Optimized and Validated HPLC Method for α- and γ-Tocopherol Measurement in *Laurus nobilis* Leaves. New Data on Tocopherol Content

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Extraction and chromatographic conditions for measuring tocopherols from *Laurus nobilis* were optimized. Newly harvested leaves were dried in a microwave oven and crushed; then, α- and γ-tocopherol and tocol, added as internal standard, were directly extracted from portions of ground material with acetone, by probe sonication. After centrifugation and filtration, the acetonic extract was directly analyzed by HPLC using a gradient elution with a Discovery C18 column (25 × 0.46 cm) at 35 °C. UV and fluorescence detections were employed simultaneously. Validation parameters of the method for linearity, accuracy, and precision can be considered to be adequate for both detection modes. After validation, a number of samples selected from different geographical areas in the Iberian peninsula were measured, and results compared with those in the literature gave surprisingly high values.

KEYWORDS: Vitamin E; tocopherol; HPLC; bay; *Laurus nobilis*

INTRODUCTION

Vitamin E, which is synthesized only in plants, is both an essential micronutrient and a phytochemical. Because of the abundance of plant-derived compounds in our diets, we easily meet the U.S. recommended daily allowance (RDA) for the micronutrient function of vitamin E, which is 10−13.4 IU of vitamin E. However, it has been found that daily intake of vitamin E in excess of the RDA (100−1000 IU) lowers the risk of heart disease and some cancers, improves immune function, and slows the progress of a number of degenerative diseases such as Alzheimer's disease (*1−3*). Easily obtaining the therapeutic amounts of vitamin E from the average diet is nearly impossible.

In 1998, Burton et al. reported that the retention of natural vitamin E is at least double that of the synthetic form (*4*). Previously, it was "officially" accepted that the natural stereo-isomer (i.e., the *d* form) was only 1.36 times more potent than synthesized vitamin E< which is composed of the *d* and *l* forms. This knowledge should increase the value of natural vitamin E. Consumers will favor obtaining their nutritional supplements as a part of their regular diet in fortified foods (i.e., functional foods) as opposed to the pill form.

Owing to the emerging evidence of the physiological importance concerning the balance of the different tocopherols in biological systems, the possible benefits of using natural tocopherol mixtures from plant origin as vitamin supplements in human nutrition should be considered when such medication seems to be necessary (*5*).

Laurus nobilis (bay) is a plant that has a spicy fragrance and flavor, and the leaves are traditionally used in all types of cooking, probably due to not only their flavor but also their antioxidant properties, which protect foods from rancidity and other deleterious processes.

Methods for the quantitative determination of α-tocopherol in natural products are abundantly described, although most of them are applied in fats and oils, a very different matrix. A recent critical review of our work group (*6*) discussed them.

After measuring the α-tocopherol content in *Rosmarinus officinalis*, we could see important differences with other values described in the literature (*7*), and we decided to obtain tocopherol values for edible plants with quality to be included in a nutritional database.

Therefore, the aim of the present work was the optimization of the extraction and chromatographic conditions for measuring tocopherols from *L. nobilis*, the validation of the method, and the measurement of a representative sample obtaining reliable data.

MATERIALS AND METHODS

Samples. For method development and validation bay plants (*L. nobilis*) grown in the University's gardens were used. The leaves were collected the day of each analysis except for the stability study and during validation when a homogeneous sample pool was prepared. The α- and γ-tocopherol contents in bay leaves from the same plant, grown in the open air, were determined every month from November to August to study their seasonal variability. For the determination of α- and γ-tocopherol contents in bay leaves of the Iberian peninsula, samples of bay leaves from 26 different locations in the Iberian peninsula were collected during a period of 1 week. In any case fresh leaves did not

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stay cut for >1 week, and they were dried and ground immediately on arrival in the laboratory. After that they were conserved in a desiccator and in darkness until their analysis within 1 week, following the validated method.

Optimization of Sample Treatment. *Grinding.* To study the influence of particle size after grinding of the desiccated bay leaves, the ground material was sieved through three sieves of 0.2, 0.4, and 0.6 mm mesh. The four fractions obtained were analyzed to determine their α- and γ-tocopherol contents.

Extraction. Five different solvents were tested in triplicate for their extraction efficacy: acetone, tetrahydrofuran, 2-propanol, hexane, and sodium dodecyl sulfate (SDS) in water (0.01 mg/mL). In all cases the α- and γ-tocopherol contents in three ground bay leaf samples were measured.

