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Analysis of the Polyphenols Content in Medicinal Plants Based on the Reduction of Cu(II)/Bicinchoninic Complexes

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A spectrophotometric method is proposed for the determination of the polyphenols content in aqueous extracts of plants. The method is based on the reduction of Cu(II) to Cu(I) by polyphenols, in the presence of bicinchoninic acid in a buffered medium (ammonium acetate, pH 7.0) with the formation of Cu(I)/BCA complexes. A calibration curve of absorbance (at 558 nm) vs tannic acid concentration is linear ($r = 0.995$; $n = 7$) with tannic acid from 0.1 to 0.7 μmol L^{-1}. The limit of detection and relative standard deviation were 40 nmol L^{-1} (99% confidence level) and 3.8% (0.4 μmol L^{-1} tannic acid, $n = 7$), respectively. For the aqueous extracts of *Hamamelis virginiana* L., *Maytenus ilicifolia* Mart. ex Reissek, *Hydrocotyle bonariensis* Lam, *Annona muricata* L., *Myrciaria cauliflora* (Mart.) O. Berg., *Caesearia sylvestris Sw.*, *Schinus terebinthifolia* (Raddi), and *Stryphnodendron adstringens* (Mart.) Coville, the total polyphenol contents, expressed as tannic acid, were 3.5, 1.3, 2.0, 3.1, 15.4, 3.1, 9.1, and 6.9%, respectively.

KEYWORDS: Polyphenol determination; copper; 4,4′-dicarboxy-2,2′-biquinoline acid

INTRODUCTION

The polyphenolic compounds present in the plants are normally classified as phenolic acids, flavonoids, and tannins, among others, which usually do not have the same chemical properties. Some analytical methods have been proposed to estimate the content of total polyphenols in a variety of samples such as wine, beer, tea, plant materials, and effluents from wastewaters.

Concerning the plant aqueous extract samples, the most used method is based on the spectrophotometry measurements after the oxidation of the polyphenolic compounds with Folin−Ciocalteu (or Folin−Denis) reagent in a very alkaline medium with the formation of a blue color solution (*1*). Despite the fact that this procedure is well-established, other reducing agents, for example, sugars, present in the plant materials may interfere. Furthermore, this method promotes the consumption of phosphomolybdic or phosphotungstic acids, resulting in waste that is usually not recycled. Thus, simpler and more rapid methods are required for routine analysis and quality control purposes.

Bicinchoninic acid [4,4′-dicarboxy-2,2′-biquinoline acid or 2-(4-carboxyquinolin-2-yl)quinoline-4-carboxylic acid], or simply BCA, is a weak organic acid ($pK_{a1} = 1.87$; $pK_{a2} = 2.85$) (*2*) derived from quinoline (**Figure 1**). This reagent is employed to determine the total level of proteins (*3*), some reducing sugars (*4*), ascorbic acid (*5*), and recently tannic acid in beverages (*6*). BCA was also used to determine copper in different samples like alloys (*7*), minerals (*8*), blood (*9*), and effluents (*10*) by using hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) or hydrazine sulfate ($H_2NNH_2 \cdot H_2SO_4$) to reduce Cu(II) to Cu(I) in a buffered solution (pH 6.0−8.0).

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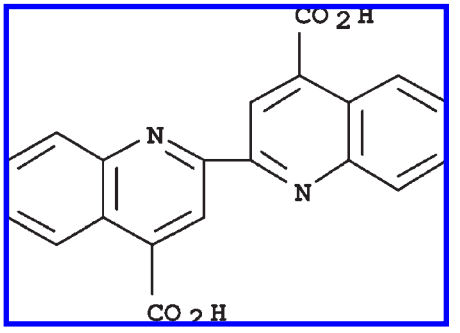

Figure 1. Structure of BCA.

