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Effects of Temperature and Time on Polyphenolic Content and Antioxidant Activity in the Pressurized Hot Water Extraction of Deodorized Thyme (*Thymus vulgaris*)

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Supporting Information

ABSTRACT: The effects of temperature (50−200 °C) and contact time (5−30 min) on the pressurized hot water extraction of deodorized thyme were explored for antioxidant activity, polyphenol profiles, and total antioxidants. Six not previously reported polyphenolic compounds were identified in thyme. An inverse correlation was found between the antioxidant activity and total antioxidants with the amount and diversity of polyphenols. The highest total extract yield and antioxidant activity were obtained at 200 °C, although maximum polyphenol extraction yields of hydroxycinnamic acids, flavones, flavonols/flavanones, and total polyphenols were detected at 100 °C and 5 min. Higher temperatures and longer exposure times reduced extract polyphenol diversity. Dihydroxyphenyllactic acid was the only phenolic compound for which extraction yield increased with temperature, probably as a product of the thermal degradation of rosmarinic acid. Consequently, for extracting phenolics from thyme, 100 °C and 5 min would be appropriate operating conditions, whereas antioxidant-active nonphenolic compounds were favored at higher temperatures and exposure times.

KEYWORDS: *pressurized hot water, thermal degradation, rosmarinic acid, Thymus vulgaris, LC-MS*

INTRODUCTION

Supplementing human diets with natural extracts and foods rich in antioxidants protects against several oxidation-related diseases.[1] These beneficial effects are mainly attributed to polyphenols, the most abundant secondary metabolites present in fruits, vegetables, herbs, and spices.

Natural polyphenols show a large diversity of structures, ranging from fairly simple molecules to complex polymers, with or without glycosylation and/or esterification.[2] Polyphenols may be classified into different groups depending on the number of phenol rings and the structural components that bind them together. Four main classes are (i) phenolic acids, (ii) flavonoids, (iii) stilbenes, and (iv) lignans.[3] Their antioxidant activity depends on many structural factors such as number and position of the hydroxyl groups and the degrees of glycosylation, esterification, and polymerization.[4]

Organic solvents, given their solvating properties, are commonly used to extract polyphenols from plants at large scale. Such processes are not environmentally friendly, because it is difficult to eliminate all trace solvent from the extracts obtained. Water, instead, is a nonflammable, nontoxic, and readily available solvent; hence, safe extracts can be attained through environmentally friendly processes. Yet water is not commonly used as an extraction solvent for plant materials because, as a polar solvent, it cannot efficiently dissolve most organic compounds found in plants.[5] Indeed, given the prevalence of nonionic bonds in their organic structure, most typical polyphenols have relatively low solubility in water at ambient conditions.[6] Nevertheless, it is possible to manipulate the properties of water by changing the temperature and thereby improving extractability.[7,8] The method involves raising the water temperature to between 100 and 374 °C while applying sufficient pressure to maintain water in a liquid state, pressurized hot water. The polarity of water declines dramatically with increasing temperature, due to the breakdown of its hydrogen bonds, and reaches values comparable to those of organic solvent−water mixtures. The lower viscosity and surface tension of water at high temperatures also increase mass transfer rates of compounds from the matrix.[8] Both temperature and pressure play a significant role in the disruption of surface equilibrium, decreasing the activation energy required for the desorption process,[5] and such behavior affords a basis for using pressurized hot water to replace organic solvents in the extraction processes.[8]

In the pressurized hot water extraction (PHWE) of polyphenols from plants, the relationship between antioxidant activity and polyphenol content is disputed, however.[9,10] The different positions may be due to diverse phenomena, such as thermal degradation, selective extraction of polyphenols, or the formation of neo-antioxidant compounds, which are highly dependent on temperature and extraction time.[11,12] Irrespec-

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tive, depending on the PHWE conditions used, it is possible to obtain extracts with different compositions, chemical activities, and, consequently, different bioactive properties. To define suitable operating conditions for a given PHWE process, it is necessary to predetermine the qualitative and quantitative effects of a wide spectrum of extraction conditions.

Despite the many works in the literature on PHWE of spices,[10,11,13] there is no comprehensive study that analyzed the impact of extraction conditions on both antioxidant activity and content of a wide spectrum of polyphenols.

In this research, a widely consumed spice, thyme (*Thymus vulgaris* L.), was considered as a case study. Biofunctional properties of thyme (antimicrobial, antifungal, and antioxidant) are generally attributed to its essential oil content.[14,15] Around 65 different polyphenolic compounds have been identified in the genus *Thymus*.[16] Even after extraction of the essential oil from thyme, the remaining solid contains polyphenolic compounds offering biological activity,[17] ranging from nutritional[18] to strong anticarcinogenic properties.[19,20] Previous research works that applied water to extract polyphenols from thyme leaves[21,22] and deodorized thyme leaves[17] limited the maximum extraction temperature to 100 °C.

In this work we aim to unmask the underlying relationship between the antioxidant capacity and polyphenolic composition of deodorized ground *T. vulgaris* extracts obtained under several PHWE conditions.

MATERIALS AND METHODS

Chemicals. All organic solvents were of HPLC gradient grade. Methanol and formic acid was purchased from Merck (Darmstadt, Germany), acetonitrile was obtained from Scharlab (Barcelona, Spain). Reagents and standards used were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin−Ciocalteu reactive, and sodium carbonate were purchased from Merck. Tripyridyl triazine (TPTZ), $FeCl_3(6H_2O)$, fluorescein (FL,), pyrogallol red (PGR), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, gallic acid, caffeic acid, and quercetin were obtained from Sigma (St. Louis, MO, USA). Apigenin was purchased from Extrasynthese (Genay, France).

Thyme Origin and Hydrodistillation Treatment. Thyme (*T. vulgaris* L.) was obtained from an organic farm in region X, Chile. A thyme herb sample (1 kg) was submitted to a process of steam distillation with distilled water for 3 h using a modified essential oil extractor (Steam Distillation Essential Oil Extractor, Figmay S.R.L.). Deodorized thyme from the hydrodistillation was separated into leaves and branches. The leaves were dried to reach equilibrium humidity (10% w/w) in a drying cabinet with forced ventilation at ambient temperature for 2 days. The dried leaves were ground in a four-knife mill to a particle size below 355 μm. The powder was stored at −4 °C until extraction.

