Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E[1]

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A spectrophotometric method has been developed for the quantitative determination of antioxidant capacity. The assay is based on the reduction of Mo(VI)to Mo(V) by the sample analyte and the subsequent formation of a green phosphate/Mo(V) complex atacidic pH. The method has been optimized and characterized with respect to linearity interval, repetitivity and reproducibility, and molar absorption coefficients for the quantitation of several antioxidants, including vitamin E. The phosphomolybdenum method, in combination with hexane monophasic extraction, has also been adapted for the specific determination of vitamin E in seeds. The results obtained with the proposed method were validated by comparison with a standard HPLC method. The phosphomolybdenum method is routinely applied in our laboratory to evaluate the total antioxidant capacity of plant extracts and to determine vitamin E in a variety of grains and seeds, including corn and soybean.

There is an increasing interest in the use and measurement of antioxidants in food, pharmaceutical, and cosmetic industries. This interest is rooted in the cumulative evidence that connects oxidative stress with numerous degenerative disorders ranging from premature aging, prostaglandin-mediated inflammatory processes, cancer, and a long series of diseases in which free radicals are implicated (1, 2). In addition, many states implement very rigorous regulations on the use of food preservatives, so that they only allow the use of natural antioxidants. Basically, these include vitamin C (ascorbic acid), vitamin E (tocopherols and tocotrienols), and relatively complex extracts from a number of plant species (*Rosmarinus officinalis, Nerium oleander,* and *Myrtus comunis*).

The early works by Chipault *et al.* (3–5) were the precursors of many studies on the antioxidant capacity of a number of plant extracts with potential applications as preservatives in the food, cosmetics, and pharmaceutical industries (6–12). The current interest of our laboratory in the biosynthesis of tocopherols in plants and in the identification of alternative sources of natural antioxidants prompted us to develop a method for the evaluation of water-soluble and fat-soluble antioxidant capacity. The phosphomolybdenum method that we propose in this article is now systematically used by us in extensive screenings of samples of very different origins and composition in our search for natural sources of vitamin E and other powerful antioxidants.

MATERIALS AND METHODS

Chemicals and reagents. Ammonium molybdate; ascorbic acid; reduced glutathione; butyl hydroxytoluene (BHT);[3] α-, γ-, and δ-tocopherol; and the internal standard α-tocopherol acetate were obtained from Sigma (St. Louis, MO). HPLC grade methanol, hexane, ethanol, dimethyl sulfoxide, and analytical grade sodium phosphate and sulfuric acid were from Merck (Darmstadt, Germany). All other chemicals 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 chromatograph was a System Gold (Beckman, Palo Alto, CA) consisting of a 20-µl injection valve (Model 7125 from Rheodyne, Cotati, CA), a 126 solvent delivery module, and a 168 diode array detector with simultaneous detection at 260 and 295 nm. Alternatively, a JASCO FP-920 fluorescence detector (Jasco, Tokyo, Japan) a JASCO FP-920 fluorescence detector (Jasco, Tokyo, Japan)

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[3] Abbreviation used: BHT, butyl hydroxytoluene.

was used with excitation at 296 and emission at 340 nm. Reversed-phase chromatography was run on a stainless steel analytical column packed with Spherisorb ODS2 (25 cm 3 4.6 mm i.d.) from Teknocroma (Barcelona, Spain). For tocopherols determination, elution was performed with a mixture of ethanol: water (96:4, v/v) at a flow rate of 2 ml/min.

Sample preparation. Stock solutions of ascorbic acid and reduced glutathione were prepared in water just before use. Stock solutions of BHT; α-, γ-, and δ-tocopherol; and α-tocopherol acetate were prepared in ethanol or hexane and kept at 220°C. Exact concentrations were determined spectrophotometrically on the basis of the absorption coefficients from the literature.