Samples were prepared by adding to 250 mg of ground bay leaves 200 μL of tocol, as internal standard (0.130 mg/mL in acetone), and 3.8 mL of the corresponding solvent. Samples were processed simultaneously and in parallel with the corresponding standards diluted in acetone. In the case of the samples extracted with hexane, to avoid the incompatibility with the mobile phase, 2 mL of the extract was evaporated under nitrogen and redissolved in 2 mL of mobile phase.

Antioxidants. To evaluate the efficacy of different antioxidants in the prevention of vitamin E oxidation, extracts with no antioxidant added, with 100 μL of butylated hydroxytoluene (BHT) (13 mg/mL), with 100 μL of ethylenediaminetetraacetic acid (EDTA) (23 mg/mL), or with 100 μL of both solutions were tested. They were added to 250 mg of ground bay leaves and 200 μL of tocol (internal standard) and made up to 4 mL with acetone. These samples were injected in the HPLC system the same day that they were prepared, 1 week, 2 weeks, and 1 month later, having been kept at −20 °C until then.

Conservation. A homogeneous pool of dry ground bay leaves was prepared and kept in closed plastic containers, half in a refrigerator and the other half in a desiccator and darkness. The α-tocopherol content was determined on the same day the pool was prepared and 1 week, 2 weeks, 1 month, and 3 months later using standards freshly prepared on the day of the analysis.

Final Quantification Procedure. *Reagents.* All solvents were of HPLC grade quality purchased from Scharlau (Barcelona, Spain). α-Tocopherol, lycopene, β-carotene, and chlorophylls *a* and *b* were from Fluka (Buchs, Switzerland), and γ-tocopherol and lutein were from Sigma (St. Louis, MO).

Stock and Working Standards. Individual stock solutions of ∼9 mg/ mL α- and γ-tocopherol as external standards and a stock solution of 2 mg/mL tocol as internal standard were prepared in ethanol. These solutions were stored in aluminum foil-covered containers and kept at −20 °C. The day of the assay, the exact concentration of α- and γ-tocopherol was determined spectrophotometrically in an ethanol dilution 1:250 (v/v). Detection was at 295 nm for α-tocopherol (ϵ = 3058 L/mol·cm) and at 298 nm for γ-tocopherol (ϵ = 3810 L/mol· cm). Meanwhile, dilutions in acetone 1:6 (v/v) of α-tocopherol, 1:800 of γ-tocopherol, and 1:15 of tocol from the corresponding stocks were prepared. Finally, working standards consisted of a mixture of 200 μL of diluted tocol and 300 μL of each diluted tocopherol made up with acetone to a final volume of 4 mL in glass tubes. These solutions were probe sonicated and centrifuged to follow the same procedure as with samples.

Sample Treatment. Approximately 5 g of fresh bay leaves was weighed before and after drying in a microwave oven at 900 W for 1 min to calculate their water content. After grinding until a mean particle size of <0.4 mm (milled at 20000 rpm for 15 s) was reached, 250 mg of the powder was weighed in a glass tube and 200 μL of diluted tocol and 3.8 mL of acetone were added. Samples were extracted by probe sonication for 1 min, centrifuged, and filtered through 0.45 μm nylon filters to HPLC vials.

Chromatographic Conditions. The chromatographic system was a Beckman (Fullerton, CA) HPLC system equipped with a 125 pump, an automatic injector (507e), a 168 diode array detector, a Gold System data processor, and an analogue interface (406) for the fluorescence detector from Waters (Milford, MA). Chromatographic analysis was performed on a 5 μm particle C$_{18}$ Discovery (25 × 0.46 cm) kept in a Bio-Rad column oven at 35 °C. A C$_{18}$ precolumn was used to protect

Table 1. α- and γ-Tocopherol Contents of Desiccated and Ground Bay Leaf Fractions with Different Mean Particle Sizes

fraction	α-tocopherol (mg/100 g of fresh leaves)	γ-tocopherol (mg/100 g of fresh leaves)
>0.6 mm	76.38	0.76
0.6–0.4 mm	93.00	0.81
0.4–0.2 mm	95.19	0.82
<0.2 mm	144.45	1.27

the column from less polar compounds. Purified-for-HPLC water (Milli-Q, Waters) was employed as mobile phase A and acetonitrile/methanol (70:30, v/v) with 0.1% (v/v) of acetic acid added as mobile phase B. From $t = 0$ to $t = 24$ min the eluent composition varied in a linear gradient from 85% B to 100% B at a flow rate of 2 mL/min. At $t = 24$ min the flow was increased to 3 mL/ min in 1 min to clean the column of less polar compounds. At $t = 35$ min the system returned to initial conditions in 1 min, which marked the end of the run. α-Tocopherol was detected by absorbance at 295 nm, and both tocopherols were detected by fluorescence excitation at 295 nm and emission at 350 nm.