PHWE. A sample of 5.00 g of dry leaf powder was mixed with neutral quartz sand to disperse the powder, reduce the volume of water used for the extraction, and avoid filter clogging in the extraction cell. The mixture was then placed in a 100 mL stainless steel extraction cell. The plant material was extracted with about 100 mL of Milli-Q water in an Accelerated Extraction Equipment (ASE 150, Dionex, Sunnyvale, CA, USA). We performed a full factorial design in triplicate at 1500 psi with two factors, temperature and time, with four and three levels, respectively. The operating conditions assessed were extraction temperature (50, 100, 150, and 200 °C) and extraction time (5, 15, 30 min). After extraction, the cell content was rinsed with 100 mL of Milli-Q water and purged for 360 s by applying pressurized nitrogen (150 psi). Finally, the extracts were freeze-dried and stored in amber vials at −20 °C until analysis. A 1 g/L dried extract solution was prepared for analysis.

Determination of DPPH Radical Scavenging Activity. Extract antiradical capacity was determined using the DPPH radical-scavenging method.[23] Initially, a stock solution was prepared by dissolving 23.5 mg of DPPH reagent in 100 mL of methanol. The DPPH solution was prepared by diluting (1:10 v/v) the stock solution with methanol. A volume of 50 μL of extract solutions at different concentrations was mixed with 2 mL of DPPH solution. The bleaching of DPPH was measured at 516 nm (DR 2000 spectrophotometer, Hach) until the absorbance remained unchanged (approximately 30 min) in the dark and at room temperature. The effective concentration of thyme extract to reach a 50% of inhibition of DPPH radical absorption, IC_{50} (mg/L), was calculated. Then, the antioxidant capacity was compared with that of Trolox, a synthetic hydrophilic vitamin E analogue, using the Trolox equivalent antioxidant capacity (TEAC) equation: TEAC = IC_{50} Trolox/IC_{50} sample.[24] DPPH values were expressed as milligrams of Trolox equivalent (TE) per gram of dry plant (dp).

Determination of Ferric Reducing Antioxidant Power (FRAP). The FRAP test offers a putative index of antioxidant or reducing capacity of antioxidants in the sample.[25] A working solution was prepared by mixing 300 mM acetate buffer (pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and a freshly prepared 20 mM $FeCl_3(6H_2O)$ solution in 10:1:1 (v/v) proportion. For the assay 3 mL of working reagent was mixed with 100 μL of sample or calibration standard. Absorbance was measured at 593 nm (DR 2000 spectrophotometer, Hach) after a resting period of 30 min.[26] A calibration curve was constructed using ascorbic acid (0.1−0.8 mM). The regression coefficient of ascorbic acid was 0.9989. The results were expressed as ascorbic acid equivalent (AAE) per gram of dry plant.

Determination of Oxygen Radical Absorbance Capacity (ORAC). Consumption of the probe molecule, FL or PGR, associated with its incubation in the presence of a peroxyl radical source, AAPH, was estimated from fluorescence (F) and absorbance (A) measurements, respectively.[27] Values of (F_i/F_0) or (A_i/A_0) were plotted as a function of time, where F_0 and A_0 are the initial fluorescence and absorbance, respectively, and F_i and A_i are the fluorescence and absorbance reading at time i, respectively. Integration of the area under the curve (AUC) was performed up to a time such that (F_i/F_0) or (A_i/A_0) reached a value of 0.2. Stock solutions of PGR (1×10^{-4} M) or FL (1×10^{-5} M) were prepared daily in 75 mM phosphate buffer, pH 7.4. A reaction mixture containing AAPH (10 mM) and PGR (5 mM), with or without the tested sample, in phosphate buffer, was incubated at 37 °C in the thermocuvette of a UV−visible spectrophotometer (Hewlett-Packard 8453). PGR consumption was evaluated from the progressive absorbance decrement measured at 540 nm. A similar procedure was carried out employing FL (70 nM), but in this case, consumption was assessed from the decrease in sample fluorescence intensity (excitation, 493 nm; emission, 515 nm) in a spectrofluorometer (Aminco-Bowman series 2). A phosphate buffer was used as a blank, and different Trolox concentrations (12.5, 25, 50, and 100 μM) were used to prepare a standard curve.

The TEAC value was calculated as

$$\mathrm{TEAC} = \left[\frac{(\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}})}{(\mathrm{AUC}_{\mathrm{Trolox}} - \mathrm{AUC}_{\mathrm{blank}})}\right] \times \left(\frac{\mathrm{concn\ of\ Trolox}}{\mathrm{concn\ of\ sample}}\right)$$

Results were expressed as Trolox equivalent per gram of dry plant.

Determination of Total Polyphenols (TA) by Folin Assay. Given that Folin values systematically exceed the total polyphenol content values, the results obtained using the Folin−Ciocalteu assay[28] were considered in this work as total antioxidant values.[29] A volume of 4.25 mL of phenolic extract (1 mg/mL) and 0.25 mL of Folin−Ciocalteu reactive were diluted with distilled water (1:1 v/v) and 0.5 mL of a sodium carbonate solution (10% w/v). Absorbance was measured at 765 nm (DR 2000 spectrophotometer, Hach) after a reaction time of 1 h at room temperature. A calibration curve was constructed using gallic acid as the calibration standard (20−90 mg/L). The regression coefficient of gallic acid was 0.9987. Results were expressed as gallic acid equivalent (GAE) per gram of dry plant.