Seed samples (corn and soybean seeds) were frozen under liquid nitrogen and ground with pestle and mortar to a fine powder. A volume of solvent (1 ml/g) was added, and the suspension was homogenized, transferred to polypropylene tubes, and shaken for 1 h at room temperature in the dark. When required, the extraction solvent was supplemented with BHT (1 mg/ ml) or spiked with α-, γ-, and δ-tocopherol or the internal standard α-tocopherol acetate. After centrifugation at 10,000*g* for 15 min, the supernatant was transferred to new tubes. The pellet was resuspended and homogenized in another volume of solvent and centrifuged once more. The supernatant was combined with the first extract and kept at 4°C until immediate use in HPLC or spectrophotometric determinations. The rest of the samples were subjected to the same treatment, except that the freezing and grinding processes were omitted.

Evaluation of total antioxidant capacity. An aliquot of 0.1 ml of sample solution containing a reducing species (in water, methanol, ethanol, dimethyl sulfoxide, or hexane) was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. For samples of unknown composition, lipidsoluble and water-soluble antioxidant capacities were expressed as equivalents of α-tocopherol and ascorbic acid, respectively.

Determination of vitamin E. A sample volume of 0.1 ml of hexane extract of seeds containing vitamin E was mixed in a test tube with 1 ml of reagent solution and incubated at 37°C for 90 min with vigorous shaking. Absorbance of the aqueous phase at 695 nm was measured against the appropriate blank. A typical blank contained 1 ml of reagent solution and 0.1 ml of pure hexane, and it was incubated under the same conditions as the samples.

Quantitation. Quantitation of vitamin E and other reducing species was based on the molar absorption coefficient of the phosphomolybdenum complex. Linearity was evaluated by obtaining calibration curves with multiple standards of the appropriate reducing species in parallel with the samples. Standardization of vitamin E determination in seeds was based on the analysis of samples spiked with known quantities of δ-tocopherol.

Method validation. The recovery of vitamin E from seeds was determined by supplementing the samples with the different vitamin E isomers or α-tocopherol acetate as internal standard and applying both the proposed method and a standard HPLC assay (13). The reference value (100% recovery) was assigned to an extract that was supplemented with the analyte and the internal standard just before determination. The linearity of the method was evaluated by supplementing samples with the analyte and the internal standard using at least 5 concentrations and 4 replicates, as proposed by the NCCLS guideline (14). The within-day and day-to-day reproducibility was evaluated by analyzing seven samples with different concentrations on three different occasions. The limit of quantitation was estimated by analyzing samples spiked with known amounts of α-tocopherol.

RESULTS AND DISCUSSION

The formation of a green-colored complex of phosphate and Mo(V) was presented by Fiske and Subbarrow (15) as the basis of a spectrophotometric method to determine inorganic phosphate. This method was later revised and modified by Chen *et al.* (16). The requirement of a reducing agent to produce Mo(V) from the Mo(VI) supplied with the reagent mixture suggested to us the modification of this method for the determination of any reducing species.

A preliminary study was done to optimize the formation and determination of the green phosphomolybdenum complex with α-tocopherol as the reducing species. As shown in Fig. 1, the UV/visible spectrum of the phosphomolybdenum complex had a characteristic maximum at 695 nm. This absorbance maximum was subsequently used for spectrophotometric determinations.

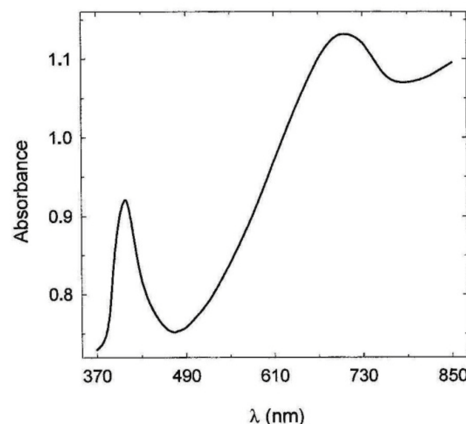

FIG. 1. UV/visible spectrum of the green phosphomolybdenum complex. The spectrum was recorded after subtraction of the appropriate blank made with the reaction mixture.

