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Investigation on phenolic compounds stability during microwave-assisted extraction

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Abstract

The stability of 22 phenolic compounds of different families (benzoic acids, benzoic aldehydes, cinnamic acids, catechins, coumarins, stilbens and flavonols) has been studied under conditions of microwave-assisted extraction. The influence on the stability affected by the working temperature between 50 and 175 °C has been evaluated, and it has been concluded that all the compounds studied are stable up to 100 °C, whereas at 125 °C there is significant degradation of epicatechin, resveratrol and myricetin. Conclusions have been drawn on the relationship between the chemical structure and the stability of the compounds; it has been found that those that have a greater number of hydroxyl-type substituents are more easily degraded under the extraction conditions.

Keywords: Phenolic compounds; Stability; Microwave-assisted extraction

1. Introduction

Phenolic compounds are one of the main groups of micronutrients present in the plant world, and form an important part of human and animal diet. They present diverse chemical structures and activity, and comprise more than 8000 different compounds. The majority of these compounds are powerful antioxidants necessary for the correct functioning of plant cells. They are found in fruits and vegetables, for example, in grape, apple and onion, and in drinks like tea and wine [1]. Given their role as antioxidants, numerous methods for their analysis have been developed in recent years.

The extraction methods for simple phenolic compounds (benzoic acids, benzoic aldehydes, cinnamic acids and catechins) from solid or semi-solid materials have been focused on maceration using organic solvents [2,3]. However, these methods involve long extraction times, which give rise to possible degradations. The process of degradation can be originated by both external and internal factors. Light, together with air and temperature, are the most important factors that facilitate degradation reactions. The temperature of extraction usually needs to be high in order to minimise the duration of the process. The enzymes present in the sample, principally oxidative enzymes, that are liberated during the extraction can also promote these degradation reactions.

In recent years, several faster and more automatic extraction techniques for solid samples have been replacing conventional techniques like Soxhlet. Among the modern techniques are extraction by supercritical fluids (SFE), extraction by pressurised liquids (PLE) and extraction assisted by microwave (MAE). These alternatives techniques considerably reduce the consumption of solvents, increase the speed of the extraction process, and simplify it.

The principal advantage of SFE is that practically no organic solvents are utilised, although this technique is limited to compounds of low or medium polarity. The literature contains descriptions of extraction methods for polyphenols by SFE, the main characteristics of which are the need for high percentages of organic modifier; this usually means that the process takes place under subcritical conditions [4,5].

PLE has been shown to be effective as a method for the extraction of polyphenols, and rapid methods, taking 10 min, have

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been developed [6,7] that utilise high temperatures (150 °C) to accelerate the process.

In respect of MAE, this technique has been applied in the development of methods for the extraction of organic compounds from matrices of soils [8], sediments [9], seeds [10] and foods [11,12]. These studies show that these compounds are extracted more effectively when the energy provided by microwaves is employed. Nevertheless, no examples have been found in the literature on the application of microwave-assisted extraction to the determination of polyphenols in solid food samples employing temperatures above the boiling point of the solvent. References have been found in which temperatures lower than the boiling point have been applied [13]. However, the possibility of developing MAE in closed systems would considerably extend its possibilities, by being able to utilise superheated liquids.

As occurs with SFE and PLE, MAE makes it possible to perform extractions in the absence of light. Phenolic compounds are very sensitive to this factor, therefore these techniques represent a great advantage. Compounds like resveratrol can be found in two isomeric forms (*cis* and *trans* configurations), but only one of these, *trans*-resveratrol, presents biological activities. Light can catalyse the transformation from the active to the inactive form [14,15]. In addition, the short extraction times that these techniques present (less than 1 h) reduce the adverse effect of the enzymatic activity.

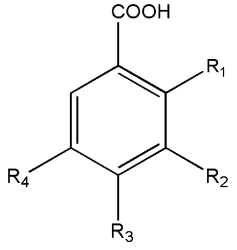
Before proposing an extraction method in the determination of polyphenols, it is necessary to verify that these compounds are stable under the extraction conditions. The purpose of this study is to examine the stability of phenolic compounds during MAE, utilising methanol as the extraction solvent, at various temperatures up to 175 °C.

Subsequently, the same extraction conditions have been applied to the grape skin (marc) and seeds, in order to test the viability of the extraction of phenolic compounds under these conditions.