Qualitative and Quantitative Polyphenol Analysis by HPLC-ESI-Q-TOF Mass Spectrometry. HPLC-ESI-Q-TOF equipment was

Table 1. Extraction Yield and Antioxidant Activities Obtained at the Different Extraction Conditions of *Thymus vulgaris* Polyphenols with Pressurized Hot Water[a]

temp (°C)	time (min)	extraction yield (%)	TA (mg GAE/g dp)	DPPH (mg TE/g dp)	FRAP (mg AAE/g dp)	ORAC-FL (mg TE./g dp)	ORAC-PGR (mg TE./g dp)
50	5	33.6 ± 0.8 a	131 ± 15 a	14.2 ± 2.0 a	96 ± 10 a	61.7 ± 1.5 cd	40.9 ± 2.2 ab
	15	35.5 ± 3.8 a	159 ± 18 ab	14.0 ± 1.2 a	112 ± 8 abc	49.3 ± 0.4 ab	43.5 ± 2.4 b
	30	34.7 ± 1.6 a	150 ± 7 ab	13.8 ± 0.2 a	107 ± 11 ab	42.9 ± 1.7 ab	39.2 ± 3.7 ab
100	5	37.4 ± 4.2 a	168 ± 12 b	15.2 ± 0.3 abc	118 ± 2 abc	45.3 ± 2.7 ab	38.4 ± 4.3 ab
	15	39.1 ± 2.1 a	168 ± 7 b	14.9 ± 0.7 abc	123 ± 14 bc	48.5 ± 4.4 ab	34.3 ± 3.7 ab
	30	38.2 ± 4.8 a	160 ± 13 ab	14.7 ± 0.6 ab	111 ± 4 abc	41.1 ± 1.4 a	32.5 ± 1.2 a
150	5	48.9 ± 0.7 b	199 ± 3 c	17.1 ± 1.6 bcd	131 ± 12 bcd	53.5 ± 4.9 bc	56.6 ± 2.4 c
	15	54.8 ± 0.8 bc	202 ± 3 c	17.7 ± 0.2 cd	132 ± 10 cd	61.1 ± 0.6 cd	54.4 ± 2.0 c
	30	56.7 ± 1.6 c	221 ± 2 cd	18.1 ± 0.3 d	156 ± 14 e	63.9 ± 2.9 cd	62.0 ± 5.7 c
200	5	59.0 ± 1.7 c	260 ± 0 e	21.4 ± 1.4 e	152 ± 3 de	69.7 ± 6.4 d	64.8 ± 3.9 cd
	15	60.1 ± 1.0 c	262 ± 6 e	19.0 ± 0.9 de	166 ± 8 e	68.7 ± 2.0 d	72.5 ± 5.5 d
	30	55.8 ± 2.3 c	247 ± 22 de	19.9 ± 1.3 de	155 ± 6 e	89.6 ± 9.4 e	73.1 ± 1.5 d

[a]Values with the same letter (a−e) in each column showed no statistically significant difference at the confidence interval of 95%. AAE, ascorbic acid equivalents; GAE, gallic acid equivalents; TE, trolox equivalents; dp, dry plant.

used to identify compounds by MS/MS analysis and for the relative quantification of polyphenol subclasses. The ESI-Q-TOF instrument was a quadrupole time-of-flight QStar Elite (AB Sciex) combined with a high-performance liquid chromatography Agilent 1200 RRLC system (Agilent, Waldbronn, Germany). Separations were conducted using a Luna C18, 3.5 μm particle size, column (50 × 2.1 mm i.d.; Phenomenex, Torrance, CA, USA). Solutions for the mobile phase were 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The following gradient was applied at a flow rate of 0.40 mL/min: 0 min, 8% B; 10 min, 23% B; 15 min, 50% B; 20 min, 50% B; 30 min, 70% B; 31 min, 100% B; 35 min, 100% B; and finally, 36 min, 8% B. The final condition was held for 9 min as a re-equilibration step. The injection volume was 25 μL. For MS analysis, data acquisition was performed using negative ion mode; the mass range was from 70 to 600 Da. Data were acquired using a declustering potential of −180 V, focusing potential of −190 V, second eclustering potential of −10 V, and source temperature of 400 °C. TOF mode was used to determine relative quantification of phenolic compounds, and MS/MS experiments were performed in the information-dependent acquisition (IDA) mode, with a collision energy of 35 V. Analyst 2.0 software from AB Sciex was used for data acquisition and processing. Peak identity was established by comparing the molecular formula proposed by the software for the different peaks obtained in the MS experiments with those included in the Phenol-Explorer database,[3] as well as with specific papers on thyme polyphenols. These identities were confirmed from the MS/MS experiments, comparing the main fragments with those previously described for the same compound[30,31] and using the general fragmentation patterns reported for polyphenols.[32] Compounds identified by LC-MS/MS analysis were grouped into four subclasses. These were quantified relatively using a corresponding standard: gallic acid (structurally related to hydroxyphenylpropanoic acids) for hydroxyphenylpropanoic acids ($y = 5244.1x$, $R^2 = 0.9781$, LOD = 20.9 μg/L, LOQ = 69.5 μg/L); caffeic acid for hydroxycinnamic acids ($y = 10026x$, $R^2 = 0.9834$, LOD = 2.2 μg/L, LOQ = 7.4 μg/L); apigenin for flavones ($y = 11093x$, $R^2 = 0.9795$, LOD = 4.2 μg/L, LOQ = 14.1 μg/L); and quercetin for flavonols and flavanones ($y = 6214x$, $R^2 = 0.9560$, LOD = 3.7 μg/L, LOQ = 12.5 μg/L). The extraction yield of each polyphenol subclass was expressed as milligrams of standard per gram of dry plant. The values of the different subclasses at each extraction condition were added to obtain the total polyphenol extraction yield.

Statistical Analysis. Extraction and analysis were performed in triplicate with the data presented as mean ± SD values. StatgraphicsPlus for Windows 4.0 (Herndon, VA, USA) was used for statistical analyses. To study the effects of the temperature and extraction time on extraction performance as well as the interaction between these two factors, analysis of variance (factorial ANOVA) and least significant difference (LSD) tests were applied on the response variables with a significance of $p \leq 0.05$.

RESULTS AND DISCUSSION

To have a common base for comparison, results of all analyses of the extracts are expressed in terms of mass of dried plant (dp) before extraction.

Effect of Temperature and Time on Total Extract Yield. Powdered thyme leaves were extracted at four temperatures, 50, 100, 150, and 200 °C, and at three extraction times, 5, 15, and 30 min. The dry extracts obtained were weighed, and the total extract yield (TEY) was expressed as the percentage of extract recovered per dp processed (Table 1).