In the present work, the reduction of Cu(II) to Cu(I) by polyphenols in the presence of BCA in a buffered medium with ammonium acetate (pH 7.0) was used to determine the total polyphenols in aqueous extracts of eight medicinal plants. In this reaction, two molecules of BCA react with a single Cu(I) ion, forming a purple water-soluble complex with absorption at 558 nm. The calibration curve, expressed as the absorbance values vs tannic acid concentration, is linear. This is the first report of an analytical alternative method for polyphenols quantification in aqueous extracts of medicinal plants based on the reduction of Cu(II) in a buffered medium containing this specific chelator for Cu(I).

MATERIALS AND METHODS

Reagents and Solutions. All solutions were prepared from analytical-grade chemicals with deionized water. A 2.3 mol L^{-1} $Cu(ClO_4)_2$ stock solution was prepared by reaction of Cu(II) carbonate ($CuCO_3$, Fluka A. G. Chemie) with a small excess of perchloric acid ($HClO_4$, Merck) to avoid

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hydrolysis of the metal ion. A diluted 1.0×10^{-2} mol L^{-1} Cu(II) working solution was prepared by accurate dilution.

A 1.0×10^{-3} mol L^{-1} tannic acid ($C_{76}H_{52}O_{46}$, J. T. Baker) fresh solution (0.17 g/100 mL) and a 2.0 mol L^{-1} ammonium acetate ($C_2H_7NO_2$, Fluka, A.G. Chemie) stock solution (pH 7.0) were both prepared by dissolution. A 3.0×10^{-2} mol L^{-1} BCA (1.165 g/100 mL) stock solution was prepared by dissolution of the dissodium salt of 4,4′-dicarboxy-2,2′-biquinoline ($Na_2C_{20}H_{10}N_2O_4$, Sigma).

The Folin−Ciocalteu reagent was prepared dissolving 20 g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$, Synth), 4.0 g of phosphomolybdic acid ($H_3Mo_{12}O_{40}P \times H_2O$, Sigma), and 10 mL of phosphoric acid (H_3PO_4, Merck) in 150 mL of water. This mixture was heated under reflux for 2 h. After it was cooled at room temperature, the mixture was diluted to 200 mL with water. The reagent was kept at 8 °C, and when it acquired a greenish color, it was regenerated by boiling with some drops of bromine, Br_2, until the yellow color returned.

Sample Preparation. The initial procedure described in the European Pharmacopoeia was employed to extract the total hydrosoluble polyphenols from plants samples (*1*). A 0.75 g amount of dry material of each plant [leaves for *Hamamelis virginiana* L., *Maytenus ilicifolia* Mart. ex Reissek, *Hydrocotyle bonariensis* Lam, *Annona muricata* L., *Myrciaria cauliflora* (DC.) Berg, *Casearia sylvestris*, *Schinus terebinthifolia* (Raddi), and bark for *Stryphnodendron adstringens* (Martius) Coville] was transferred to a 250 mL erlenmeyer, containing 150 mL of water, which was maintained on a water bath for 30 min at 80−90 °C.

After it was cooled, the mixture was transferred to a 250.0 mL volumetric flask, made up with water, and left the plant material was decanted. This solution was then filtered by discarding the first 50 mL of the filtrate.

For total polyphenols quantification, 5 mL of the above filtrate was transferred to a 25.0 mL volumetric flask and completed with water. For unprecipitated polyphenols evaluation, 0.15 g of casein in 20 mL of the filtrate was added, and the solution was shaken for 60 min. After this, the mixture was centrifuged, and 5 mL of this solution was transferred to a 25.0 mL volumetric flask and made up with water (*11*).

Apparatus. All absorbance measurements were carried out using a HPUV 8453. A 1.0 cm optical path length glass cell was used in all measurements.

Reference Procedure. The Folin−Ciocalteu procedure was carried out as described in the Brazilian Pharmacopoeia for total polyphenols (*11*), with a 10-fold reduction of all solution volumes. A typical calibration curve was obtained by mixing aliquots of 5−25 μL from a 1.0×10^{-3} mol L^{-1} tannic acid standard solution with 200 μL of Folin−Ciocalteau reagent and was completed to 5.00 mL with a 10% sodium carbonate in a volumetric flask. After 3 min, the absorbance was measured at 715 nm.

