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Rapid Determination of Polyphenols and Vitamin C in Plant-Derived Products

STEPHANE GEORGÉ,*,† PIERRE BRAT,‡ PASCALINE ALTER,‡ AND MARIE J. AMIOT§

Centre Technique de la Conservation et des Produits Agricoles (CTCPA), Site Agroparc, F-84911 Avignon Cedex 9, Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Département FLHOR, TA50/16, F-34398 Montpellier Cedex 5, and Human Nutrition and Lipids, Faculté de Médecine, UMR U-476 INSERM (National Institute of Health and Medical Research)/U-1260 INRA (National Institute for Agronomic Research), 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France

Polyphenols, widely spread in our diet by the consumption of plant food products, are commonly determined using Folin−Ciocalteu reagent that interacts with other different reducing nonphenolic substances and leads to an overestimation of polyphenol content. In this paper we report an optimized Folin−Ciocalteu method to specifically determine the contents of total polyphenols and vitamin C. After the optimal conditions for the colorimetric assay were set, solid-phase extraction (Oasis HLB (hydrophilic−lipophilic balance)) was carried out to eliminate the water-soluble reducing interferences including vitamin C. Colorimetric correction was thus performed by subtracting interfering substances contained in the water washing extract from the raw extract. Moreover, vitamin C present in the water washing extract can be destroyed by heating and thus colorimetrically deduced. This procedure was set up with synthetic solutions and validated on different extracts from fruit products.

KEYWORDS: Polyphenols; vitamin C; Folin−Ciocalteu; colorimetry; solid-phase purification; fruit; puree; juice

INTRODUCTION

During the past decade the interest in polyphenols, including flavonoids, has considerably increased, especially by nutritionnists, epidemiologists, and food manufacturers. This is mainly due to the discovery of various biological properties, namely, their antioxidant effects and thereby their possible role in the prevention of several chronic diseases involving oxidative stress (*1*, *2*). Polyphenols are the most abundant antioxidants in our diet, since the average daily intake is about 1 g, which is almost 10-fold the intake of vitamin C, 100-fold the intake of vitamin E, and 500-fold the intake of carotenoids (*3*). Foods and beverages rich in polyphenols may have a large potential with respect to prevention of diseases. This is the case for fruits and vegetables associated with the prevention of stroke (*4*) and cancers (*5*) and for tea (*6*) and soy that may protect breast cancer (*7*). A food datababase on flavonoids, a large class of polyphenols, was recently published by the USDA, http://www.nalusda.gov/fnic/foodcomp, based on the quality evaluation system reported by Holden et al. (*8*). Such a database is extremely useful for epidemiological studies on the relationship between dietary flavonoids and health. However, it remains extremely difficult to estimate the average content daily intake of total polyphenols. In fact, polyphenols include other subclasses besides flavonoids, such as phenolic acids, stilbenes, lignans, tannins, and oxidized polyphenols. Many of these compounds display a large diversity of structures and escape quantification, usually carried out by HPLC and diode array detection (*9*), because (i) there is lack of commercial standards and (ii) numerous structures are not yet elucidated. In these cases, the total polyphenol content is usually underestimated. Recently, Vinson et al. (*10*−*12*) reported data on the total polyphenol content of various fruits and vegetables. This content was colorimetrically measured by the Folin−Ciocalteu reaction after correction for ascorbic acid contribution. In fact, ascorbic acid is certainly the main reducing agent, which can interfere in the Folin−Ciocalteu reaction. However, other reducing substances such as some sugars and amino acids could also interfere.

The aim of this study was to set up a rapid colorimetric method to quantify total polyphenols in various fruits, vegetables, and derived products (purees, juices). The initial step was the optimization of the colorimetric method using Folin−Ciocalteu reagent. To quantify total polyphenols, solid-phase extraction was performed. An additional step is proposed to estimate vitamin C content. Results obtained with synthetic solutions were validated with some foods and beverages.

MATERIALS AND METHODS

Sample Preparation. All of the food products (apple purees and juices) were purchased at a local market and stored at 4 °C until analysis.