Extraction temperature had a statistically significant effect (p value < 0.0001) on TEY, even though yields obtained at 50 and 100 °C were similar. Increasing the extraction temperature from 100 to 200 °C resulted in a >2-fold increase in TEY. This effect of temperature confirms previous observations[33,34] in which two main phenomena are involved: an increase in solubility and hydrolytic reactions. The use of higher temperatures increases the capacity of water to solubilize analytes.[5] At temperatures of 160 °C and higher, pressurized hot water is even able to solubilize hemicellulose[35] and lignin.[36] Moreover, during a water-based thermal process, part of the hemicellulose is hydrolyzed and forms acids. These acids are assumed to catalyze the hydrolysis of remaining hemicelluloses.[37] Effects of these reactions can be observed clearly at 150 °C, at which the extraction yield increased over time. Hydrolysis of lignocellulosic material in PHWE of polyphenol-rich materials, which is usually overlooked, may contribute to the release of phenolic cell wall-associated compounds.[38] It may also release other compounds, such as reducing sugars, which may alter the Folin assay measurement.[39]

Extraction time had no statistically significant effect on total extraction yield. In addition, no correlation on the extraction yield was detected in the interaction between temperature and time factors. Little to no influence of PHWE time on extraction yield has been reported previously for a similar biomass.[40]

Effect of Temperature and Time on Total Antioxidants and Antioxidant Activity. Extraction temperature was the only factor that had a statistically significant effect (all p values < 0.0001) on TA and antioxidant activity tests. The results are listed in Table 1. TA values increased with temperature. A temperature increment from 50 to 200 °C resulted in a 2-fold increase in TA from 131 to 260 mg GAE/g dp. This temperature effect on TA has been well reported in PHWE of other plants.[33,41] In addition, an increase in extraction temperature from 50 to 200 °C resulted in an increase of DPPH radical scavenging and ferric ion reducing activities of >50%. These results are in agreement with the findings of similar research in which the temperature was found to be the dominant factor in attaining high antioxidant activity.[41] The effects of temperature on ORAC-FL and ORAC-PGR assays (scavenging of peroxyl radicals) were the same as those on DPPH and FRAP assays (reducing activity). Therefore, the antioxidants extracted were equally efficient under different experimental setups, and both the amount of antioxidants and their reactivity increased with temperature.[27]

Correlation coefficients between TA and antioxidant activity obtained from the different assays are shown in Table 2.

Table 2. Pearson Correlation Coefficients between Antioxidant Activity Assays and Total Antioxidants

correlated assays	correl coeff
DPPH−FRAP	0.90
DPPH−ORAC(FL)	0.80
DPPH−ORAC(PGR)	0.90
FRAP−ORAC(FL)	0.72
FRAP−ORAC(PGR)	0.90
ORAC(FL)−ORAC(PGR)	0.87
TA−DPPH	0.96
TA−FRAP	0.97
TA−ORAC(FL)	0.75
TA−ORAC(PGR)	0.92

Despite these assays being based on different chemical mechanisms, a strong correlation was observed between them all; corroborating that temperature has the same effect on TA as it has on antioxidant activity. This observation agrees with previous findings.[9,42]

Under most conditions studied, TA and antioxidant activity values increased with increasing extraction temperature; the highest values for all assays were obtained at the maximum temperature (Table 1). The antioxidant activities obtained at 50 and 100 °C were similar and significantly increased above 100 °C. The increase in antioxidant activity and TA obtained at high temperatures might be due to the increase in TEY. However, apart from an enhancement in solubility and diffusion rate, several reactions may generate compounds with antioxidant activities at temperatures above 100 °C.[34,43]

Polyphenol Profile of Extracts Obtained under Different Extraction Conditions. *Compound Identification.* Mass spectral data for compounds identified in negative ionization mode are listed in Table 3, where a summary of the proposed identification is presented. Seventeen phenolic compounds were identified from the extracts obtained, among which six compounds have not been reported previously in the genus *Thymus*. 3,4-Dihydroxyphenyllactic acid (DHPLA) was the only compound identified as hydroxyphenylpropanoic acid. This compound has not been reported previously in the genus *Thymus*, despite being one of the *o*-diphenol moieties constituting rosmarinic acid. Four compounds belonging to hydroxycinnamic acids were identified; caffeic and rosmarinic acids and two compounds not reported previously in the genus *Thymus*: caffeic acid 4-*O*-glucoside and dihydrocaffeic acid. In the flavone subclass, four glycosylated compounds (apigenin 6,8-di-*C*-glucoside, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, and apigenin 7-*O*-glucuronide), one aglycone (luteolin), and three methoxylated flavones (cirsimaritin, cirsilineol, and 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone, not previously reported in *Thymus*) were detected. Also identified were two glycosylated flavonols (quercetin 3-*O*-glucoside and dihydrokaempferol 3-*O*-glucoside), for which only the aglycone form had been previously reported in thyme. Finally, one glycosylated flavanone, eriocitrin, and one phenolic terpene, carnosol, were identified. Some of the newly identified compounds are glycosylated conjugates of already described aglycones. The more hydrophilic conjugates may have been concentrated in our starting material, which is the remaining leaves from steam distillation of the hydrophobic essential oil. The distillation at 100 °C might have also concentrated the methoxylated flavones in the leaves. The new methoxylated flavones might have been more efficiently extracted with pressurized hot water that shows a dielectric constant and polarity lower than those of steam.

With regard to the six new compounds found in thyme, it is worth pointing out that knowledge of the polyphenol composition of many plants is often limited to one or a few varieties. Furthermore, numerous other factors may affect the polyphenol content of plants; ripeness at the time of harvest, environmental factors, processing (pretreatment, extraction method, and drying) and storage.[2] In our study, a comprehensive revision of the results of LC-MS/MS analysis was conducted in an attempt to identify a wide spectrum of polyphenols and not just specific given compounds.

Identification of these compounds was confirmed by comparison between observed and calculated mass (errors below 10 ppm) and by the fragmentation pattern obtained when MS/MS analysis was performed (Table 3). For instance, for phenolic compounds found in thyme, either glucosylated or glucuronidated, aglycone fragments (e.g., the fragment at m/z 285 for both luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide) were detected, and in the case of phenolic acids (e.g., caffeic acid or dihydrocaffeic acid) losses of CO_2 were observed upon fragmentation.