For the sample analyses, the aliquots of tannic acid standard solution were replaced by a volume (50 or 375 μL) of the diluted aqueous extracts of medicinal plants. The Folin−Ciocalteu reagent used was the reference solution.

Proposed Procedure. The calibration curve was obtained by mixing in a 5.00 mL volumetric flask the reagents in the following order: 250 μL of a 1.0×10^{-2} mol L^{-1} Cu(II) and 1.0 mL of 2.0 mol L^{-1} ammonium acetate solutions. After homogenization, aliquots from 0.5 to 3.5 mL of a 1.0×10^{-6} mol L^{-1} tannic acid standard solution were added followed by 250 μL of a 3.0×10^{-2} mol L^{-1} BCA. The volume was subsequently completed to 5.00 mL with the same ammonium acetate solution. The absorbance measurements were recorded at 558 nm.

For the sample analyses, the aliquots of tannic acid standard solution were replaced by a volume (50 or 375 μL) of the diluted aqueous extracts of medicinal plants. A mixture containing 0.5×10^{-3} mol L^{-1} Cu(II), 0.4 mol L^{-1} ammonium acetate, and 1.5×10^{-3} mol L^{-1} BCA was used as a reference solution. In both Folin−Ciocalteu and BCA methods, the concentration of tannic acid in the aqueous extracts of the plant sample solutions was calculated by the multiple standard addition method.

RESULTS AND DISCUSSION

Chemical Aspects of the Cu(II)/BCA Reaction. The Cu(I)/BCA violet complexes formed, after reduction of Cu(II) by hydroxylamine hydrochloride in BCA medium, have an absorption peak at 558 nm with a molar absorptivity of 7.7×10^3 L mol^{-1} cm^{-1} (*2*). According to the literature, two molecules of BCA coordinate to one Cu(I) (*2*). However, for analytical purposes, the BCA must be kept 3-fold in excess over Cu(II), so the color of the solution remains constant for at least 1 week (*6*, *12*).

Our previous work showed that polyphenols can reduce Cu(II) to Cu(I) in a buffered ammonium acetate solution (pH 7.0) containing BCA. In that work, an alternative analytical method, using flow injection analysis, was proposed for polyphenols determination in wines and teas, and the calculated detection limit was 10 nmol L^{-1} (99.7% confidence level) with a linear range from 0.5 to 5 μmol L^{-1}. In that procedure, it was possible to analyze 50 samples per hour with a low consumption of Cu(II) and BCA being 20 and 230 μg per determination, respectively (*6*).

In the present work, for the determination of the polyphenols in aqueous extracts of plants, the ratio 1:3 of metal ion:ligand was maintained, and the highest absorbance value was attained using a working solution containing Cu(II) 0.5×10^{-3} mol L^{-1} and BCA 1.5×10^{-3} mol L^{-1} (**Figure 2**). At higher concentrations such as 1.0×10^{-3} Cu(II) and 3.0×10^{-3} mol L^{-1} BCA, the formation of a light green precipitate of Cu(II) with BCA was observed. It must be emphasized that the order of the addition of the reagents, described in the experimental part, must be followed to get the highest sensitivity and reproducibility, also avoiding the precipitation of Cu(II)/BCA.

In all of these tests, a 0.4 mol L^{-1} ammonium acetate solution was employed to keep the working solution at pH 7.0. At this pH value, BCA ($pK_{a1} = 1.87$; $pK_{a2} = 2.85$) is totally deprotonated, being a specific chelator agent for Cu(I) (*2*). Following these conditions, a violet solution is formed, which remains stable for more than 1 week when protected from light and stored at 8 °C.

Analytical Features. A typical calibration curve of absorbance value at 558 nm was linear for the tannic acid concentration from 0.1 to 0.7 μmol L^{-1}, and the limit of detection was calculated, defined as three times the standard deviation of the linear coefficient value divided by the angular coefficient value, and was 40 nmol L^{-1} (0.068 μg mL^{-1}) with a 99% confidence level (**Figure 2**, inset). This curve is described by the regression equation as $A = 0.0179 + 235998 \times [TA]$ ($r = 0.996$; $n = 7$), where A is the absorbance value (at 558 nm) and [TA] is the concentration of tannic acid in mol L^{-1} (**Figure 2**, inset). The relative standard deviation was estimated as 3.8% for seven measurements of a 0.4 μmol L^{-1} tannic acid solution.