Peak identification was performed by comparing the retention times with pure standards and confirmed with characteristic spectra obtained from the photodiode array detector, which also permitted the confirmation of the purity of the peaks.

Validation Assay. The low levels of γ-tocopherol in samples made its UV detection impossible, so the method was validated for this analyte only with fluorescence detection.

Standards linearity was tested by analyzing in triplicate standards at five levels of concentration from 0.022 to 0.223 mg/mL for α-tocopherol and from 1.9×10^{-4} to 1.9×10^{-3} mg/mL for γ-tocopherol to cover the expected range of concentrations for both tocopherols in samples. Two hundred microliters of diluted tocol as internal standard were added to each tube, and the volume was made up to 4 mL with acetone.

Sample linearity was verified in triplicates of 125 mg of ground bay leaves to which 0, 60, 180, 300, or 450 μL of both diluted tocopherols, 200 μL of diluted tocol, and acetone to make a final volume of 4 mL were added.

For accuracy, the recovery assay was developed simultaneously with the sample linearity in which samples were prepared from 50% of the amount of ground bay leaves (125 mg), and therefore 50% of the endogenous tocopherol present in a normal sample, and increasing it by adding working standards as described before.

Instrumental precision was determined by analyzing the same sample six times, whereas the repeatability or *intra*-assay precision and intermediate precision of the method were obtained by processing two series of six samples on two different days.

Finally, limits of quantification of the technique were obtained according to the EURACHEM method (*8*). For this purpose, a new calibration curve for both tocopherols was prepared ranging from 0.1 to 20% of their theoretical content in samples by dilution from the medium standard (100%). Simultaneously samples (100%) were diluted in the same way. Both standards and samples were analyzed six times. Relative standard deviations (RSD) were represented versus concentrations, and 10% of RSD was selected to establish the limits of quantification.

RESULTS AND DISCUSSION

Method development related to sample pretreatment started with the experience accumulated in our work group with rosemary leaves (*7*). In that work it was established that the microwave oven was the best option for drying the samples and, therefore, with bay leaves, it needed only to be proved that the conditions were adequate to obtain a constant weight.

Grinding dried material is necessary to facilitate the contact between the extractive solvent and the desiccated bay leaves. **Table 1** shows the results obtained for α- and γ-tocopherol contents in each fraction with different mean particle sizes. As

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Table 2. α- and γ-Tocopherol Mean Contents Obtained with Each Extraction Solvent (1, Acetone; 2, Tetrahydrofuran; 3, 2-Propanol; 4, Hexane; 5, Water with SDS) and the Student–Newman Mean Comparison Study Results

			α-tocopherol			γ-tocopherol	
		mean (mg/100 g)	RSD (%)	homo-geneous group	mean (mg/100 g)	RSD (%)	homo-geneous group
group	1	111.67	1.77	A	0.93	1.96	A B
	2	110.34	0.94	A	0.94	1.15	A
	3	110.67	0.77	A	0.88	4.67	A B
	4	109.58	3.47	A	0.87	5.38	B
	5	82.50	3.26	B	0.77	2.70	C

		α-tocopherol		γ-tocopherol	
		difference	significance (95%)	difference	significance (95%)
contrast	1–2	1.326	no	−0.011	no
	1–3	1.002	no	0.045	no
	1–4	2.090	no	0.052	no
	1–5	29.168	yes	0.160	yes
	2–3	−0.324	no	0.056	no
	2–4	0.764	no	0.064	yes
	2–5	27.842	yes	0.170	yes
	3–4	1.088	no	0.008	no
	3–5	28.167	yes	0.114	yes
	4–5	27.078	yes	0.106	yes

could be expected, the extraction efficacy increased with smaller particle size, and that increase was more pronounced at smaller sizes. This could be explained by a different composition in this fraction that could be enriched in the softer parts of the plant, which include the chloroplasts, where tocopherols are synthesized (*9*, *10*). Therefore, if this fraction were selected, the sample could be shifted. Finally, as sieving increased considerably the analysis time and experimentally particles >0.4 mm were well recognized, they were discarded de visu and a homogeneous sample was taken of the rest.