Effect of Extraction Temperature and Time on Qualitative Polyphenol Profile. Different extraction conditions tested caused differences in the phenolic profiles of the extracts (Table 4). Some of the phenolic compounds (apigenin 7-*O*-glucoside, luteolin, cirsimaritin, cirsilineol, and 5,6′-dihydroxy-7,8,3′,4′-tetramethoxyflavone) were not detected at 200 °C for any extraction time, whereas other compounds (caffeic acid 4-*O*-glucoside, quercetin 3-*O*-glucoside, and eriocitrin) were detected only at shorter extraction times. Dihydroxykaempferol 3-*O*-glucoside was especially labile; hence, it was detected only in the 5 min extractions at 100 and 150 °C. Dihydrocaffeic acid, in turn, was detected only at 200 °C and 30 min, and it is possible that the appearance of this compound is due to the loss of unsaturation (the double bond) in the caffeic acid chain from its exposure over a long time at high temperature. Finally, luteolin 7-*O*-glucuronide was not detected at 50 °C and 5 min

Table 3. Polyphenols Found by HPLC-ESI-Q-TOF in *Thymus vulgaris* Extracts Obtained with Pressurized Hot Water[a]

compound	t_R (min)	$[M-H]^-$ experimental	$[M-H]^-$ calculated	ppm[b]	characteristic MS/MS ions m/z		reported in genus *Thymus*
3,4-dihydroxyphenyllactic acid	1.4	197.0432	197.0450	−9.1	135	$[M-H-H_2O-CO_2]$	no
					123	$[M-H-C_3H_6O_2]$	
caffeic acid 4-*O*-glucoside	2.9	341.0862	341.0873	−3.1	179	[M − H − Gluc]	no (aglycone only)
					135	$[M-H-Gluc-CO_2]$	
caffeic acid	4.1	179.0342	179.0344	−1.3	135	$[M-H-CO_2]$	ref 31
dihydrocaffeic acid	4.9	181.0503	181.0500	1.2	137	$[M-H-CO_2]$	no
apigenin 6,8-di-*C*-glucoside	5.4	593.1504	593.1506	−0.4	503	[M − H − 90][c]	ref 16
					473	[M − H − 120][c]	
					383	[A + 113][c]	
					353	[A + 83][c]	
quercetin 3-*O*-glucoside	7.5	463.0914	463.0877	8.1	301	[M − H − Gluc]	no (aglycone only)
eriocitrin	8.4	595.1707	595.1663	7.4	287	[M − H − 308][d]	ref 53
					147		
dihydrokaempferol 3-*O*-glucoside	8.7	449.1085	449.1084	0.3	287	[M − H − Gluc]	no (aglycone only)
					151		
					135		
luteolin 7-*O*-glucoside	9.6	447.0896	447.0927	−7.0	285	[M − H − Gluc]	ref 31
luteolin 7-*O*-glucuronide	10.2	461.0737	461.0720	3.7	285	[M − H − Glur]	ref 54
apigenin 7-*O*-glucuronide	12.6	445.0753	445.0771	−4.0	269	[M − H − Glur]	ref 53
rosmarinic acid	12.8	359.0785	359.0767	5.0	197	[DHPLA − H]	ref 31
					179	[caffeic acid − H]	
					135	$[\text{caffeic acid} - H - CO_2]$	
luteolin	16.5	285.0394	285.0399	−1.8	199	$[M-H-CH_2O_2-CO_2]$	ref 53
					175	$[M-H-C_3O_2-CH_2O_2]$	
					151	lactone form A^- ring	
					133	B^- ring	
cirsimaritin	19.2	313.0703	313.0712	−2.9	298	$[M-H-CH_3]$	ref 16
					283	$[M-H-CH_3-CH_3]$	
cirsilineol	19.5	343.0812	343.0817	−1.7	328	$[M-H-CH_3]$	ref 16
					313	$[M-H-CH_3-CH_3]$	
					298	$[M-H-CH_3-CH_3-CH_3]$	
5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone	20.1	373.0899	373.0923	−6.5	343	$[M-H-CH_3-CH_3]$	no
					328	$[M-H-CH_3-CH_3-CH_3]$	
carnosol	20.9	329.1766	329.1752	4.0	285	$[M-H-CO_2]$	ref 31

[a]Gluc, glucoside; A, aglycone; Glur, glucoronide; DHPLA, 3,4-dihydroxyphenyllactic acid. [b]Data from the extract obtained at 100°C and 5 min was used to calculate the error value (ppm). [c]Typical of di-*C*-glycosylflavonoids. [d]Associated with a loss of a disaccharide.

extraction, which would appear to indicate that the conditions were not sufficiently conducive.

In summary, high extraction temperatures and, in some cases, long extraction times produce extracts with a small number of polyphenols, notably methylflavones and flavonols.

Effect of Temperature and Time on the Extraction of Polyphenol Subclasses. The effects of temperature and extraction time on the polyphenol extraction yields were assessed through the relative quantification of the different polyphenol subclasses (Figure 1; Table 5). Temperature, time (except for flavones), and the interaction of these factors had a statistically significant effect on extraction yield of all polyphenol subclasses analyzed. Temperature showed p values <0.0001 for all subclasses and total polyphenols. Extraction time showed p values <0.0001, 0.0040, <0.0001, and 0.0126 for flavonols, hydroxyphenylpropanoic acids, hydroxycinnamic acids, and total polyphenols, respectively. The interaction of temperature and time factors showed p values 0.0014, 0.0002, 0.0095, <0.0001, and 0.0021 for flavones, flavonols, hydroxyphenylpropanoic acids, hydroxycinnamic acids, and total polyphenols, respectively.