* To whom correspondence should be addressed. Phone: +33(0) 490841709. Fax: +33(0) 490841726. E-mail: sgeorge@ctcpa.org.
† CTCPA.
‡ CIRAD.
§ UMR U-476 INSERM/U-1260 INRA.

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Material and Solvent Standards. All solvents were of the highest analytical grade. Reference compounds were from Extrasynthèse (Genay, France) and reagents and solvents from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France).

Cartridges. The SPE cartridge was an Oasis HLB from Waters (Milford, MA). HLB is an acronym for hydrophilic−lipophilic balance, the solid-phase being a copolymer [poly(divinylbenzene-*co*-*N*-vinylpyrrolidone)]. After any analysis, the cartridge was conditioned with 4 mL of pure methanol and rinsed with 2 × 4 mL of water.

Preparation of Raw Extracts. Purees or juices (10−20 g) were homogenized with the same extraction solution (acetone/eau, 7/3, v/v) for 30 min. Mixture supernatants were then recovered by filtration (Whatman, England) and constituted the raw extracts (REs).

Separation of Polyphenol and Other Water-Soluble Components by Solid-Phase Extraction. REs were added with distillated water to reduce the proportion of acetone to 7%. Diluted REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (reducing sugars, ascorbic acid) were recovered with 2 × 2 mL of distillated water. The recovered volume of the washing extract (WE) was carefully measured.

Elimination of Vitamin C from WE. Heating was carried out on the washing extract (3 mL) for 2 h at 85 °C (Fisons Haake N2 oil bath) and led to the heated washing extract (HWE).

Folin−Ciocalteu Assay. All extracts (RE, WE, and HWE) were submitted to the Folin−Ciocalteu method (*13*), adapted, and optimized: A 2.5 mL sample of water-diluted Folin−Ciolcateu reagent (1/10) was added to the different extracts. The mixture was incubated for 2 min at room temperature, and 2 mL of sodium carbonate (75 $g \cdot L^{-1}$) was added. The mixture was incubated for 15 min at 50 °C and finally cooled in a water−ice bath. The specific absorbance at 760 nm was immediately measured.

Determination of Total Polyphenols and Vitamin C and Expression of the Results. Total polyphenols, determined by subtracting gallic acid equivalent from RE from that of WE, were expressed as mg of gallic acid/100 g of product (slp = 0.012, $R^2 = 0.99$). Linearity was obtained between 50 and 500 mg/L corresponding to absorbance values between 0.1 and 0.6. Vitamin C, determined by subtracting ascorbic acid equivalent from WE from that of HWE, was expressed as mg/100 g of product (slp = 0.008, $R^2 = 0.99$). Linearity was obtained between 50 and 1000 mg/L corresponding to absorbance values between 0.1 and 0.6.

All the experiments were performed in triplicate.

HPLC Analysis. Vitamin C was quantified by HPLC (Waters system) using an isocratic gradient equipped with a reversed-phase C_{18} column (Waters, Spherisorb ODS 2) (5 μm packing) (250 × 4.6 mm id). Ascorbic acid was eluted under the following conditions: injected volume 20 μL; oven temperature 30 °C; solvent mixture K_2HPO_4 (0.1 M), KH_2PO_4 (0.08 M), MeOH (55/25/20, v/v/v). The flow rate was 1.5 mL min^{-1}, and the total elution time was 10 min. Detection was performed by a III-1311 Milton Roy fluorimeter (Ivyland, PA) with $\lambda_{excitation} = 350$ nm and $\lambda_{emission} = 430$ nm. Quantification was carried out by external calibration with ascorbic acid. The calibration curve was set from 1 to 7 μg/mL ascorbic acid.

An HPLC instrument (Agilent 1100 Series) with diode array detectors was used to check phenolic compounds in each extract according to the following method: column, 4.6 × 150 mm C_{18} (Alltima 5 μm, Alltech) with a C_{18} column guard with the same packing; polyphenols were eluted with a gradient of water (0.5% (v/v) formic acid) and acetonitrile. In the first 10 min, acetonitrile was 10%, from 10 to 35 min, acetonitrile increased from 10% to 50% and from 35 to 50 min acetonitile increased to 100%. The total elution time was 50 min, and the flow rate was 1.0 mL min^{-1}.