Samples must be submitted to stages of extraction and/or cleaning prior to their injection in the chromatographic system. Saponification was discarded because it is time-consuming, its severe conditions can degrade tocopherols, and it has been previously demonstrated that it is not necessary if adequate extraction and chromatographic conditions are employed (*7*, *11*, *12*). For extraction four different solvents were tested simulta-neously with acetone, which had previously shown its adequacy with rosemary leaves (*7*). Mean values for both tocopherols with each solvent and their RSD (percent) can be observed in **Table 2**. Results were analyzed with a Student−Newman mean comparison study, and the homogeneous group to which results of each solvent belong is also indicated. There were no significant differences for α-tocopherol contents except for SDS, which was lower, and the four first solvents (tetrahydrofuran, acetone, 2-propanol, and hexane) belonged to the same homo-geneous group. γ-Tocopherol contents were slightly higher with tetrahydrofuran and acetone, but differences with hexane and 2-propanol were not significant due to the higher RSDs of the values obtained with these solvents.

Finally, acetone was chosen because, although it gave the same results as tetrahydrofuran, it is less expensive and there is experience with its behavior.

Vitamin E is easily oxidable. Oxidation losses can be induced by heat, light, alkaline pH, and the presence of free radicals or other components in samples that can oxidize vitamin E during the extraction process and in the extract until its final analysis.
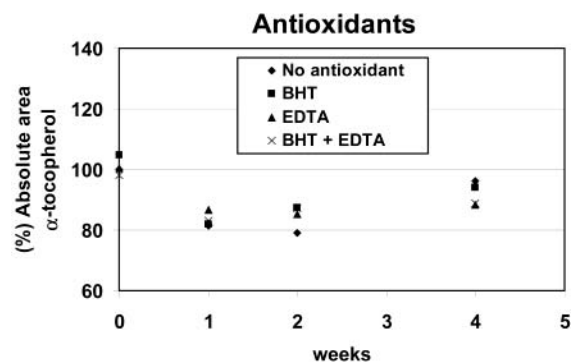

Figure 1. α-Tocopherol absolute areas variation in samples with and without different antioxidants added.
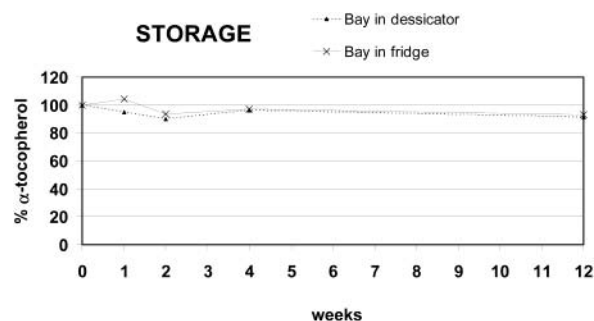

Figure 2. α-Tocopherol content variation in desiccated and ground bay leaves stored in a refrigerator or in a desiccator in darkness.

The addition of antioxidants is not considered to be necessary, except in the case of simultaneous analysis of more labile compounds, special matrices, or when saponification is applied (*6*). In the case of plants the presence of metal ions such as iron or copper could promote the oxidation of lipids and tocopherols. This could be avoid by adding a chelating agent such as EDTA and/or a low-polar antioxidant such as BHT (*11*). For this reason we tested the action of these two antioxidants separately and simultaneously not only during the extraction but also through 4 weeks. Results of the injection of the different samples are represented in **Figure 1** in percentage of absolute areas related to those values obtained in samples without any antioxidant at the initial time. As it was a single measure of area in each time, small and random variations can be attributed to the actual measurement and not to a real difference due to the presence of BHT and/or EDTA. Therefore, samples can be considered to be stable after extraction and without the addition of antioxidants at least for 1 month.

