Eurosemillas (Córdoba, Spain). Tomato fruits, lettuce, red peppers, peas, and sunflower seeds were acquired from a local market in Córdoba. Food samples were frozen under liquid nitrogen and ground with a pestle and mortar to a fine powder. A volume of hexane (4 mL/g) was added, and the suspension was homogenized, transferred to glass tubes, and shaken for 1 h at 4 °C in the dark. When required, the extraction solvent was spiked with α-, γ-, and δ-tocopherol or α-tocopherol acetate. After centrifugation at 6000*g* for 10 min, the supernatant fraction was transferred to new tubes and kept at 4 °C in the dark for immediate use. Hexanic sample extracts were desiccated and redissolved in methanol prior to HPLC analysis.

Detection of Antioxidant Species. Sample extracts of 200 μL containing putative antioxidant species were chromatographed by RP-HPLC at 2 mL/min, and the eluant was mixed with the phosphomolybdenum reagent at a flow of 1 mL/min. After formation of the phosphate/Mo(V) complex along a linear reactor heated to 95 °C, peaks representing antioxidant species were identified with a diode array detector at 598 nm.

Determination of Tocopherols. An HPLC method with minor modifications was used (*25*). A gradient of methanol in water was applied to separate 200 μL samples containing α-, γ-, and δ-tocopherol by RP chromatography on a Spherisorb ODS2 column (25 cm × 4.6 mm i.d.). The mobile phase was 90% methanol in water at time zero. A linear gradient from 90 to 100% methanol in water was applied in 2 min, and 100% methanol was kept during 10 min. Initial conditions were recovered in 4 min. Peaks corresponding to the different isomers were identified directly by fluorescence, and their identity was confirmed by their absorbance spectra. Alternatively, tocopherols were determined indirectly at 598 nm after postcolumn formation of the phosphate/Mo(V) complex. Either with the HPLC fluorescence method or when the proposed method was applied, tocopherols were quantitated with the following formula, on the basis of the response factor obtained for each isomer:

$$XT = RFT \times AT \times (XIS/AIS)/RFIS$$

where XT is the amount of tocopherol, RFT is the response factor for the tocopherol, AT is the peak area of tocopherol, XIS is the amount of internal standard, AIS is the peak area of internal standard, and RFIS is the response factor of the internal standard (*9*).

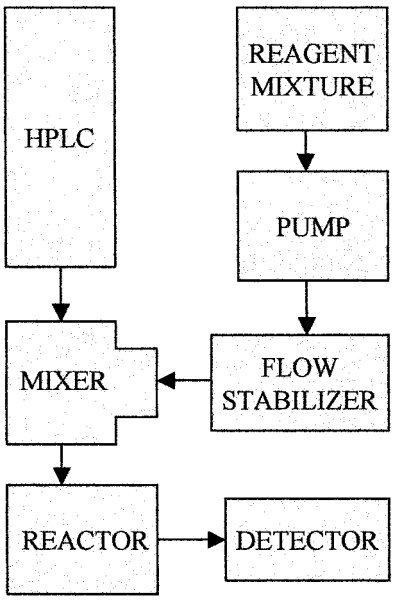

Figure 1. Diagram of a postcolumn reaction system for the on-line detection of antioxidants coupled to HPLC.

Method Validation. To determine tocopherol recovery from food samples, these were supplemented either with different isomers or with α-tocopherol acetate as an internal standard, and tocopherols were determined by means of the proposed method and by an HPLC method with fluorometric detection (*25*). The reference value (100% recovery) was assigned to a sample extract that was supplemented with the analyte and the internal standard just before determination. Linearity was established by supplementing samples with the analyte and the internal standard using at least five different concentrations and four replicates, as proposed by the NCCLS guideline (*26*). Within-day and day-to-day reproducibilities were evaluated by analyzing seven samples with different concentrations on three different occasions. The detection limit was estimated by analyzing samples spiked with known quantities of α-, γ-, and δ-tocopherol.