RESULTS AND DISCUSSION

Folin−Ciocalteu Optimization. Folin−Ciocalteu reagent, a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol oxidation. This reaction occurring under alkaline conditions is carried out with sodium carbonate. Blue coloration is followed at 760 nm and reflects the quantity of polyphenols usually expressed as gallic acid equivalent (GAE) or catechin equivalent. The first step of our study was to optimize the experimental conditions of this reaction: influence of the extraction solvent of polyphenols in the colorimetric reaction mixture, reaction kinetics after addition of the reagents (FC and sodium carbonate) under different temperatures. Generally, polyphenols are extracted with methanol/water or acetone/water (*14*). The proportion of the two organic solvents, methanol and acetone, were tested in FC reaction. Dilution was necessary prior to the colorimetric reaction. In fact, the maximum organic solvent (methanol or acetone) percentage was 7% without interference on the colorimetric reaction. Finally, all the extractions were performed with acetone/water (70/30, v/v), and then the mixture was water diluted 10-fold before colorimetric reaction. The kinetic reaction (from 2 to 20 min) after addition of FC reagent (10-fold diluted in water) was tested. Two minutes at room temperature was fixed for the reaction time after addition of FC reagent. After addition of sodium carbonate, the maximum stable color response was obtained for a 15 min reaction time at 50 °C (water bath). It could be stressed that a decline of the coloration started after 30 min.

Table 1. Colorimetric Response (Absorbance due to Folin–Ciocalteu Reaction) before and after Methanol Elution of Different Polyphenol Solutions Using the Oasis HLB Cartridge

phenolic compound	quantity (μg)	initial absorbance	absorbance after methanol elution	percentage of recovery	no. of assays
gallic acid	420	0.383	0.348	91	4
caffeic acid	330	0.236	0.222	94	3
chlorogenic acid	459	0.248	0.213	86	3
catechin	345	0.362	0.279	77	4
polymer	183	0.141	0.091	65	4
naringenin	390	0.087	0.087	99	3
naringin	390	0.085	0.084	98	3
phloretin	954	0.493	0.351	71	3
phloridzin	486	0.174	0.154	88	3
quercetin	462	0.394	0.315	80	3
quercitrin	255	0.174	0.138	79	3
rutin	424	0.207	0.183	88	3
luteolin	17	0.145	0.139	96	3
luteolin 7-glucoside	34	0.045	0.048	100	3
cyanidin	148	0.076	0.054	71	4
cyanin	216	0.355	0.338	95	4

Results Expression. Under this optimized reaction, different standards (phenolic or not) were tested at different concentrations. In reference to gallic acid, catechin, caffeic acid, naringenin, and rutin gave similar responses while glyscosides of naringenin and hesperetin (naringin and hesperidin) displayed a lower absorbance (~2-fold lower). Such behavior could lead to an underestimation of these compounds. Vitamin C exhibited a slightly lower response than gallic acid (~80% of the gallic acid absorbance at the same concentration). By contrast, carotenoids appeared to exhibit a higher response value (~2- or 3-fold higher than that of gallic acid). We can conclude that, in extracts rich in carotenoids, Folin−Ciocalteu could more or less overestimate the polyphenol content. However, carotenoids are poorly extracted with more polar solvents, suggesting their low contribution in the colorimetric assay.

Solid-Phase Extraction. Different cartridges were assayed as described by Antolovich and Robards (*15*), and only the Oasis HLB gave exploitable results. Different polyphenols were tentatively eluted with methanol after their adsorption on the solid phase of Oasis HLB (**Table 1**). The recovery percentage greatly varied according to the structures. For naringenin and its glycoside naringin, the recovery reached close to 100%. By contrast, catechin was less eluted; 23% of catechin was