Due to the characteristics of tocopherols and because sometimes samples cannot be analyzed on the same day that they were collected, a stability assay was performed to determine the best method of sample storage and the stability of desiccated samples. In **Figure 2** the percentage of α-tocopherol related to the initial content is represented along the weeks for samples stored in the refrigerator and for those in the desiccator and darkness. Recoveries were very similar in both cases, but we observed that the refrigerated samples became damp and moldy. Therefore, collected samples were kept dry and ground in the desiccator and in darkness as a storage condition.

The next step was the optimization of the chromatographic conditions. The critical point was improving the resolution between α-tocopherol and chlorophyll *b* in UV without too much increase in analysis time. Starting conditions were those previously employed with rosemary extracts (*7*), with minor
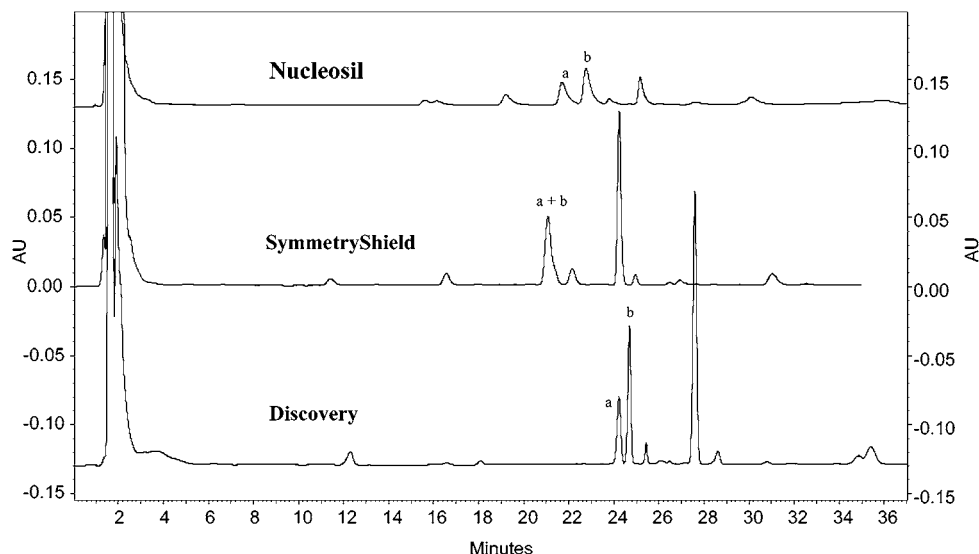
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Figure 3. Chromatograms of bay leaves obtained with different columns (a, α-tocopherol; b, chlorophyll *b*).
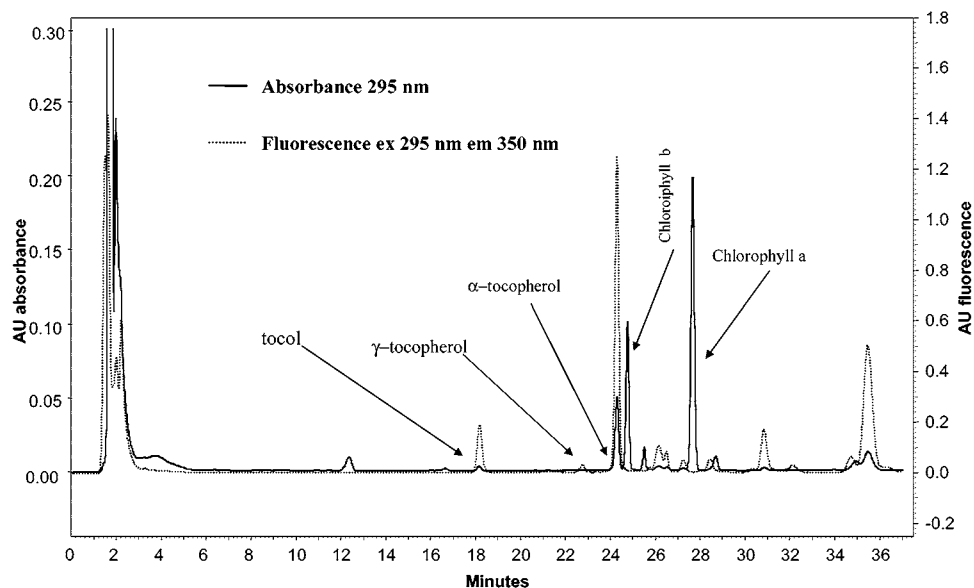