RESULTS AND DISCUSSION

Postcolumn derivatization is commonly used in combination with HPLC to increase sensitivity or selectivity in the detection of many analytes. Our approach to this technique was aimed at the selective detection of tocopherols and other antioxidants present in a complex extract by the on-line monitoring of their reducing power after their separation by any liquid chromatographic system. We set up a postcolumn reaction system coupled to conventional HPLC, as described in **Figure 1**. The phosphomolybdenum reagent was supplied by a pump, and its flow was stabilized before being mixed with the eluant coming from a conventional HPLC system. The reaction mixture was later pumped through a thermostated linear reactor, where the blue-green phosphomolybdenum complex was formed before being detected with an on-line absorbance detector at 598 nm. This wavelength was chosen as a compromise option to avoid any possible interference at lower wavelengths (i.e., chlorophylls). This wavelength also facilitates detection, because on-line absorbance detectors seldom reach as far as 695 nm, the major

Table 1. Optimal Conditions for the On-line Detection of α-Tocopherol by the Proposed Method after RP-HPLC[a]

variable	interval studied	optimum
reagent input flow (mL/min)	0.3–2.0	1.0
sodium phosphate (mM)	32×10^{-3}–32	32.0
ammonium molybdate (mM)	1.3–8.0	4.0
sulfuric acid (M)	0.06–1.2	0.6
reaction temperature (°C)	27–95	95

[a] Samples containing 10 nmol of α-tocopherol were chromatographed by RP-HPLC with a gradient of methanol/water at a flow of 2 mL/min.

absorbance maximum of the phosphomolybdenum complex, the method sensitivity being highest at this wavelength (*21*).

All of the relevant parameters of the process were studied to achieve an optimized method. These parameters included HPLC flow and phosphomolybdenum reagent input flow, composition of the reaction mixture, and reaction temperature. RP chromatography of samples containing a standard of α-tocopherol was used for this purpose, and the yield of the formation of blue-green phosphomolybdenum complex was estimated. The relative flows of HPLC mobile phase and phosphomolybdenum reagent showed an optimum that reflects both the resolution needs for the chromatography and the sensitivity of the detection method, which in turns depends on the amount of derivatization reagent mixed with the eluant. A flow increase from 0.3 to 1 mL/min caused a 40% higher yield of phosphomolybdenum complex formation, whereas the observed yield showed a 40% reduction at 2 mL/min.

The compatibility of several organic solvents with the formation of the phosphomolybdenum complex was previously tested in batch applications (*21*), and none of the inorganic or organic solvents that are commonly used for extraction and chromatography were found to interfere significantly; these include phosphate buffers, acetonitrile, methanol, ethanol, dimethyl sulfoxide, dimethylformamide, and hexane. We did optimize the composition of the phosphomolybdenum reagent, and a saturation effect was found for ammonium molybdate and sodium phosphate. Sulfuric acid concentration, however, did not show a saturation effect but an optimum that is probably related to a pH optimum for the formation of phosphomolybdenum complex: a 60% lower yield was observed with 1.2 M sulfuric acid when compared with 0.6 M. The reaction of phosphomolybdenum complex formation was temperature-dependent, and a 30% increase was found between 27 and 95 °C. **Table 1** contains a summary of this analysis with the optimal conditions that would be used for further experiments. **Figure 2** shows a typical chromatogram of a sample containing a standard of α-tocopherol.

Once the method was set up and all of the relevant variables optimized, it was applied to the detection of tocopherols and other antioxidant species in complex mixtures such as those obtained from food samples. **Figure 3** shows typical HPLC chromatograms with multiple peaks of absorbance at 595 nm. The correlation of those peaks (absorbance at 595 nm) with the formation of phosphomolybdenum complex due to the presence of reductants (antioxidant species) in the eluate was checked by monitoring the absorbance spectra of the peaks with a diode array detector. The spectra corresponded with those of the phosphomolybdenum complex (not shown). To further demonstrate that the peaks of absorbance at 595 nm really correlate with the presence of antioxidant species, we identified some of those putative antioxidants, as indicated in **Figure 3**. Tocopherol and carotenoid peaks were assigned by their