Table 2. Total Polyphenols and Vitamin C Content as the Mean of Three Replicates (mg (100 g of fresh weight)$^{-1}$) in the Different Products

food and beverage	total polyphenols[a]	vitamin C[a]	vitamin C[b]	vitamin C[c]
apple puree 1	96.0 (97.7–94.3)[d]	29.3 (30.1–28.5)	40.9 (42.1–39.7)	43.5 (45.6–41.3)
apple puree 2	63.5 (65.2–62.0)	10.4 (11.8–9.0)	14.5 (15.9–12.9)	14.9 (15.5–14.3)
apple juice	57.1 (59–55.2)	24.4 (25.7–23.1)	34 (35.5–32.5)	37.2 (39.1–35.3)
orange juice	19.5 (20.9–18.1)	20.7 (22.1–19.3)	28.8 (30.8–27.0)	34.6 (35.9–33.2)
tomato juice	18.4 (20.1–16.7)	4.1 (5.8–2.4)	5.6 (7.9–3.3)	3.2 (3.4–3.0)

[a] Expressed in gallic acid equivalent (GAE) and determined by Folin–Ciocalteu method. [b] Expressed in ascorbic acid equivalent and determined by Folin–Ciocalteu method. [c] Expressed in ascorbic acid equivalent and determined by HPLC. [d] Values in parentheses are the minimum and maximum values.

retained on the column. The retained percentage was greater for polymerized catechins (procyanidins), 35%. In addition, other flavonoids as aglycon forms (phloretin, quercetin, cyanidin) appeared to be poorly eluted with methanol. Other elution solvents (acetonitrile, methanol/methyl tributyl ether, dichloromethane) were tested without more success. We conclude that the normal protocol using the Oasis cartridge cannot be applied for the complete desorption of total polyphenols, especially when plant food products are particulary rich in tannins. In regard to these results, the total polyphenol content was only assayed by subtracting the interfering reducing substances from the Folin−Ciocalteu response. Because in fruits and their derived products, vitamin C is known to be the main interfering reducing substance in the colorimetric assay, the efficiency of vitamin C washing by water was checked using the Oasis cartridge. Vitamin C was totally eluted with 2 × 2 mL of water. The WE did not contain any polyphenol tested (caffeic and gallic acid, cyanidin, and catechin), which was confirmed by HPLC. In a manner similar to that of vitamin C, cysteine was totally eluted during the washing step. In addition, Oasis HLB cartridges can be used 5-fold after its regeneration with 3-fold of 2 mL of methanol followed by 2-fold of 2 mL of water.

Colorimetric Assay of Vitamin C Using Folin−Ciocalteu. As previously shown, ascorbate could interfere in the Folin−Ciocalteu reaction. Its concentration cannot be directly measured by the FC method on the washing extract, which could contain other reducing substances. As reported by Vinson et al. (*12*), vitamin C could be separately assayed by HPLC. However, because vitamin C is known to be highly thermolabile, a thermal treatment was chosen as an easier way for its determination. By subtracting the FC response of the HWE from that of the WE, we thus obtained the concentration of vitamin C. Optimal conditions to eliminate the totality of vitamin C in synthetic solutions were 2 h of heating at 85 °C. It could be noted that no browning color was developed during such heating conditions. To quantify total polyphenols and vitamin C, we propose the scheme reported in **Figure 1**.

Application to Food Products and Beverages. Correction by subtracting interfering substances appeared necessary to get appropriate values for polyphenol determination. In fact, for apple purees and juice, the contribution of the interfering substances varied from 31% to 48% (**Figure 2**). This contribution was higher for orange juice (77%) and tomato juice (70%). As expected, numerous reducing compounds could interfere in the quantification of polyphenols by the FC method. Among them, vitamin C was supposed to have the major contribution. This was the case in one-apple derived product (enriched in vitamin C). However, the contribution of vitamin C was 32% of the total interferences for orange juice and 46% for apple juice (**Table 2**). The contribution of vitamin C was remarkably