Discussion. In this study, tannic acid (J. T. Baker Chemical, United States) was used as a standard compound to express the total polyphenols content found in the aqueous extracts of plants due to its more complex chemical composition. Moreover, simpler phenolic compounds like dihidroxy phenolic compounds

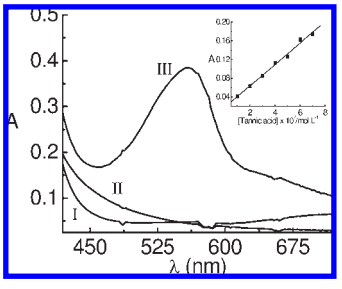

Figure 2. Absorption spectra of I = 5 mM tannic acid; II = 0.5 mM Cu(II) + 1.5 mM BCA + 0.4 mol L^{-1} ammonium acetate; and III = 0.5 mM Cu(II) + 1.5 mM BCA + 0.6 mol L^{-1} ammonium acetate + 0.5 μM tannic acid. Inset figure: Calibration curve for tannic acid using the absorbance at 558 nm of the Cu(I)/BCA complex (b = 1.0 cm; reference solution, 0.5 mM Cu(II) + 0.4 mol L^{-1} ammonium acetate + 1.5 mM BCA).

Table 1. Total Polyphenols and Unprecipitated Polyphenols Quantification by the Present and the Official Methods Expressed as Tannic Acid Percentage

	tannic acid (%)[a]					
	reference method (*1*)			present method		
plants	total polyphenols	unprecipitated polyphenols[b]	tannins	total polyphenols	unprecipitated polyphenols[b]	tannins
H. virginiana L. (leaves)	12.9 ± 2.7	4.7 ± 0.4	8.2 ± 2.7	3.5 ± 0.4	0.74 ± 0.02	2.8 ± 0.4
S. adstringens (Mart.) Coville (bark)	12.2 ± 2.9	3.2 ± 0.6	9.0 ± 3.0	6.9 ± 0.7	1.8 ± 0.4	5.1 ± 0.8
M. ilicifolia Mart. ex Reissek (leaves)	4.6 ± 0.7	3.5 ± 0.4	1.1 ± 0.8	1.3 ± 0.2	0.13 ± 0.01	1.2 ± 0.2
H. bonariensis Lam (leaves)	4.5 ± 0.9	3.9 ± 0.3	0.6 ± 0.4	2.0 ± 0.4	1.2 ± 0.02	0.8 ± 0.4
A. muricata L. (leaves)	10.6 ± 1.3	5.2 ± 0.6	5.4 ± 1.4	3.1 ± 0.7	2.0 ± 0.05	1.1 ± 0.7
M. cauliflora (Mart.) O. Berg. (leaves)	15.9 ± 2.7	3.1 ± 0.7	12.8 ± 2.8	15.4 ± 2.2	3.8 ± 0.2	11.6 ± 2.2
C. sylvestris Sw. (leaves)	4.4 ± 0.55	0.87 ± 0.03	3.5 ± 0.6	3.1 ± 0.1	0.26 ± 0.06	2.8 ± 0.1
S. terebinthifolia (Raddi) (leaves)	12.9 ± 1.9	2.6 ± 0.2	10.3 ± 1.9	9.1 ± 0.8	1.6 ± 0.07	7.5 ± 0.8

[a] Results obtained with the average of three analyses representing g of tannic acid per 100 g of dry material (leaves or bark). [b] After precipitation with casein.