Figure 4. Chromatograms with UV absorbance detection at 295 nm and fluorescence detection at excitation of 295 nm and emission of 350 nm of bay leaf samples with the following final chromatographic conditions: C_{18} Discovery (25 × 0.46 cm) column at 35 °C and mobile phases A [purified-for-HPLC water (Milli-Q, Waters)] and B [acetonitrile/methanol (70:30, v/v) with 0.1% (v/v) of acetic acid added using the gradient described in the text].

modifications to improve the detection of other compounds in the chromatogram. The Nucleosil C_{18} column was substituted by a SymmetryShield RP_{18} of the same size. This column presents similar hydrophobicity but lower silanol activity (manufacturer data). Simultaneously a low proportion of ethyl acetate was added to mobile phase B because, as previously reported (*13*), this modifier improved the elution of carotenes and tocopherols. In these conditions at 295 and 450 nm as detection wavelengths, other compounds such as lutein, β-carotene, chlorophylls *a* and *b*, and γ-tocopherol were also identified. γ-Tocopherol was resolved and identified in the chromatogram, but the UV detector was not sensitive enough to detect it. In these conditions α-tocopherol appeared after chlorophyll *b*. As this situation of a minor compound appearing behind another with a greater absorbance is not desirable, we returned to initial conditions and tested three different columns of the same size: a Phenomenex Nucleosil 5 μm C_{18} (25 × 0.46 cm), a Waters SymmetryShield RP_{18}, 5 μm (25 × 0.46 cm), and a Supelco Discovery 5 μm C_{18} (25 × 0.46 cm). Chromatograms obtained from bay leaf samples at 295 nm with these columns are shown

in **Figure 3**. The Discovery column was able to separate α-tocopherol from chlorophyll *b* and provided higher efficiency. Tocol was chosen as internal standard because it can be detected as much in UV as in fluorescence, which is needed for γ-tocopherol measurement. After the development, the method was validated for the determination of α-tocopherol (by UV and fluorescence) and γ-tocopherol (only by fluorescence) in bay leaves. **Figure 4** shows a typical chromatogram obtained with the final conditions. β- and γ-tocopherol cannot be separated with conventional C_{18} columns and, therefore, if there was some β-tocopherol in samples, it would be included in the γ-tocopherol values.

Validation results appear in **Table 3**. Both standards and samples show a good linearity for both analytes and both detectors, with correlation coefficients >0.99 and slopes statistically different from zero (*t* test, $p > 0.95$). Although the intercept for γ-tocopherol in samples does not include the zero value, it is mainly due to the narrow limits of confidence, due to the good fit of the points to the regression line, but it does not pose any bias about recoveries of the extreme values.

Table 3. Mean Statistical Validation Results

			α-tocopherol abs	α-tocopherol fluor	γ-tocopherol fluor
linearity	standards	range (mol/L)	5.186×10^{-5}–5.186×10^{-4}	5.186×10^{-5}–3.890×10^{-4}	4.496×10^{-7}–4.496×10^{-6}
		R	0.997	0.998	0.999
		$b \pm tS_b$	60502 ± 2866	20569 ± 970	60949 ± 1197
		$a \pm tS_a$	0.4 ± 0.9	0.0 ± 0.2	-0.002 ± 0.003
	samples	range (mol/L)	1.350×10^{-4}–5.247×10^{-4}	1.350×10^{-4}–3.855×10^{-4}	1.216×10^{-6}–4.593×10^{-6}
		R	0.999	0.996	0.999
		$b \pm tS_b$	61245 ± 1383	21780 ± 1463	61308 ± 718
		$a \pm tS_a$	-0.3 ± 0.4	0.0 ± 0.4	-0.0023 ± 0.0021
accuracy	standards	range (mol/L)	5.186×10^{-5}–5.186×10^{-4}	5.186×10^{-5}–3.890×10^{-4}	4.496×10^{-7}–4.496×10^{-6}
		R (%) $\pm tS_R/n^{1/2}$	100 ± 3	98 ± 5	99 ± 2
	samples	range (mol/L)	1.350×10^{-4}–5.247×10^{-4}	1.350×10^{-4}–3.855×10^{-4}	1.216×10^{-6}–4.593×10^{-6}
		R (%) $\pm tS_R/n^{1/2}$	102 ± 1	105 ± 3	100 ± 1
precision mean (mol/L); RSD (%)	standards	instrum precision	2.73×10^{-4}; 1.96	2.24×10^{-4}; 2.47	2.12×10^{-6}; 2.27
		repeatability	2.68×10^{-4}; 1.58	2.68×10^{-4}; 4.03	2.19×10^{-6}; 2.20
		intermed precision	2.64×10^{-4}; 2.23	2.64×10^{-4}; 4.16	2.22×10^{-6}; 2.80
	samples	instrum precision	2.64×10^{-4}; 1.55	2.07×10^{-4}; 1.12	2.12×10^{-6}; 1.82
		repeatability	2.60×10^{-4}; 1.81	2.53×10^{-4}; 3.08	2.20×10^{-6}; 2.19
		intermed precision	2.57×10^{-4}; 2.24	2.49×10^{-4}; 4.11	2.21×10^{-6}; 3.22