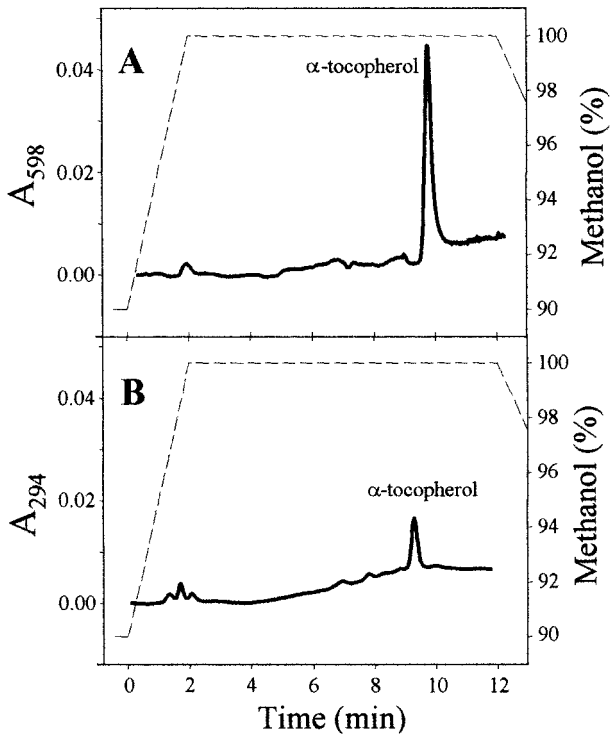

Figure 2. Detection of α-tocopherol by the proposed method after RP-HPLC. A 20 nmol sample of α-tocopherol was subjected to RP-HPLC using a gradient of methanol/water (dash line) and detected at 598 nm after postcolumn formation of phosphomplybdenum complex (A). A duplicate sample was chromatographed under the same conditions and detected directly by its absorbance at 294 nm (B).

coelution with standards and by their spectra, which were identical to the spectra of pure standards (not shown).

The literature on procedures for antioxidant detection and quantitation is very abundant. However, most methods are designed for individual sample analysis, making their application to the screening of food materials in search of new antioxidants very hard and time-consuming. Several of these techniques can be applied to the evaluation of total antioxidant capacity, that is, the chemiluminescence assay (*20*) and ESR spectroscopy (*19*). These methods have the disadvantage of requiring sophisticated instruments that make the assays very expensive. Some of them are based on chemical reactions that are poorly understood, making them less reliable, and there are also a number of bioassays that are even more complex, time-consuming, and expensive (*27*). The methods commonly used to determine total antioxidant capacity (*16*) have the drawback of not being applicable to the continuous on-line detection of reducing species after their separation by conventional chromatographic techniques. An on-line method to measure the antioxidant capacity can be very useful for monitoring the purification of potential antioxidant compounds.

After the optimal conditions were established for the on-line detection of antioxidant species such as tocopherols and carotenoids by the proposed method, we studied its potential application as a quantitative method for the determination of specific antioxidants after their separation by HPLC. Standards of α-tocopherol (vitamin E) were chromatographed, and the on-line phosphomolybdenum method was applied to determine its linearity range and precision, two parameters that are essential to validate a quantitative method. We performed three groups of measurements with three different groups of α-tocopherol standards within the interval 0.1−100 nmol. Repeatability

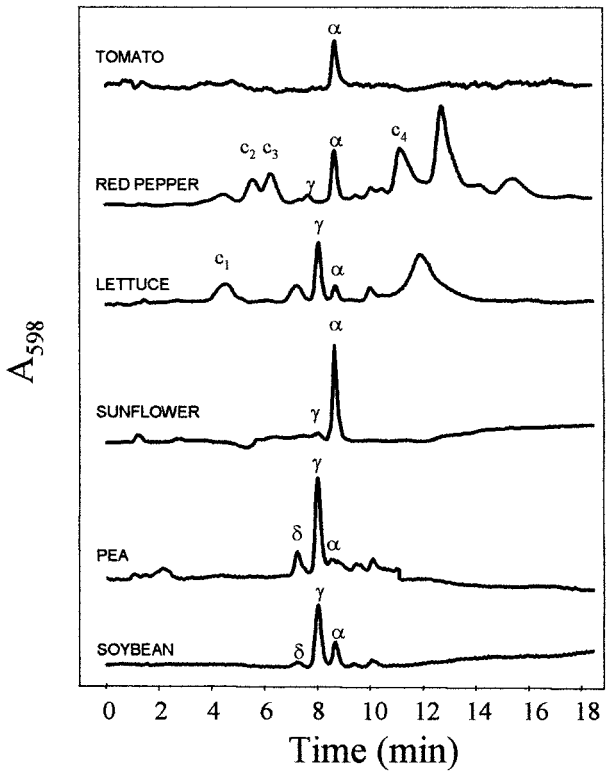

Figure 3. On-line detection of antioxidants in food samples. The indicated samples were extracted with hexane, and after changing the solvent by methanol, they were chromatographed by RP-HPLC. The presence of several antioxidants in the extracts was detected on-line by monitoring the postcolumn formation of phosphomolybdenum complex (A_{598nm}) at 95 °C. Some of the peaks were identified as tocopherol isomers (α, γ, δ) and carotenoids (c_1, c_2, c_3, c_4).