Table 2. Parameters of the Linear Regression of the Calibration Curves for Some Compounds ($y = a + b \times [C]$)[a]

			reference method (*1*)						present method[b]					
compound	FPH	FW	LR	*n*	*a*	$b \times 1000$	*r*	LD	LR	*n*	*a*	$b \times 1000$	*r*	LD
tannic acid	12	1701.2	1−5	5	0.089	117	0.999	0.023	1−6	6	0.097	470	0.996	0.040
(−)-epigallocatechin gallate	8	458.4	1−5	4	0.010	37.6	0.999	0.086	2−6	5	0.039	78.2	0.999	0.39
quercetin	4	302.2	1−6	5	0.010	34.2	0.998	0.46	1−6	5	0.0066	64.1	0.999	0.30
gallic acid	3	188.1	1−6	5	0.012	19.6	0.987	1.06	1−6	5	0.010	80.5	0.997	0.50
resveratrol	3	228.2	1−6	6	2.2×10^{-4}	25.8	0.997	0.44	1−6	6	0.015	13.9	0.992	0.74
pyrogallol	3	126.1	1−6	5	0.0032	20.8	0.997	0.59	1−5	4	0.0026	37.0	0.999	0.17
phloroglucinol	3	162.1	1−5	5	0.032	16.6	0.992	0.75	2−6	5	0.0022	14.1	0.999	0.30
o-pirocathecol	2	110.1	1−5	5	0.0044	13.1	0.991	0.80	2−6	5	0.0060	19.8	0.992	0.94
hydroquinone	2	110.1	1−6	5	0.043	7.48	0.998	0.16	1−5	5	0.0056	46.8	0.989	0.86
resorcinol	2	110.1	1−6	5	0.020	15.8	0.996	0.59	1−6	6	0.0049	10.6	0.997	0.44
β-caroten	0	536.9	1−5	4	1.1×10^{-3}	3.16	0.998	0.47	1−6	6	3.8×10^{-5}	6.68	0.990	0.83

[a] FPH, free phenolic hydroxyl group; FW in g mol $^{-1}$; LR (linear range) $\times$ 1.10^6 mol L^{-1}; LD (limit of detection) in μmol L^{-1}; *n* = number of points. Stock solutions (1 mM) prepared in water except quercetin and resveratrol (50:50 v/v water:ethanol) and β-caroten (ethanol). [b] Path length = 0.5 cm.

such as cathecol and 4-methyl cathecol decompose rapidly in alkaline medium (*13*).

Table 1 reports the results for all samples using the BCA and the Folin−Ciocalteu methods. One series of experiments was carried out to determine the total polyphenols contents, and another was made to evaluate the unprecipitated polyphenols after precipitation with casein (*11*) (see the Materials and Methods). The tannins present in these samples were obtained from the difference between the total polyphenols and the unprecipitated polyphenols values. The values obtained for *H. virginiana* L., *S. adstringens* (Mart.) Coville, and *M. ilicifolia* Mart. ex.Reissek using the Folin−Ciocalteu method agree with those of the literature (*11*). For the others samples [*H. bonariensis* Lam, *A. muricata* L., *M. cauliflora* (Mart.) O. Berg., *C. sylvestris Sw*., and *S. terebinthifolia* (Raddi)], the values for total polyphenols, unprecipitated polyphenols and tannins were not still standardized and consequently not available (*11*). From **Table 1**, it can be also seen that for *M. cauliflora* (Mart.) O. Berg. the results obtained by both methods are in very good agreement, although tannin values were similar only for *M. ilicifolia* Mart. ex Reissek, *H. bonariensis* Lam, *M. cauliflora* (Mart.) O. Berg., and *C. sylvestris Sw*.

To find the reasons for such differences, calibration curves with several standard polyphenols and β-caroten solutions were obtained by using BCA and Folin−Ciocalteu methods (**Table 2**). As the BCA method is more sensitive, a cell with a path length of 0.5 cm was used, and for easier comparison, the angular coefficients (*b*) of all of the calibration curves were multiplied by two.