The kinetics of the formation of the phosphomolybdenum complex was monitored at different temperatures with α-tocopherol as the reducing agent (Fig. 2). A reaction time of around 90 min seemed to be enough to achieve a maximum in the production of phosphomolybdenum complex. Though the formation of the green complex could proceed at room temperature, this reaction showed a positive dependence on temperature, so that a significant yield increase was observed at higher temperatures. Stability of the green complex at room temperature (25° C) was also monitored. No changes in the spectrum of the complex were seen, and only a negligible increase of absorbance at 695 nm occurred after several days (not shown).

We analyzed the compatibility of several organic solvents with the formation of the phospholybdenum complex. Methanol, ethanol, dimethyl sulfoxide, and hexane, the solvents typically used in the extraction of α-tocopherol and other lipid-soluble antioxidants, did not show any interference that could invalidate the method (not shown). Even a nonmiscible solvent like hexane could be used as carrier of the reducing species. In this case, the only requirement was a vigorous agitation to facilitate the transfer of the reducing species to the aqueous phase and the subsequent formation of the green complex.

Standards of α-tocopherol in ethanol were used to determine the molar absorption coefficient, the concentration range for linearity and validity of the Beer's law, and the precision of the method, by measuring the absorbance at 695 nm (A_{695}). Spectrophotometric measurements were performed on three different occasions at several-day intervals on seven analyte groups whose A_{695} varied within the range 0.15–0.85. Repeatability (within-run precision) and reproducibility (between run precision) were estimated by regression analysis, and they were expressed as relative standard deviation (RSD, %) of the slope (molar absorption coefficient) of A_{695} vs molar concentration curves. The mean molar absorption coefficient obtained from the overall linear regression relating A_{695} vs concentration of 21 values at the 95% confidence level was $\epsilon = (4.0 \pm 0.3) \times 10^3\ M^{-1}\ cm^{-1}$ with an RSD = 1.3, 2.2, and 2.4% in each case and a $r^2 = 0.997$ (reproducibility). Linearity was observed between 2×10^{-5} *and* 2×10^{-4} α-tocopherol. The precision of the phosphomolybdenum method was compared with a standard HPLC method. An overall standard deviation (SD) was calculated from the standard deviations associated to each method (SD_1 and SD_2). As illustrated in Table 1 the Student t was smaller than the critical value $t_{0.95}$, which means that there is no significant difference between the phosphomolybdenum method and the HPLC method at a 95% confidence level. Other vitamin E isomers (γ- and δ-tocopherol and tocotrienols) were indistinguishable from α-tocopherol by the proposed method, and no significant differences were found among their respective molar absorption coefficients (not shown). These results are consistent with the fact that the method is based on the reducing potential of the hydroxyl group of the 6-hydroxychroman ring, which is shared by all vitamin E isomers. We also applied the phosphomolybdenum method to the determination of ascorbic acid, reduced glutathione and BHT.

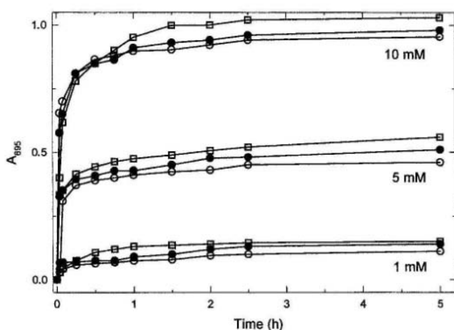

FIG. 2. Kinetics of the formation of the phosphomolybdenum complex. Complex formation was followed with three different concentrations of α-tocopherol and the assays were run at three different temperatures: 25°C (○), 37°C (●), and 95°C (□). Absorbance at 695 nm was monitored at the times indicated. Each point represents the average of three different measurements, and deviations were always below 1%.