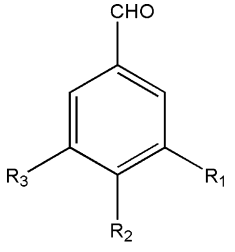
2. Materials and methods

The methanol and acetic acid (Merck, Darmstadt, Germany) used were of HPLC grade. Water was supplied using a Milli-Q water purifier system from Millipore (Bedford, MA, USA). Phenolic standards were obtained from Sigma (St. Louis, MO, USA). The standards used were: *p*-hydroxybenzoic acid, vanillic acid, veratric acid, vanillin, veratric aldehyde, umbelliferone, scopoletin, *p*-coumaric acid, ferulic acid, gentisic acid, gallic acid, (+)-catechin, (−)-epicatechin, protocatechuic aldehyde, syringaldehyde, esculetin, 4-hydroxycoumarin, caffeic acid, sinapic acid, resveratrol, myricetin and kaempferol, all with a purity higher than 95%. The chemical structures are shown in Fig. 1.

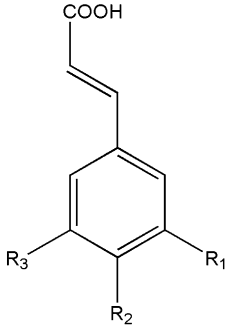
Stock standard solutions of each family of the phenolic compounds were prepared in methanol and water 50:50 (v/v) and stored in a freezer at −20 °C. The stability of the stock solutions was controlled during the stability study and no change in concentrations was observed.



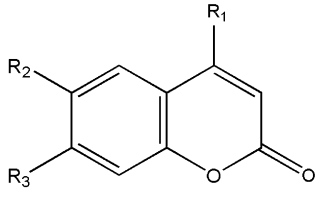
R_1, R_2, R_4 = H; R_3= OH	p-Hydroxybenzoic acid
R_1, R_4 = OH; R_2, R_3 = H	Gentisic acid
R_1,R_4 = H; R_2= OH; R_3 = OCH_3	Vanillic acid
R_1, R_4 = H; R_2, R_3 = OCH_3	Veratric acid
R_1 = H; R_2, R_3, R_4 = OH	Gallic Acid



R_1, R_2 = OH; R_3 =H	Protocatechuic aldehyde
R_1 = OH; R_2 = OCH_3; R_3= H	Vanillin
R_1, R_2 = OCH_3; R_3=H	Veratric aldehyde
R_1, R_3 = OCH_3; R_2 = OH	Syringaldehyde

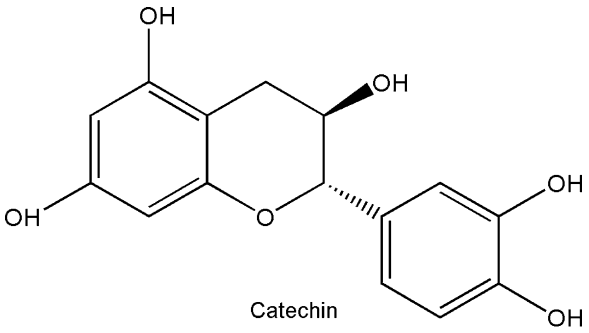


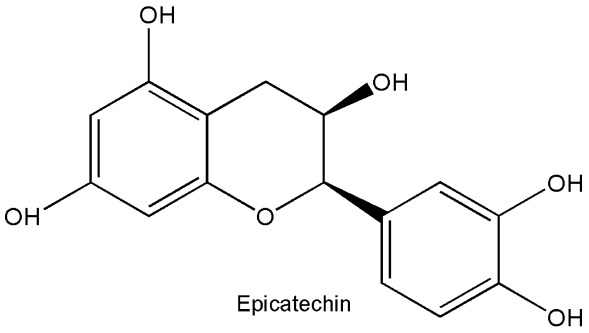
	R_1, R_3 = H; R_2= OH	p-Coumaric acid
	R_1, R_2 = OH; R_3 = H	Caffeic acid
	R_1= OCH_3; R_2 = OH; R_3 = H	Ferulic acid
(a)	R_1, R_3 = OCH_3; R_2 = OH	Sinapic acid