It is possible to verify that for both methods (Folin−Ciocalteu and BCA), the sensitivity increases with the number of free phenolic hydroxyl (FPH) groups, as can seen by comparing the (*b*) values, as it was observed in another study dealing with Folin−Ciocalteu reagent (*14*). It can also be noted that, except for the resveratrol, BCA is more sensitive than the Folin−Ciocalteu method for polyphenols with FPH group ≥3 [tannic acid, (−)-epigallocatechin gallate, quercetin, gallic acid, and pyrogallol] and also for β-carotene. On the other hand, the difference in the (*b*) values found between (−)-epigallocatechin gallate and quercetin in both methods was not so different considering only the FPH group, showing that the sensitivity may also be related with

Table 3. Recovery Rates from Some Aqueous Extracts of Plant Samples Obtained with Different Quantities of Tannic Acid Added and with BCA Assay

	tannic acid concentration (μmol L^{-1})			
plants	diluted aqueous extracts sample	added	found	recovery (%)
H. virginiana L.	0.31	0.50	0.58	72
		0.60	0.79	87
S. adstringens (Mart.) Coville	0.61	0.40	0.93	92
		0.50	1.04	94
		0.60	1.07	88
M. ilicifolia Mart. ex Reissek	0.10	0.40	0.46	92
		0.50	0.67	111
		0.60	0.74	106
H. bonariensis Lam	0.38	0.50	0.71	80
		0.60	0.88	90
A. muricata L.	0.22	0.40	0.55	89
		0.50	0.70	97
		0.60	0.77	94
M. cauliflora (Mart.) O. Berg	0.81	0.50	1.02	78
		0.60	1.19	85
C. sylvestris Sw.	0.81	0.50	1.16	89
		0.60	1.38	98
S. terebinthifolia (Raddi)	0.80	0.20	0.21	107
		0.30	0.29	97
		0.40	0.38	99

Table 4. Recent Selected Spectrophotometric Studies of Polyphenolic Compounds Determination in Plants Extracts

material	remarks	ref
Acacia mangium Willd., *Aleurites moluccanus* (L.) Willd., *Anthocephalus cadamba* (Roxb.) Miq., *Artocarpus integrifolia* L.f., *Astronium* sp., *Ceiba pentandra* (L.) Gaertner, *Cocos nucifera* L., *Curculigo capitulata* (Lour.) Kuntze, *Diospyros* sp., *Dipterocarpus* sp., *Dryobalanops* sp., *Durio zibethinus* Rumph. ex Murrray, *Dyera costulata* Hook.f., *Gonystylus bancanus* (Miq.) Kurz, *Knema* sp., *Manilkara* sp., *Parashorea* sp., *Populus nigra* L., *Shorea leprosula* Miq., *Shorea* (*Rubroshorea*) sp., *Shorea* spp., *Sindora walichi* Benth., *Tamarindus indica* L., and *Vatairea* sp.	qualitative analysis of TPC[a] with FC[b]; flavonol with vanillin-HCl; standards used are not mentioned	*18*
Olea europea L.	detection of TPC in *n*-hexane with FC expressed as mg/kg of caffeic acid	*19*
Actinidia deliciosa (A. Chev.) C. F. Linag & A. R. Ferguson	detection of TPC with FC, expressed as mg of gallic acid; flavonoids with $NaNO_2/AlCl_3$, expressed as mg of (+)-catechin	*20*
Arrabidaea chica var. *cupnea* (Cham.) Bur. & K. Schum., *Bauhinia forficata* var. *latifolia* Benth., *Bauhinia macrostachya* Benth., *Byrsonima crassifolia* (L.) Kunth., *Cecropia obtusa* Trécul, *Cecropia palmata* Willd., *Cedrela odorata* L., *Cordia exaltata* Lam., *Dalbergia monetaria* L.f., *Dalbergia subcymosa* Ducke, *Davilla kunthii* A. St. Hil., *Davilla rugosa* Poir., *Eugenia patrisii* Vahl, *Inga edulis* Mart., and *Stryphnodendron barbatimam* Mart.	detection of TPC with FC, expressed as mg of gallic acid; flavonoids using *p*-dimethylamino cinnamaldehyde	*21*
Dicksonia sellowiana Hook	detection of TPC with FC, expressed as mg of gallic acid	*22*
Quercus robur L.	analysis of TPC with FC, expressed as gallic acid; butanol-HCl assay for proanthocyanidins	*23*
Quercus alba L., *Quercus coccinea* Muenchh., *Quercus rubra* L., *Quercus velutina* Lam., *Acer saccharum* Marshall, *Acer rubrum* L., *Acer pensylvanicum* L., *Betula lutea* F. Michx., *Ostrya virginiana* (Mill). K. Koch, *Fraxinus americana* L., *Cornus florida* L., *H. virginiana* L., *Viburnum acerifolium* L., and *Vaccinium angustifolium* Aiton	comparative study using FD[c], FC, and PB[d] in the detection of TPC, with three kinds of purified tannins	*24*
Hordeum vulgare L.	detection of TPC using FC in hydroethanolic medium, expressed as mg of gallic acid/kg material	*25*
Helichrysum arenarium (L.) Moench	detection of TPC with FC, expressed as pirogallol; total flavonoids with $AlCl_3$ in ethyl acetate medium	*26*
Cynara scolymus L.	detection of the TPC with FC, in ethanol medium, expressed as mg of cathecol/100 g material	*27*
Ocimum basilicum L.	detection of the TPC with FC, in ethanol medium, expressed as mg of cathecol/100 g material	*28*
Plantago L. species	detection of the TPC with FC, in methanol medium, expressed as mg of gallic acid/100 g material	*29*
olive mill wastewater	detection of the TPC with FC, in methanol medium expressed as % of tannins	*30*
H. virginiana L., *M. ilicifolia* Mart. ex Reissek, *H. bonariensis* Lam, *A. muricata* L., *M. cauliflora* (Mart.) O. Berg. *C. sylvestris Sw*., *S. terebinthifolia* (Raddi), and *S. adstringens* (Mart.) Coville	detection of the TPC with BCA test in aqueous medium, expressed as % of tannic acid	this work