Table 2. Validation of the Proposed Method: Comparative Analysis of the Precision of α-Tocopherol Determination by the Proposed Method and by an HPLC Method with Fluorescence Detection[a]

	amount of α-tocopherol detected (nmol)	
sample	proposed method	HPLC fluorescence
1	5.35	5.18
2	5.90	4.95
3	5.42	5.67
4	5.15	4.86
5	5.24	5.28
6	5.01	5.20
7	5.28	5.06
8	4.98	4.94
9	4.83	5.16
10	5.34	5.08
11	4.88	4.91
standard deviation	$SD_1 = 0.27$	$SD_2 = 0.24$
mean	$X_1 = 5.25$	$X_2 = 5.09$
degrees of freedom	$n_1 + n_2 - 2 = 20$	
Student's t	1.43	
critical value of Student's t (95% confidence level)	2.09	

[a] The actual amount of α-tocopherol present in the samples was 5 nmol.

(within-run precision) and reproducibility (between-run precision) were calculated by regression analysis and expressed as relative standard deviation (RSD, percent) of the slope of peak area versus nanomoles of α-tocopherol curves. RSD values of 0.7, 1.2, and 1.7% were estimated when the postcolumn reaction

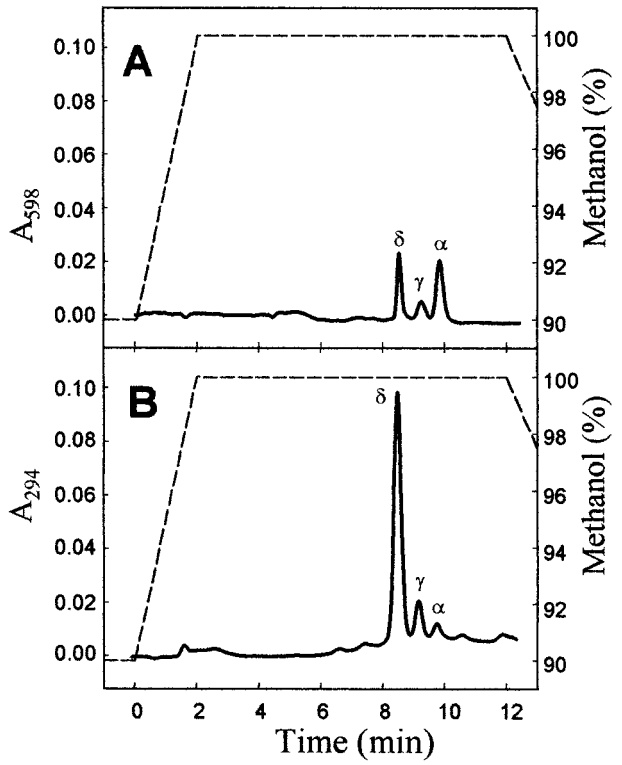

Figure 4. Simultaneous determination of tocopherol isomers by the proposed method after separation by RP-HPLC. A sample containing 20 nmol of α-tocopherol, 100 nmol of γ-tocopherol, and 1000 nmol of δ-tocopherol was subjected to RP-HPLC using a gradient of methanol/water (dash line) and detected at 598 nm after postcolumn formation of phosphomplybdenum complex (A) at 95 °C. A duplicate sample was chromatographed under the same conditions, and the different tocopherol isomers were detected directly by their absorbance at 294 nm (B).

Table 3. Summary of Statistical Parameters of the Proposed Method Applied to the Determination of Tocopherols

analyte	reaction temp (°C)	calibration curve[a]	linearity interval (nmol)	r^2	RSD[b] (%)
α-tocopherol	27	$y = 0.0 + 0.48x$	1.0–20.0	0.988	1.8
	95	$y = -0.1 + 0.60x$	0.5–20.0	0.998	1.7
γ-tocopherol	27	$y = -2.0 + 0.04x$	50–300	0.920	25.0
	95	$y = 0.4 + 0.51x$	0.5–25	0.995	1.8
δ-tocopherol	27	$y = -4.0 + 0.015x$	250–500	0.810	26.7
	95	$y = 1.2 + 0.049x$	10–200	0.985	0.4

[a] Calibration curves represent peak area vs amount of analyte in nmol. Calibration curves were obtained with 18 different points at 27 °C and with 21 points at 95 °C. [b] RSD: relative standard deviation of the slope.

was performed at 27 °C. The overall linear regression relating peak area versus nanomoles of α-tocopherol of 21 values at 95% confidence level was $y = 0.0 + 0.48x$, with an $r^2 = 0.988$ (reproducibility). Linearity was observed between 1 and 20 nmol.