The impact of temperature on the extraction yield of total polyphenols, hydroxycinnamic acids, flavones, and flavonols/flavanones is similar (Figure 1). The lowest yields were obtained at 200 °C and the highest at 100 °C. At 50 and 150 °C the yields were also affected by exposure time. The extraction yields of flavones and total polyphenols at 50 °C increased with time, whereas those of flavonols and hydroxycinnamic acids remained practically the same. In turn, at 150 °C all yields were negatively affected by the exposure time, except for hydroxyphenylpropanoic acids. In fact, this subclass presents a completely different pattern (Figure.1). No statistical difference in yields obtained at 50 and 100 °C for any exposure time was observed. In addition, at 150 and 200 °C the extraction yield increased with exposure time.

In summary, the highest extraction yields were achieved at 100 °C and 5 min for hydroxycinnamic acids, flavones, flavonols/flavanones, and total polyphenols (27.4 mg poly-

Table 4. Polyphenols Identified in Extracts Obtained at Different Extraction Conditions of Temperature and Time, Analyzed by HPLC-ESI-Q-TOF[a]

	extraction conditions											
	50 °C			100 °C			150 °C			200 °C		
compound	5 min	15 min	30 min	5 min	15 min	30 min	5 min	15 min	30 min	5 min	15 min	30 min
Polyphenol Subclass: HPPA												
DHPLA	X	X	X	X	X	X	X	X	X	X	X	X
Polyphenol Subclass: Hydroxycinnamic Acids												
caffeic acid 4-*O*-glucoside	X	X	X	X	X	X	X	X	X	X	X	
caffeic acid	X	X	X	X	X	X	X	X	X	X	X	X
dihydrocaffeic acid												X
rosmarinic acid	X	X	X	X	X	X	X	X	X	X	X	X
Polyphenol Subclass: Flavones												
apigenin 6,8-di-*C*-glucoside	X	X	X	X	X	X	X	X	X	X	X	X
luteolin 7-*O*-glucoside	X	X	X	X	X	X	X	X	X	X	X	X
luteolin 7-*O*-glucuronide		X	X	X	X	X	X	X	X	X	X	X
apigenin 7-*O*-glucuronide	X	X	X	X	X	X	X	X	X			
luteolin	X	X	X	X	X	X	X	X	X	X	X	X
cirsimaritin	X	X	X	X	X	X	X	X	X			
cirsilineol	X	X	X	X	X	X	X	X	X			
DHTMF	X	X	X	X	X	X	X	X	X			
Polyphenol Subclass: Flavonols												
quercetin 3-*O*-glucoside	X	X	X	X	X	X	X	X	X	X	X	
DHKG	X	X	X	X			X					
Polyphenol Subclass: Flavanones												
eriocitrin	X	X	X	X	X	X	X	X	X	X		
Polyphenol Subclass: Phenolic Terpenes												
carnosol	X	X	X	X	X	X	X	X	X	X	X	X

[a]Symbol X indicates presence of the compound in the corresponding extraction condition. HPPA, hydroxyphenylpropanoic acid; DHPLA, 3,4-dihydroxyphenyllactic acid; DHTMF, 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone; DHKG, dihydrokaempferol 3-*O*-glucoside.

phenols/g dp). The extraction yield of hydroxyphenylpropanoic acids showed a maximum at 200 °C and 15 min.

Flavonoids are known to degrade in water at temperatures of 100 °C and above.[44,45] The number and type of substituents as well as the position of the hydroxyl group affect flavonoid thermal stability, as compounds possessing a smaller number of substituents are less stable at high temperatures.[46] In the case of phenolic acids the effect of temperature is especially marked. For example, caffeic acid and DHPLA are the two hydrolyzed degradation products of rosmarinic acid.[47] As seen in Figure 2, the extraction yield of rosmarinic acid is lowest at 200 °C and at 150 °C is highly sensitive to the exposure time, suggesting degradation. On the other hand, the extraction yield of DHPLA at 150 °C increased gradually with extraction time, whereas its extraction yield was highest at 200 °C. To the best of our knowledge, there are no prior studies on the formation of DHPLA from polyphenolic degradation of rosmarinic acid at high temperatures. However, only a slight increase in the extraction yield of caffeic acid with temperature was found, reaching a maximum at 150 °C. Hossain et al.[41] also observed a slight decrease of extraction yield of rosmarinic acid and an increase of caffeic acid using pressurized hot methanol/water extraction at a temperature of around 150 °C. In an attempt to explain this behavior, some researchers have reported the formation of simple catechol monomers such as 4-vinylguaiacol and 4-vinylphenol from the thermal decarboxylation of caffeic acid in an aqueous system at temperatures between 90 and 100 °C.[48] Moreover, caffeic acid subjected to mild pyrolysis (225−226 °C) under vacuum resulted in rapid decarboxylation and the formation of tetraoxygenated phenylinadan isomers, showing strong antioxidant activity.[49] Therefore, it seems that the caffeic acid formed after rosmarinic acid decomposition is rapidly degraded and transformed into other derived compounds.

Correlation between Polyphenol Subclasses, Total Antioxidants, and Antioxidant Activity. Quantification results obtained from LC-MS were correlated with both antioxidant and TA analysis using all of the values (corresponding to the 12 extraction conditions). The results obtained (Supporting Information, Table 1S) show a strong negative correlation between chemical antioxidant assays and hydroxycinnamic acids, flavones, flavonols/flavanones, and total polyphenols (R values ranging from −0.592 to −0.898), but a strong positive correlation with DHPLA (R values ranging from 0.838 to 0.918). However, the increase in the DHPLA does not in itself explain the increase in antioxidant activity because in chemical assays phenolic acids present antioxidant activities similar to those of flavonoids,[4] and even DHPLA has shown less antioxidant activity than rosmarinic acid.[50] This suggests that factors other than the amount of polyphenols in the extracts play a significant role in determining antioxidant activity and TA.