[a] Total polyphenol content. [b] Folin−Ciocalteu reagent. [c] Folin−Denis reagent. [d] Prussian blue reagent.

the reactivity of the phenolic hydroxyl group. In fact, taking into account the benzenetriols (FPH = 3), it was pointed out that pyrogallol (1,2,3-benzenetriol and reacting group = 2) is 1.9 more sensitive than phloroglucinol (1,3,5-benzenetriol and reacting group = 1) when the Folin−Ciocalteu reagent was used (*14*). For the BCA assay, this ratio increases to 2.6.

Considering still the benzenetriols, no significant difference on the (*b*) values was found for phloroglucinol (1,3,5-benzenetriol) using both methods, while for pyrogallol (1,2,3-benzenetriol), the BCA assay is about 1.8 times more sensitive than the Folin−Ciocalteu.

For the benzenediols (FPH = 2), a higher (*b*) value was found for hydroquinone (1,4-benzenediol) with BCA assay. The order of the sensitivity for these diphenolic compounds for BCA assay is hydroquinone > pirocathecol > resorcinol, but the opposite is found by using the Folin−Ciocalteu reagent (resorcinol > pirocathecol > hydroquinone). The difference of the sensitiveness for these compounds with Folin−Ciocalteu and BCA methods may explain the divergent results obtained with the most of the samples studied.

To verify the consistency of the BCA assay, the recovery rates of some tannic acid spiked (0.20, 0.30, 0.40, 0.50, and 0.60 μmol L^{-1}) to eight samples [*H. virginiana* L., *M. ilicifolia* Mart. ex Reissek, *H. bonariensis* Lam, *A. muricata* L., *M. cauliflora* (Mart.) O. Berg., *C. sylvestris Sw*., *S. terebinthifolia* (Raddi), and *S. adstringens* (Mart.) Coville] were estimated. The data presented in **Table 3** show recoveries within 72−111%, with a mean value of 92.3%.

To better understand the matrix effect in the present method, the calibration curves obtained by the multiple standard additions were compared with the analytical curves. Parallel straight lines were obtained with angular coefficient variations of 1.0% (*H. bonariensis* Lam), 2.3% [*M. cauliflora* (DC.) Berg], 3.7% (*A. muricata* L.), 3.8% [*S. terebinthifolia* (Raddi)], 6.4% (*H. virginiana* L.), 8.0% (*C. sylvestris*), 12% [*S. adstringens* (Martius) Coville], and 14% (*M. ilicifolia* Mart. ex Reissek). These results indicate that the proposed method can be used as an alternative for polyphenols estimation in aqueous extracts of plants rich in polyphenols.