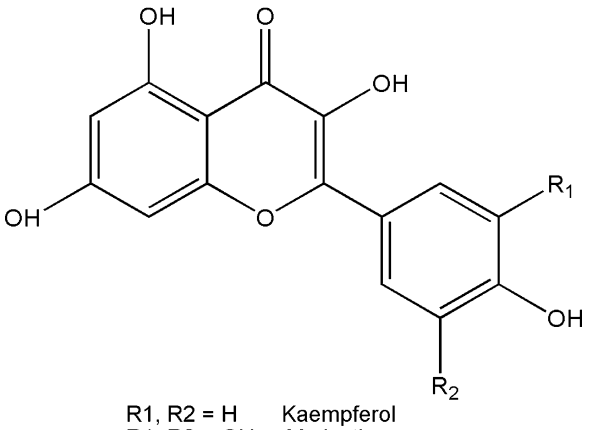


R_1 = OH; R_2, R_3 = H	4-Hydroxycoumarin
R_1, R_2 = H; R_3 = OH	Umbelliferone
R_1 = H; R_2, R_3 = OH	Esculetin
R_1 = H; R_2 = OCH_3; R_3 = OH	Scopoletin

Fig. 1. (a and b) Chemical structures of the phenolic compounds studied.







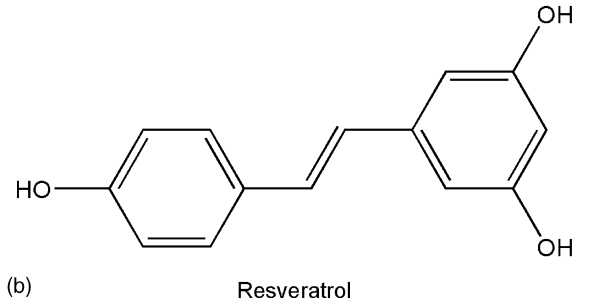


Fig. 1. (*Continued*).

The microwave system used was the ETHOS-1600 (Milestone, Shelton, CT, USA) closed vessel oven system equipped with 10 perfluoro alkoxy (PFA) vessels. Extractions were performed at 500 W. Temperatures ranging from 50 to 175 °C were evaluated. The extraction protocol used was the following: 1 mL of solution of each family of phenolic compounds in approx. Twenty milliliter of the extraction solvent (methanol) for 20 min.

After each extraction, the volume of extract was made up to 25 mL with the solvent. All samples were filtered through a 0.45 μm nylon syringe filter (Millipore) before chromatographic analysis. All extractions were performed in triplicate.

2.1. Preparation of samples

The grape skin and seeds were obtained from grapes of the Red Globe table variety (red), Napoleón (red) and Moscatel (white), the seeds were separated from the skin, and then the seeds and skin of each variety were milled individually in a tabletop mill for 2 min, in bursts of 15 s at a time to avoid heating the samples. The milled samples were kept at −20 °C until analysis.

2.2. High-performance liquid chromatography

Chromatographic analyses were performed using a HPLC system with a photodiode array detector (PDA-100, Dionex) and a fluorescence detector (RF-2000, Dionex). UV–vis detection was used for benzoic acids (280 nm), benzoic aldehydes (280 nm), cinnamic acids (320 nm) and flavonols (360 nm). Sample injection volume was 10 μL. Fluorescense detection was used for catechins (excitation 280 nm and emission 310 nm), coumarins (excitation 340 nm and emission 426 nm) and resveratrol (excitation 310 nm and emission 403 nm). The software for control of equipment and data acquisition was Chromeleon Version 6.60.

Phenolic compounds were separated on a monolithic column (Chromolith TH Performance RP-18e, 4.6 mm, 100 mm; Merck). Two solvents were used: solvent A (10% methanol and 2% acetic acid in water) and solvent B (90% methanol and 2% acetic acid in water) at a flow rate of 5.0 mL min^{-1}. The gradient applied was as follows: (time, solvent A): 0 min, 100%; 1 min, 94%; 4 min, 94%; 5 min, 87%; 6 min, 60%; 9 min, 50%; 14 min, 0%.

A series of working standard solutions of phenolic compounds found in real samples was prepared by dilution. These were used to prepare the calibration curve. Table 1 presents the properties of the calibration curves of those phenolic compounds. The limits of detection and of quantification have been calculated using the ALAMIN software [16].

3. Results and discussion

In this study we evaluate the stability of phenolic compounds with the same method of extraction and with the same solvent (methanol) at different temperatures: 50, 75, 100, 125, 150 and 175 °C. All the extractions have been performed in triplicate and the extracts have been analysed by HPLC.