TABLE 1

Comparative Analysis of the Precision of α-Tocopherol Determination by the Phosphomolybdenum Method and by a Standard HPLC Method (13)

	Amount of α-tocopherol detected (μmol)	
	Phosphomolybdenum method	HPLC method
Mean	$x_1 = 0.133$	$x_2 = 0.134$
No. of samples	$n_1 = 13$	$n_2 = 13$
Standard deviation	$SD_1 = 0.021$	$SD_2 = 0.012$
Degrees of freedom	$n_1 + n_2 - 2 = 24$	
Student *t*	$t = 0.12$	
Critical value of Student *t* (95% confidence level)	$t_{0.95} = 2.09$	

Note. The amount of tocopherol present was 0.135 μmol.

As shown in Fig. 3, there was an evident correlation between the reducing power of the species and the kinetics of the green complex formation. Unlike ascorbic acid or α-tocopherol, similar concentra- tions of BHT and reduced glutathione were undetectable when the reaction was run at low temperature (37°C). For the determination of reduced glutathione and BHT, the formation of the green complex required a high temperature (95°C). Interestingly, this fact would allow the determination of vitamin E in the presence of BHT and ascorbic acid in the presence of reduced glutathione. Following the procedure described for α-tocopherol, we determined the molar absorption coefficient, the concentration range for the validity of the Beer's law, repetitivity, and reproducibility of the phosphomolybdenum method applied to the determination of ascorbic acid, reduced glutathione, and BHT (Table 2).

In an attempt to validate the proposed method in the context of more complex natural samples, we applied the phosphomolybdenum method to the determination of vitamin E in corn and soybean seeds. In conjunction with the monophasic extraction procedure described under Materials and Methods, the phosphomolybdenum method was validated with respect to linearity, recovery, and reproducibility. Samples spiked with γ-tocopherol were used to determine the molar absorption coefficient, the concentration range for linearity and validity of the Beer's law, and the reproducibility of the method. This particular isomer was chosen for the spiking because it is the most abundant in corn and soybean seeds (1), although any of the other isomers could be used. Obviously, it is common sense that other sample problems should be spiked with the most abundant zsomers. Spectrophotometric measurements were performed in three different occasions with several days interval on seven analyte groups whose A_{695} varied within the range 0.15–0.85. The monophasic extraction procedure used in our method yielded a satisfactory recovery of tocopherols (93–97%). Repeatability and reproducibility were estimated by regression analysis, and they were expressed as RSD (%) of the molar absorption coefficient. The molar absorption coefficients were $\in = (2.3 \pm 0.2) \times 10^3$, $(2.2 \pm 0.1) \times 10^3$, and $\in = (2.1 \pm 0.1) \times 10^3\ M^{-1}\ cm^{-1}$ with an RSD of 5.0, 4.9, and 4.5% in each occasion. The mean molar absorption coefficient obtained from the overall linear regression relating A_{695} vs concentration of 21 values at the 95% confidence level was $\in = (2.3 \pm 0.2) \times 10^3\ M^{-1}\ cm^{-1}$ with an RSD = 4.8% and a $r_2 = 0.998$ (reproducibility). Linearity was observed between 4×10^{-5} and 4×10^{-4} M vitamin E. Our phosphomolybdenum method was also compared with a standard HPLC method for the determination of vitamin E in seeds. Individual vitamin E isomers (α-, γ-, and δ-tocopherol) were determined by HPLC and later pooled together as total vitamin E. The amount of tocotrienols was negligible (below 1%) in the samples analyzed. As shown in Table 3, the results obtained by both methods were comparable and they show that vitamin E is mainly composed of α-, γ-, and δ-tocopherol in these samples. These results confirm that the proposed method can be an alternative to HPLC methods for the determination of total vitamin E in seeds, one of the most important natural sources of this vitamin (1).