Others reducing agents, like hydroxylamine hydrochloride, hydrazine sulfate, bisulfite, uric and ascorbic acids, and some reducing sugars reduce Cu(II) to Cu(I) in a buffered solution (pH 6.0−8.0) containing BCA. However, these species, if present in the aqueous extracts of plants, are usually in lower

concentrations than the polyphenols, and a considerable interference is not expected. In fact, in our previous work, no interference up to 50-fold of glucose, sucrose, and caffeine and 10-fold excess of ascorbic acid and bisulfite (*6*) was observed.

Some other methods for measuring polyphenols in plant extracts involve expensive techniques, like NMR (*15*) and HPLC (*16*), or take a long time for execution, like a gravimetric test (*17*), which makes the spectrophotometric analysis more attractive.

The Folin−Ciocalteu (or Folin−Denis) reagent has been suggested to determine the total polyphenols in several extract plant samples (*1*), but disagreement was pointed out concerning some experimental details such as the waiting time for the absorbance measurements time and the wavelength used (*1*, *11*).

Some procedures for the spectrophotometric determination of polyphenols in plant extracts are shown in **Table 4** (*18*−*30*). It can be seen that according to the selected study, the final values were expressed as tannic, gallic, or caffeic acids and pirogallol concentrations, so the calibration curves were obtained using different phenolic compounds to estimate the total content of polyphenols. However, the reactivity of these different standards is not the same; it means that the concentration of the total polyphenols obtained by the procedures listed may not be strictly compared, unless the standard used and the polyphenolic compounds present in the samples have identical calibration sensitivity.

Indeed, the comparative study of the total polyphenols amount in olive mill revealed that the Folin−Ciocalteu method can overestimate the polyphenols content depending on the substance used as the reference (*30*), and the same could be observed in **Table 1**. Moreover, different extraction procedures and the used solvents certainly have an influence on the polyphenolic compounds quantification of any plant material (*19*, *26*−*29*).

In addition, disagreements were observed by comparing the results obtained with the Folin−Ciocalteau and HPLC methods (*30*). In that case, a correction factor, calculated from the mean analytical response of a series of reference substances (veratric acid, vanillic acid, catechol, resorcinol, and 4-hydroxyphenylacetic), was used to achieve a more reliable estimate of total polyphenols.

Conclusions. As is well-known, the amount and the nature of the phenolic compounds found in plant species diverge from many aspects, including seasonal variation, soil composition, foliage quality, sample preparation, extracting solvent, and evaluation method (*15*). Moreover, because heterogeneous mixtures of these natural polyphenolic compounds (e.g., condensed and hydrolyzable tannins, phenolic acids, cathecol, resorcinol, flavonoids, and caffeic acid) may be present, a different chemical reactivity could be expected and thus raise some analytical difficulties.

However, in spite of the fact that one simple protocol has not been recommended to quantify the total phenolic compounds in plant materials (*15*), the procedure described here is rapid and reliable and the results expressed as tannic acid may be useful in characterizing plants rich in polyphenols.

SAFETY ASPECTS OF THE Cu(I)/BCA TEST

The BCA toxicological properties have not been totally studied, but it irritates the eyes and mucous membranes, and it is supposed to be toxic if ingested, so it must be conveniently discarded. The total waste volume for each determination consumption (5 mL) of Cu(II), BCA, and $C_2H_7NO_2$ is approximately 0.2 mg, 3 mg, and 0.7 g, respectively. However, the waste obtained after many BCA analysis can be recycled through a simple procedure. The solution should be alkalized up to pH 10 with 3.0 mol L^{-1} sodium hydroxide solution to precipitate the entire copper ion. After the copper hydroxo compounds are removed by filtration, the remaining solution must be acidified with a 2.0 mol L^{-1} hydrochloric acid solution; then, the insoluble protonate BCA is filtered and must be recrystallized twice or three times in water before new use.

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