To validate the proposed method, we compared its precision with that of a method of determination of α-tocopherol by RP-HPLC and fluorescence detection. A number of samples containing α-tocopherol were determined by both methods, and an overall standard deviation (SD) was calculated from the standard deviations associated with each method (SD_1 and SD_2). As described in **Table 2**, Student's t was smaller than the critical value $t_{0.95}$, which means that, with regard to their precision, there

Table 4. Comparative Analysis of Tocopherols in Different Food Samples

	proposed method			HPLC fluorescence		
sample	α-tocopherol	γ-tocopherol	δ-tocopherol	α-tocopherol	γ-tocopherol	δ-tocopherol
soybean seed	109 ± 14	282 ± 1	82 ± 5	69.1 ± 0.9	274 ± 3	39 ± 1
red pepper pericarp	2.6 ± 0.1	0.3 ± 0.0	nd[b]	3.4 ± 0.1	0.3 ± 0.0	nd
lettuce leaf	0.5 ± 0.1	2.1 ± 0.0	nd	0.5 ± 0.0	3.4 ± 0.1	nd
tomato pericarp	0.4 ± 0.0	nd	nd	0.5 ± 0.0	0.1 ± 0.0	nd

[a] Abundance of each isomer is expressed as nmol/g of sample fresh weight. Every sample was determined in triplicate, and mean values are given accompanied by their respective standard deviations. [b] nd, not detected.

are no significant differences between the proposed method and the HPLC/fluorescence method at a 95% confidence level.

Our method was also tested with other vitamin E isomers (γ- and δ-tocopherol), and calibration curves were obtained for the simultaneous quantitation of all three isomers after separation of standard mixtures (**Figure 4**). A study of linearity interval, repeatability, and reproducibility of the method when applied to these two vitamin E isomers revealed that sensitivity, repeatability, and reproducibility are improved by performing the postcolumn synthesis of the phosphomolybdenum complex at high temperature (**Table 3**). It is worth noticing that the effect of temperature on the yield of phosphomolybdenum complex formation is related to the nature of the antioxidant to be determined. This fact could be used to improve selectivity in the detection of strong antioxidants such as α-tocopherol. As previously described (*21*), the phosphomolybdenum method enables the determination of α-tocopherol in the presence of other weaker antioxidants such as BHT, when the reaction is performed at low temperature (27 °C). Selectivity is actually the most important advantage of our method over other methods based on UV or even fluorescence detection.

We went a step farther to validate the proposed method as a quantitative assay and applied it to the determination of tocopherols in real food samples such as soybean seeds, tomatos, red peppers, and lettuce. We set up the monophasic extraction procedure described under Materials and Methods and, after replacing the hexane by methanol, we determined the concentration of tocopherols in the samples by means of the proposed method and by an HPLC method with fluorescence detection. The method was validated with respect to linearity and recovery. Samples were spiked with α-, γ-, and δ-tocopherol, and the recovery was estimated. Measurements were performed three times with several days' interval on seven samples within the range of 1−100 nmol. The hexane extraction procedure used in our method gave a good recovery of all the tocopherols analyzed (95−100%). Linearity was found within the ranges of 0.5−20 nmol for α- and γ-tocopherol and 10−200 nmol for δ-tocopherol.

As shown in **Table 4**, our on-line method was also compared with an HPLC method for the determination of tocopherols in the same seed, fruit, and vegetable samples. The results obtained by both methods were very similar and confirm that the proposed method is suitable for the quantitative determination of tocopherols in food samples. The higher selectivity of the method when the postcolumn derivatization reaction is developed at 27 °C (the temperature at which other weaker antioxidants are not detected) makes the phosphomolybdenum reagent a good alternative for the determination of vitamin E (α-tocopherol) in a variety of samples that are relevant for the agriculture and food industry, that is, water-soluble or lipid-soluble plant extracts and vegetal oils as well as pharmaceutical and cosmetic preparations or human serum.

Our results clearly suggest that the phosphomolybdenum reagent can be used for the on-line quantitative detection of tocopherols and that selective methods can be developed in the future for the determination of not only tocopherols but also other strong antioxidants. This kind of on-line detection method can be a powerful alternative to other more laborious and expensive methods commonly used in the search for new antioxidants (*15*, *17*, *28*, *29*).

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