In the accuracy study, recoveries do not differ statistically from 100% (*t* test, $p > 0.95$) except for α-tocopherol in samples with both detection modes, but they are near enough to 100% to be considered adequate.

RSDs for the instrumental precision were lower than those for repeatability, and these were lower than those obtained for intermediate precision, as was expected. All of the RSDs were adequate for the levels of analytes in samples.

Limits of quantification (LOQ) were 1.8×10^{-6} M for α-tocopherol with UV, 1.5×10^{-7} M with fluorescence, and 1.3×10^{-7} M for γ-tocopherol with fluorescence.

Once the analytical method was validated, the stability of the analytical process assessed, and sample handling evaluated, 26 samples from around the Iberian peninsula were measured to obtain a reliable score for tocopherol content in bay leaves, able to be included in the nutritional database. The values obtained with their limits of confidence ($p > 0.95$) were 132.2 ± 22.6 mg of α-tocopherol/100 g of fresh leaves and 1.1 ± 0.20 mg of γ-tocopherol/100 g of fresh leaves. Ranges were from 30.8 to 272.8 mg of α-tocopherol/100 g of fresh leaves and from 0.3 to 2.1 mg of γ-tocopherol/100 g of fresh leaves. The value obtained for α-tocopherol in bay is considerably higher than the value obtained by Marero et al., which was 12.6 mg of α-tocopherol/100 g of fresh leaves (*14*), probably because analytical tools have improved. On the other hand, the high values obtained by Demo et al. (*15*) in a hexanic extract by HPLC with UV detection seem to be related with the possible overlapping of chlorophyll with α-tocopherol in such conditions. The value in the USDA database is 1.786 mg of α-tocopherol equiv/100 g of ground bay, but probably it is in commercially dried bay; nevertheless, in our opinion, these values ought to be reviewed.

Finally, as described above, the variation in tocopherol content in one plant in the University's gardens grown in the open air at atmospheric conditions was evaluated from November to August. Results are shown in **Figure 5**. In general terms α-tocopherol content was higher in winter than in summer, which is in agreement with previous works that showed an increase in antioxidant defense for plants grown at low temperatures (*16*).

Periodical analysis of the material has been performed, sample handling and storage have been studied, and a large enough number of samples have been measured to obtain values that could be included in a nutritional database with a high reliability
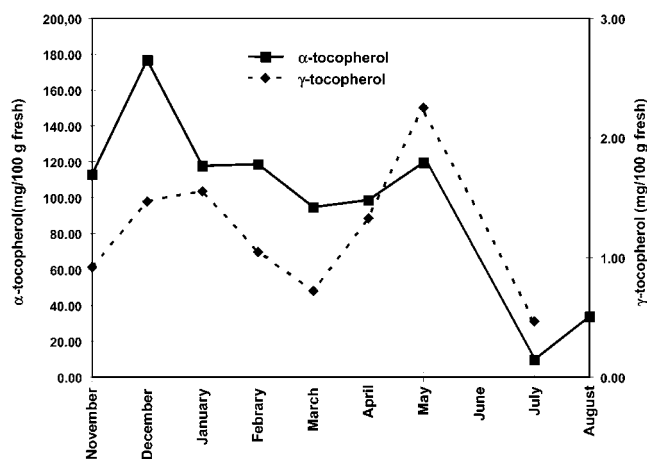

Figure 5. Variation of α- and γ-tocopherol contents obtained monthly in a bay plant.

score, on the basis of the evaluation procedure that critically reviews analytical data to be included in a database for flavonoid values for food (*17*).

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