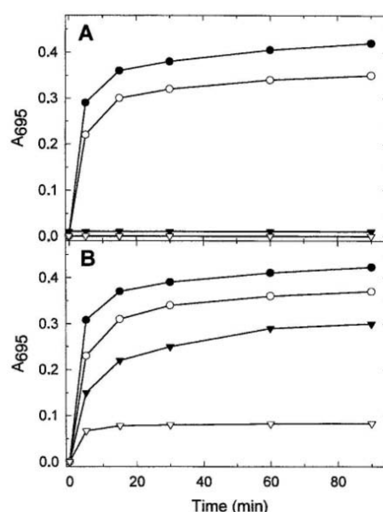

FIG. 3. Temperature dependence of the phosphomolybdenum assay for different reducing species. Identical concentrations (5 mM) of four different reducing species were assayed at 37°C (A) or 95°C (B). The formation of the phosphomolybdenum complex was monitored at the times indicated by measuring the absorbance at 695 nm. α-Tocopherol (●), ascorbic acid (○), reduced glutathione (▼), BHT (▽). All the points represent the average of three different measurements, and deviations were always below 1%.

TABLE 2

Calibration of the Phosphomolybdenum Method for Different Reducing Species

	α-Tocopherol	Ascorbic acid	Glutathione	BHT
ϵ (M^{-1} cm^{-1}):	$(4.0 \pm 0.1) \times 10^3$	$(3.4 \pm 0.1) \times 10^3$	$(2.7 \pm 0.2) \times 10^3$	$(3.6 \pm 0.3) \times 10^2$
RSD (%)	1.5	1.2	1.8	3.1
r^2	0.997	0.995	0.994	0.996
Linearity interval (M)	$2 \times 10^{-5}/2 \times 10^{-4}$	$7 \times 10^{-5}/3 \times 10^{-4}$	$2 \times 10^{-5}/3 \times 10^{-4}$	$2 \times 10^{-4}/2 \times 10^{-3}$

Total antioxidant capacity was also determined in these extracts (Table 3). These results also confirm that vitamin E is a major contributor to the overall lipid-soluble antioxidant capacity in seed extracts, as previously suggested for plant leaf lipid-soluble extracts (10). Other compounds that might contribute to the total lipid antioxidant capacity include carotenoids, flavonoids, and cinnamic acid derivatives (6). The specificity of the method at 25–37°C (temperatures at which other weaker antioxidants are not detected) makes the phosphomolybdenum method a good alternative for the determination of vitamin E in a variety of samples (plant lipid-soluble extracts, vegetal oils, butter, pharmaceutical and cosmetic preparations, human serum, etc.). Other more laborious and expensive methods are commonly used (17–20). On the other hand, determination of specific antioxidant species might be less useful than the knowledge of the total antioxidant capacity of a sample. This is the case when one wants to analyze the antioxidant contributions of specific dietary components and how this relates to the antioxidant composition and activities of the individual constituents. Other situations where the knowledge of total antioxidant activity can be useful include the analysis of changes in plasma antioxidant activity related to oxidative stress, or the understanding of structure–activity relationships of pure antioxidant species. Because of its simplicity and the cheap reagents it uses, the phosphomlybdenum method is an alternative to the methods already available for the evaluation of total antioxidant capacity (6, 21). Besides, these methods yield the results of their evaluations as a semiquantitative antioxidant activity coefficient (AAC). The phosphomolybdenum method is quantitative, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid or α-tocopherol.

TABLE 3

Comparative Analysis of Vitamin E and Total Antioxidant Capacity in Seeds

Sample	Amount of vitamin E detected (nmol/g)		Total antioxidant capacity (nmol α-tocopherol/g)
	HPLC	Phosphomolybdenum	
Corn	191.7 ± 8.5	195.5 ± 9.8	378.8 ± 20.0
Soybean	96.7 ± 13.0	101.9 ± 15.9	248.7 ± 16.6

Note. Figures represent means accompanied by their respective standard deviations. Concentrations are relative to weight of fresh tissue. Total antioxidant capacity is expressed as the equivalent of α-tocopherol in nmol of α-tocopherol per gram of fresh tissue.

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