As can be seen in Table 2, the mean recoveries of most of the phenolic compounds extracted are around 95% at 125 °C. As the temperature is increased, considerable degradation of the phenolic compounds is observed. For example, at 175 °C we obtain a mean recovery of only 34.7%.

Table 1
Analytical properties of calibration curves

	Caffeic acid	Catechin	Epicatechin	Rutin
Calibration range (mg L^{-1})	0.21–20	0.1–20	0.1–20	0.5–25
Regression equation	$y = 8044.7x - 15154$	$y = 43738x + 21262$	$y = 47438x + 31076$	$y = 7155.2x + 2580.2$
Regression coefficient	0.9990	0.9998	0.9999	0.9999
Detection limit (mg L^{-1})	0.23	0.31	0.35	0.60
Detection limit (pg)	2.3	3.1	3.5	6.0
Quantification limit (mg L^{-1})	0.62	0.60	0.77	1.28
Quantification limit (pg)	6.2	6.0	7.7	12.8

Statistical comparisons have been made to determine if there are series of data that differ significantly from the references. Among the 22 compounds studied, only epicatechin, resveratrol and myricetin have shown significant differences when submitted to the extraction conditions at a temperature of 125 °C. Of these three compounds, catechin and resveratrol are known to be easily degradable [17,18]; in fact, in previous studies on the development of extraction methods with PLE, these compounds were also found to be among those most easily oxidizable when employing high extraction temperatures, and it was not possible to utilize extraction temperatures at or above 100 °C for epicatechin [19]. No references have been found in the literature to the stability of myricetin under different extraction conditions.

All the compounds of the benzoic acid family were found to be stable up to 150 °C. However, at 175 °C, veratric acid and *p*-hydroxybenzoic acid were the only compounds that did not present recoveries significantly lower than the reference. In respect of the remaining compounds, it has been found that, the greater the number of hydroxylic-type substituents, and the smaller the number of methoxylic-type substituents, the easier it is for degradation to take place. This can be confirmed from the observation that, despite having an equal number of substituents

Table 2
Recoveries for MAE at 50, 75, 100, 125, 150 and 175 °C of 22 phenolic standards[a]

	50 °C	75 °C	100 °C	125 °C	150 °C	175 °C
Benzoic acids						
Gentisic acid	97.1 ± 6.9	102.8 ± 3.9	103.5 ± 1.7	99.4 ± 3.3	89.6 ± 0.8	15.0 ± 16.6[b]
Gallic acid	96.0 ± 6.4	102.3 ± 4.4	104.0 ± 1.3	100.4 ± 4.5	94.5 ± 0.7	17.3 ± 24.6[b]
p-Hydroxybenzoic acid	97.1 ± 5.6	102.8 ± 3.1	104.6 ± 2.0	102.7 ± 3.1	95.7 ± 0.2	94.6 ± 1.8
Vanillic acid	96.5 ± 5.3	102.4 ± 4.5	102.9 ± 0.7	100.8 ± 3.4	93.9 ± 0.4	81.6 ± 4.7[b]
Veratric acid	99.4 ± 6.4	111.1 ± 8.0	103.8 ± 2.9	101.2 ± 0.8	101.2 ± 1.8	101.2 ± 3.1
Flavan-3-ols						
(+)-Catechin	100.8 ± 6.3	103.1 ± 5.4	99.2 ± 1.4	105.6 ± 9.0	122.1 ± 4.2[b]	0.0[b]
(−)-Epicatechin	100.9 ± 2.7	96.7 ± 0.8	99.5 ± 2.3	75.9 ± 6.2[b]	52.5 ± 5.2[b]	0.0[b]
Benzoic aldehydes						
Protocatechuic aldehyde	98 ± 3.6	102.7 ± 1.3	101.1 ± 6.6	99.3 ± 9.3	78.9 ± 0.9[b]	0.0[b]
Vanillin	97.0 ± 6.1	101.6 ± 4.9	95.5 ± 6.3	104.2 ± 6.1	82.4 ± 2.8[b]	74.2 ± 1.8[b]
Syringaldehyde	100.0 ± 5.4	101.7 ± 3.3	97.2 ± 6.8	103.8 ± 5.9	88.6 ± 1.0[b]	47.5 ± 9.6[b]
Veratric aldehyde	99.0 ± 3.4	100.1 ± 2.2	97.3 ± 9.1	94.7 ± 3.3	81.7 ± 3.1[b]	84.0 ± 2.6[b]
Coumarins						
Esculetin	108.1 ± 5.9	108.5 ± 2.6	101.7 ± 2.3	102.5 ± 1.8	74.1 ± 7.5[b]	43.1 ± 48.4[b]
Umbelliferon	103.2 ± 5.6	105.6 ± 8.9	92.0 ± 0.9	105.1 ± 1.5	94.2 ± 8.1	95.2 ± 3.0
Scopoletin	102.4 ± 6.1	104.1 ± 7.9	93.6 ± 0.1	101.8 ± 1.5	91.9 ± 2.7	90.6 ± 2.5[b]
4-Hydroxycoumarin	103.1 ± 5.3	101.0 ± 6.7	94.9 ± 9.3	103.0 ± 0.1	76.0 ± 11.0[b]	71.1 ± 2.2[b]
Cinnamic acids						
Caffeic acid	97.9 ± 10.2	96.2 ± 3.8	95.7 ± 1.2	96.9 ± 0.4	83.7 ± 3.0	1.9 ± 42.1[b]
p-Coumaric acid	98.6 ± 7.4	97.2 ± 2.5	99.5 ± 2.4	102.2 ± 1.8	92.7 ± 1.2	99.4 ± 5.5
Ferulic acid	96.9 ± 7.7	93.4 ± 0.7	100.8 ± 0.8	104.9 ± 2.2	89.1 ± 1.5[b]	0.0[b]
Sinapic acid	96.8 ± 7.3	89.0 ± 1.6	98.5 ± 6.4	97.6 ± 1.7	78.8 ± 1.0[b]	0.0[b]
Estilben						
Resveratrol	102.9 ± 4.0	94.4 ± 1.8	98.8 ± 2.7	83.0 ± 6.1[b]	61.2 ± 3.7[b]	30.1 ± 5.8[b]
Flavonols						
Myricetin	96.1 ± 3.0	96.7 ± 0.4	91.9 ± 1.5	70.6 ± 1.6[b]	0.0[b]	0.0[b]
Kaempferol	100.2 ± 1.8	97.6 ± 1.0	99.3 ± 0.5	95.0 ± 3.0	0.0[b]	0.0[b]