In aqueous systems at high temperatures many plant material reactions produce antioxidants or reducing compounds. The high antioxidant activity of extracts obtained at 150 °C and above may be associated, in part, with the generation of antioxidant-capable Maillard reaction products such as melanoidins.[41] In addition, thermal decomposition products from polyphenols, such as caffeic acid, have revealed stronger antioxidant activity than those of the polyphenols they come from.[51] The thermal decomposition products of rosmarinic acid, especially DHPLA, would also contribute to the

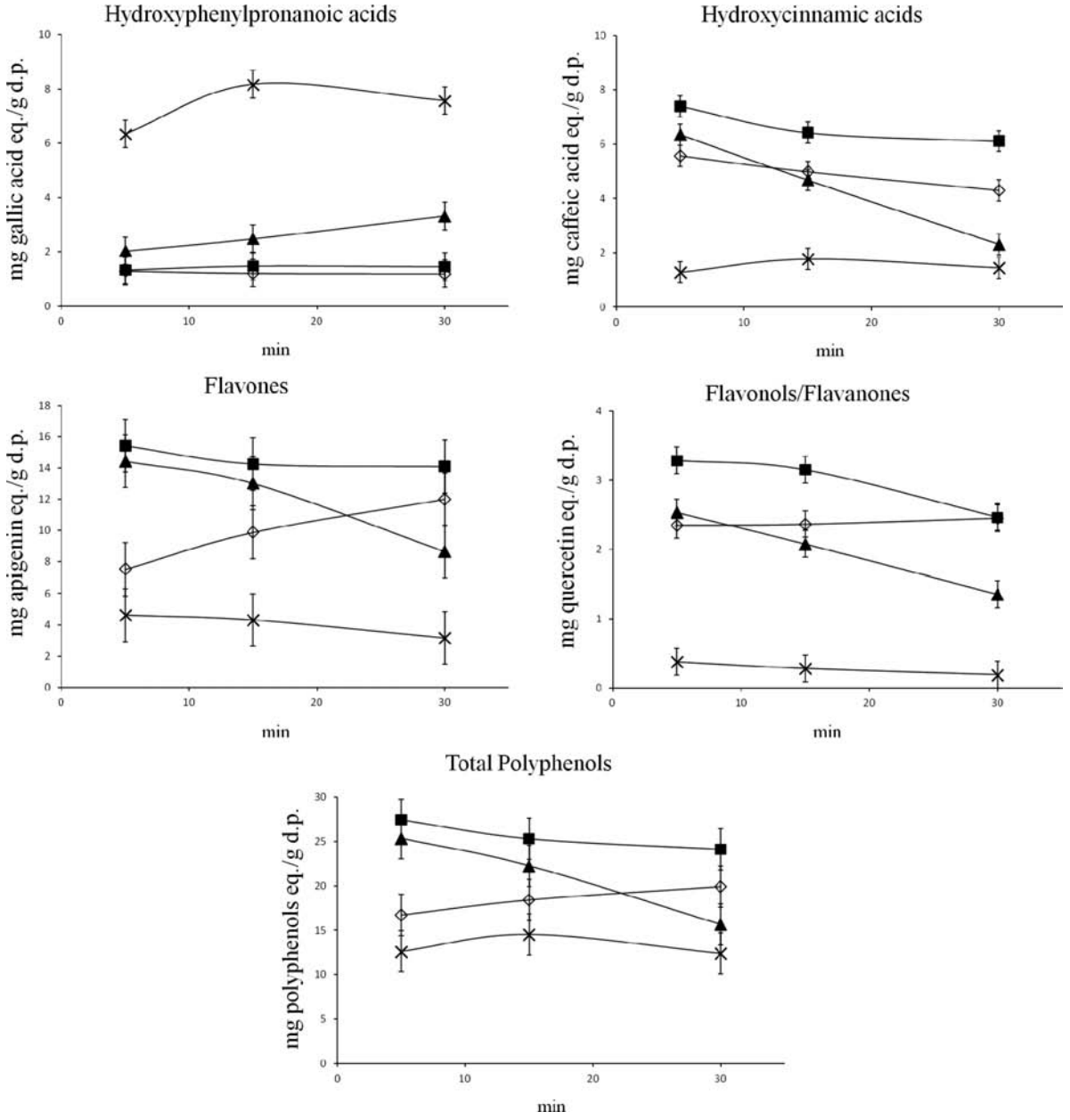

Figure 1. Extraction dynamics of polyphenol subclasses and total polyphenols. Extraction temperatures: (◇) 50 °C; (■) 100 °C; (▲) 150 °C; (×) 200 °C. Bars represent the upper and lower limits of the 95% confidence interval. Gallic acid, caffeic acid, apigenin, and quercetin were used as the relative quantification standard for hydroxyphenylpropanoic acids, hydroxycinnamic acids, flavones, and flavonols/flavanones, respectively.

antioxidant activity. Moreover, the most common products of the lignocellulose thermal degradation process are reducing agents such as sugars and phenolic compounds.[52] Therefore, the aggregated effects of formation and release of polyphenol derivates and nonpolyphenolic compounds at high temperatures would explain the negative correlation between antioxidant capacity assays and polyphenol subclasses analyzed by LC-MS, with the exception of DHPLA, the increase of which, similar to antioxidant activity, is due to degradation and hydrolytic reactions that occur at high temperatures.

In conclusion, temperature and extraction time affected polyphenol extract profiles both quantitatively and qualitatively. High temperatures and high exposure times reduced the yield of phenolic compounds detected. One exception was the yield of hydroxyphenylpropanoic acid that reached a maximum at 200 °C (the highest temperature in our study), probably due to the thermal degradation of rosmarinic acid. The clearest effect of increased exposure time occurred at 150 °C, at which flavonoids and hydroxycinnamic acids degradation and the extraction of hydroxyphenylpropanoic acids were favored. The extraction yield of flavones, flavonols/flavanones, hydroxycinnamic acids, and total polyphenols peaked at 100 °C and 5 min.