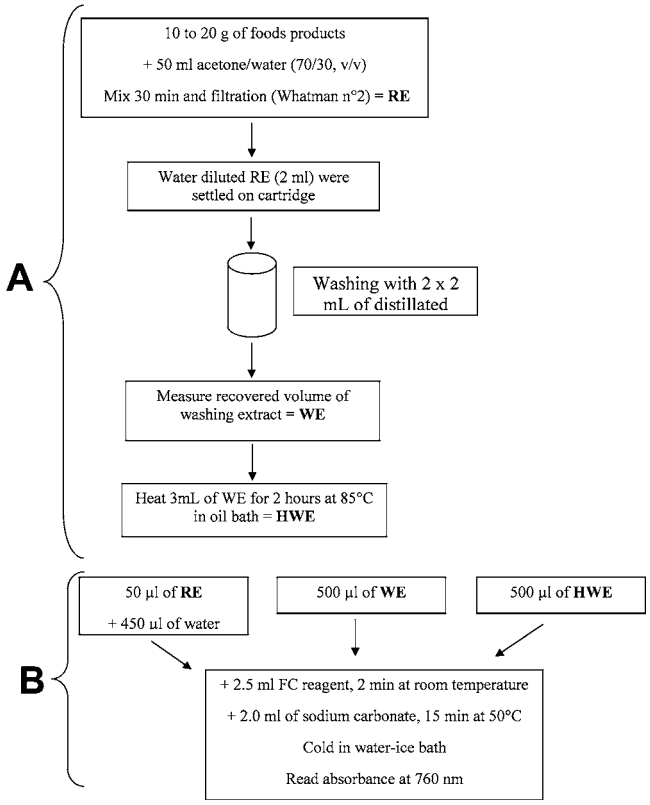

Figure 1. Flowchart of the polyphenol and vitamin C determination procedure (**A**, obtention of different extracts; **B**, Folin–Ciocalteu reaction).

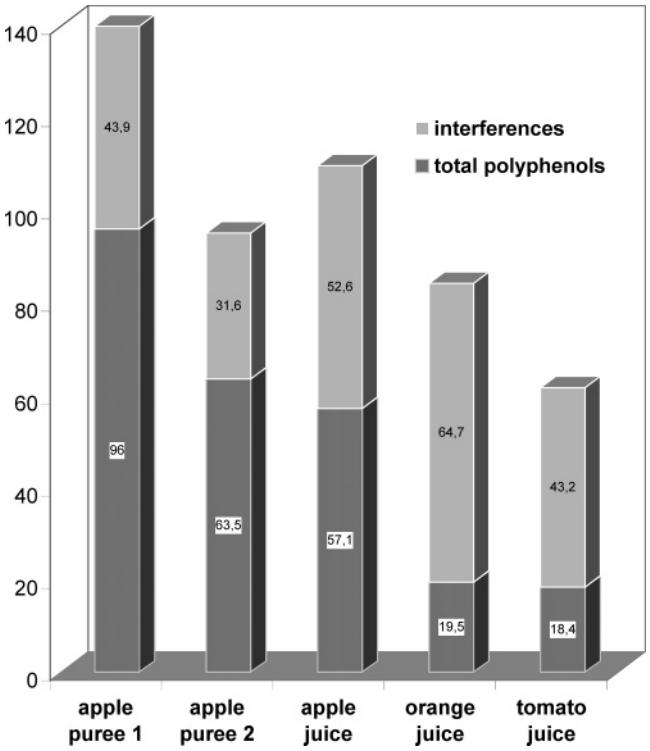

Figure 2. Total polyphenols and interferences in the different products (mg (100 g of fresh weight)$^{-1}$).

low in tomato juice, vitamin C being only 9% of the total interferences expressed as GAE.

In addition to the polyphenol quantification, the heating step in the present colorimetric procedure could be proposed to quantify vitamin C. This approach gave values which were in good agreement with those obtained by HPLC. However, it

could be underlined that for low values of vitamin C an overestimation was observed.

In conclusion, this study proposes a rapid colorimetric method using Folin−Ciocalteu reagent to estimate the total polyphenol content of foods and beverages, after removal of interfering nonphenolic reductants. Even HPLC is a suitable method to quantify each phenolic compound, it cannot correctly estimate the total polyphenol content, as previously described in the Introduction.

In the present method, food products require minimal sample preparation before polyphenol analysis, by using specific solid-phase extraction on minicartridges. In addition, vitamin C quantification could be concomitantly performed in this procedure. The proposed method could be useful for a large panel of applications (epidemiological studies, composition tables, industry, ...). This approach could also be employed for human fluids (plasmas and urines). Such studies are currently being carried out.

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