[a] Mean ± SD for recoveries relative to the reference.
[b] Significant difference at 95% confidence level.

in their aromatic ring, veratric acid is more stable than vanillinic acid (degradation of 18.5% at 175 °C).

In the case of the aldehyde derivatives of benzoic acid, the most stable was found to be veratric aldehyde, which suffered a degradation of 16% at 175 °C, while the compound with one methoxyl and one hydroxyl (vanillin) suffered a degradation of 25.8% at that temperature. Finally, the compound with two hydroxyls as substituents (protocatechuic aldehyde) suffered complete degradation at 175 °C, for no trace was found of this compound in the sample submitted to those conditions. Siringaldehyde also shows a low stability at 175 °C, this compound has the largest number of substituents in the aromatic ring, however they are both hydroxilic and methoxylic substituents.

The results obtained for the derivatives of cinnamic acid were along similar lines. Specifically, with equality in the number of substituents, the most stable compound of those assayed was the one that had the largest number of methoxyls (ferulic acid), which suffered degradation of 10.9% at 150 °C, while caffeic acid (with two hydroxyl substituents) suffered degradation of 16.3% at the same temperature. The most stable of the compounds of this family was found to be the compound with the smallest number of substituents, *p*-coumaric acid, which remained without suffering any degradation even employing a temperature of 175 °C. At the other extreme is sinapic acid (with three substituents) which suffered complete degradation at 175 °C but only 21.2% at 150 °C.

Four compounds of the coumarin family were assayed and, of these, only umbelliferone was found to be stable up to the maximum working temperature. The other coumarins can only be extracted without significant degradation at temperatures up to 125 °C. In this case the differences due to the methoxyl substituents are reflected in a reduced degradation at 175 °C; that is, degradation of 9.5% for scopoletin and of 57% for the esculetin, which is the compound with the largest number of hydroxyl substituents.

Among the flavonols, kaempferol is stable up to 125 °C and at 150 °C it is completely degraded under the extraction conditions, while myricetin already presents a significant degradation at 125 °C. The difference between these two compounds is the presence of two additional hydroxyls in the ring in the case of myricetin, and consequently, as in the previous cases, a greater degree of substitution translates into reduced stability of the compounds.