In polyphenol PHWE from thyme, the quantity of neither total polyphenols nor any particular subclass of polyphenols identified in this work, with the exception of DHPLA, determined observed antioxidant activity, as the two factors returned strong negative correlations (inverse relationships) with the latter. Antioxidant activity increased steadily as temperature rose, whereas the quantity of polyphenols extracted decreased. Reactions occurring at temperatures above 100 °C affected this behavior through polyphenol degradation and the formation and release of antioxidant-active

Table 5. Polyphenol Extraction Yield in *Thymus vulgaris* Obtained with Pressurized Hot Water Analyzed by HPLC-ESI-Q-TOF[a]

temp (°C)	time (min)	HPPA (mg gallic acid equiv/ g dp)	HCA (mg caffeic acid equiv/ g dp)	flavones (mg apigenin equiv/ g dp)	flavonols/flavanones (mg quercetin equiv/ g dp)	polyphenols (mg polyphenol equiv/ g dp)
50	5	1.29 ± 0.08 a	5.57 ± 0.06 de	7.53 ± 0.01 bc	2.35 ± 0.05 cd	16.74 ± 0.10 abc
	15	1.22 ± 0.06 a	4.98 ± 0.04 cd	9.88 ± 0.96 cde	2.37 ± 0.07 cd	18.44 ± 0.79 bcd
	30	1.20 ± 0.03 a	4.30 ± 0.32 c	11.99 ± 1.58 def	2.45 ± 0.00 cd	19.93 ± 1.92 cde
100	5	1.34 ± 0.00 a	7.39 ± 0.24 g	15.42 ± 1.04 g	3.29 ± 0.00 e	27.43 ± 1.28 g
	15	1.47 ± 0.15 ab	6.43 ± 0.22 f	14.25 ± 1.73 fg	3.15 ± 0.15 e	25.31 ± 1.52 fg
	30	1.46 ± 0.07 a	6.11 ± 0.17 ef	14.09 ± 0.84 fg	2.47 ± 0.15 d	24.11 ± 1.23 efg
150	5	2.04 ± 0.31 ab	6.34 ± 0.42 f	14.43 ± 1.23 fg	2.53 ± 0.15 d	25.34 ± 2.11 fg
	15	2.48 ± 0.07 bc	4.67 ± 0.41 c	13.01 ± 1.70 efg	2.08 ± 0.23 c	22.25 ± 2.41 def
	30	3.32 ± 0.37 c	2.31 ± 0.27 b	8.66 ± 1.22 cd	1.36 ± 0.24 b	15.65 ± 2.10 abc
200	5	6.35 ± 0.61 d	1.28 ± 0.05 a	4.61 ± 0.34 ab	0.38 ± 0.02 a	12.62 ± 0.88 a
	15	8.17 ± 0.63 e	1.77 ± 0.28 ab	4.30 ± 0.28 ab	0.28 ± 0.04 a	14.53 ± 1.23 ab
	30	7.57 ± 0.52 e	1.45 ± 0.10 a	3.17 ± 0.01 a	0.19 ± 0.06 a	12.39 ± 0.68 a

[a]Values with the same letter indicate no statistically significant difference between extraction conditions at the confidence interval of 95%. HPPA, hydroxyphenylpropanoic acids; HCA, hydroxycinnamic acids; dp, dry plant.

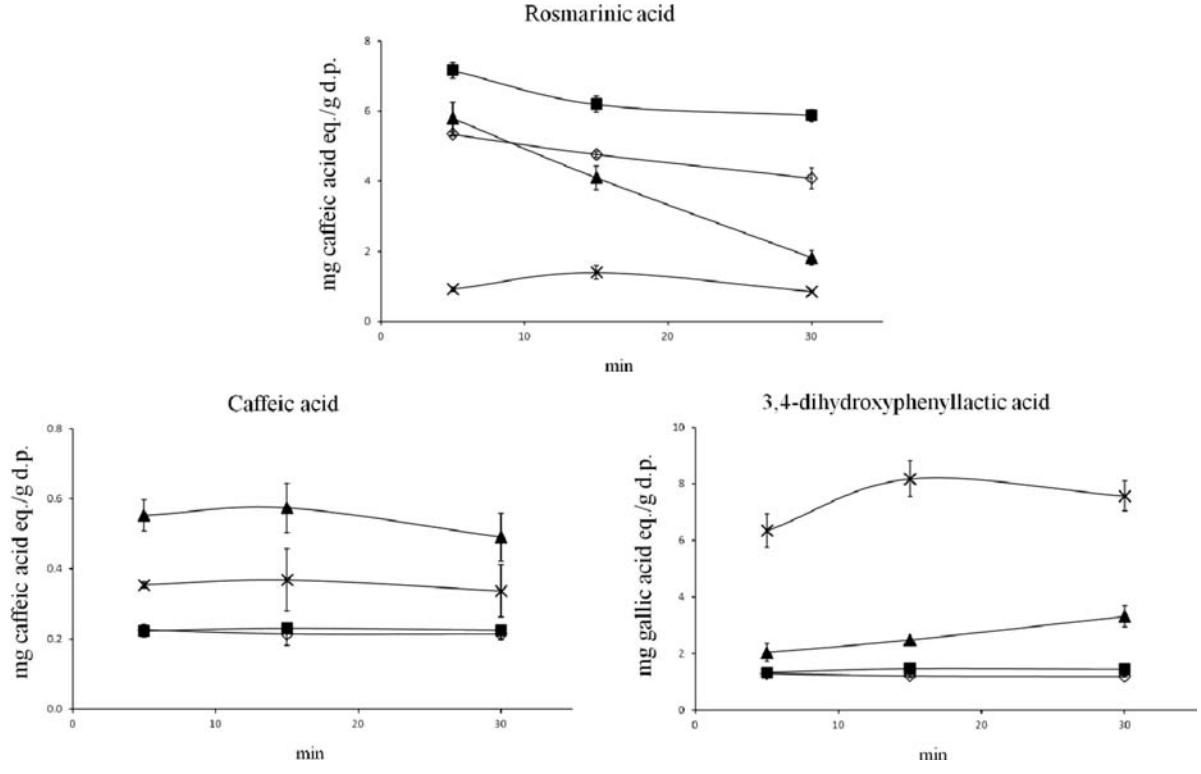

Figure 2. Extraction dynamics of rosmarinic acid, caffeic acid, and 3,4-hydroxyphenyllactic acid. Extractions temperatures: (◇) 50 °C; (■) 100 °C; (▲) 150 °C; (×) 200 °C. Bars represent the standard deviation of three replicates. Caffeic acid was used as the relative quantification standard for rosmarinic and caffeic acids, and gallic acid was used as the relative quantification standard for 3,4-dihydroxyphenyllactic.

nonpolyphenol compounds and polyphenol derivates such as DHPLA.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Pearson's correlation coefficients between the quantification results obtained from LC-MS and both antioxidant and TA analyses (Table 1S). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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