Table 3
Recoveries for MAE at 100 °C of phenolics from spiked hot peppers[a]

Phenolic compound	Recovery
Benzoic acids	
Gentisic acid	102.7 ± 7.3
Gallic acid	98.0 ± 6.7
p-Hydroxybenzoic acid	108.3 ± 3.5
Vanillic acid	107.2 ± 3.0
Veratric acid	94.3 ± 5.0
Benzoic aldehydes	
Protocatechuic aldehyde	103.7 ± 0.7
Vanillin	106.1 ± 4.2
Syringaldehyde	108.0 ± 2.6
Veratric aldehyde	102.7 ± 2.2
Cinnamic acids	
Caffeic acid	99.0 ± 3.5
p-Coumaric acid	97.9 ± 4.1
Ferulic acid	105.6 ± 2.2
Sinapic acid	102.8 ± 4.9

[a] Mean ± SD for recoveries relative to the amount spiked.

Thus the stability of all the cited phenolic compounds has been tested by submitting them to extractions at high temperatures; therefore the technique of MAE is considered viable for the extraction of these compounds at temperatures equal to or less than 100 °C for 20 min.

After checking the behaviour of the phenolic compounds at different temperatures by means of MAE, the next step was to determine the recovery of these compounds from real samples. First, the recovery of tested phenolics was studied from hot peppers. Naturally occurring phenolics in this sample are shown in Table 3. Freeze dried hot pepper (*Capsicum annuum*) was spiked with different amounts of the standards. The sample was spiked with phenolics 1 h before analysis in order to ensure interaction of standards with the sample. Recovery was obtained by dividing the amount added by the phenolic amount in the spiked sample after subtracting the amount found in the original sample. The results for each phenolic are shown in Table 3. Mean recovery for all phenolics with the sample spiked was 102.5 ± 4.4%. The lowest recovery was found for veratric acid (94.3 ± 5.0%), and the highest for *p*-hydroxybenzoic acid (108.3 ± 3.5%).

A different real sample was studied in order to determine the behaviour of another non commercially available phenolics

Table 4
Amount of phenolics (mg kg^{-1}) extracted using MAE

Grape variety		Caftaric acid[a]	Catechin	Epicatechin	Quercetin-3-gluronide[b]	Quercetin-3-glucoside[b]	Kaempferol-3-glucoside[b]
Red Globe		0.009	0.079	0.059	0.129	0.196	tr
Napoleón	Skin	0.016	0.393	0.070	0.884	0.247	0.455
Moscatel		0.025	0.414	0.082	0.810	0.671	0.224
Red Globe		nd	0.796	1.169	nd	nd	nd
Napoleón	Seed	nd	0.647	0.875	nd	nd	nd
Moscatel		nd	3.748	1.951	nd	nd	nd

Extraction conditions: temperature 100 °C; solvent, 100% methanol; extraction time 20 min; tr, traces; nd, not detected.
[a] Expressed as caffeic acid equivalents.
[b] Expresed as rutin equivalents.

during the microwave-assisted extraction at the temperature of 100 °C; these samples were two varieties of red grape and one variety of white grape. The seeds and skins were extracted separately using MAE, under the same conditions as those applied to the standards. The extracts obtained were concentrated under nitrogen, made up to a volume of 1 mL and, after filtering, were analysed by CLAE. The results are presented in Table 4.

The results for grapes are in accordance with the literature data [20]. Flavanols are the most important compounds in skins of both red and white varieties, whereas none of them are relevant in grape seeds. On the other hand, catechins are the most abundant compounds in grape seeds, while they are found in lower levels in skins. Finally, caftaric acid is the most important benzoic derivative found in grape skins, whereas it was not found in grape seeds.

In the light of the results obtained using hot peppers and grapes, it appears that a viable method of extraction could be developed, based on the application of microwaves and employing 100 °C as the extraction temperature, for sample preparation in the determination of phenolic compounds in solid samples.

4. Conclusions

From what has been presented here, if the intention is to develop a method based on MAE for these compounds, it is possible to at a temperature of up to 100 °C for 20 min without degradation of these compounds taking place. Regarding the relationships between the chemical structure and the susceptibility to degradation process, it has been found that the fewer the substituents that are present in the aromatic ring, the higher the stability of phenolic compounds during the microwave-assisted extraction. Additionally, when two compounds have an equal number of substituents in the ring, the hydroxylates will be more easily degradable than the methoxylates.

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