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Ultrasound-assisted extraction of phenolic compounds from strawberries prior to liquid chromatographic separation and photodiode array ultraviolet detection

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Abstract

Ultrasound-assisted extraction was used for the determination of phenolic compounds present in strawberries. The optimization study of the extraction was carried out using spiked samples (100 mg/kg). The sample immersed in an aqueous solution containing hydrochloric acid (0.4 M) was sonicated for 2 min (duty cycle 0.2 s, output amplitude 20% of the nominal amplitude of the converter, applied power 100 W with the probe placed 1 cm from the bottom of the water bath and 5 cm from the walls of the precipitate glass). Subsequent separation was carried out by liquid chromatography (LC) with photodiode array UV detection. Calibration curves using the standard addition in green strawberries typically gave linear dynamic ranges of 2–300 mg/l for all analytes; R^2 values exceeded 0.996 in all cases. The method was applied to two types of strawberries to demonstrate the applicability of the proposed method, which is much faster and produces less analyte degradation than methods as solid–liquid, subcritical water and microwave-assisted extraction.
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Keywords: Ultrasound-assisted extraction; LC; Phenolic compounds; Strawberries

1. Introduction

Phenolic compounds are a group of biologically active molecules present as metabolites in plants. The interest for these natural compounds has increased in the last years due to their antioxidative [1,2], and anticarcinogenic activity [3], and relationship to human health [4]. It has been demonstrated that some health benefits of food and beverages depend on the presence of these antioxidants, which occur in olives and olive oil, red wine, berries, fruits and vegetables in general. In this study, strawberries were used as sample due to the fact that a number of phenolic compounds have been detected in berries [5–8] conferring them antioxidative and anticarcinogenic properties [9,10].

Many studies deal with detection and quantification of these antioxidants.. The most widely used methods are based on liquid chromatography (LC) with photodiode array and/or mass spectrometric detection [5,11,12]. The latter, LC–MS, has become a key alternative for the determination of phenolic compounds in plants, because of the analytical complexity [13]. LC with direct injection is the technique usually applied for beverages and other liquid samples [14]. Filtration of the samples is the only pre-treatment needed. Solid foods require an appropriate prior extraction [15,16], for which solvents such as ethanol, acetone or methanol are used [17], or a water–methanol mixture which contains both hydrochloric acid and an antioxidant [12]. The extraction of phenolic compounds requires special care, because they are easily oxidized and rapidly degraded by light. Different techniques as supercritical fluid extraction (SFE) – using either pure or modified CO_2 [18] – and microwave-assisted extraction [19] have been applied. These techniques offer a better control over the extraction conditions and allow the extraction to be performed in shorter times and in a more selective way.

Ultrasonic radiation is a powerful aid to accelerate of various steps of the analytical process. This energy is of great help in the pre-treatment of solid samples as it facilitates and accelerates operations such as the extraction of organic and inorganic compounds [20,21], homogenization [22] and various others [23]. Ultrasound-assisted leaching is an effective way to extract

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analytes from different matrices in shorter time than other extraction techniques.

The research presented here is based on the application of ultrasound to leach and hydrolyze phenolic compounds. LC–UV method is used for the determination of a group of phenolic compounds present in strawberries under normal and/or stress conditions.

2. Experimental

2.1. Instruments and apparatus

Ultrasonic irradiation was applied by means of a Branson 450 sonifier (20 kHz, 100 W) equipped with a cylindrical titanium alloy probe (2.54 cm diameter) which was immersed in a water bath in which a precipitate glass with the sample was placed. A rotary-evaporator (R-200, Büchi, Switzerland) was used to release the solvent after extraction.

The LC system was an HP1100 liquid chromatograph (Agilent Technologies, Avondale, PA, USA) consisting of a G1311A high-pressure quaternary pump, a G1322A vacuum degasser, a Rheodyne 7725 high-pressure manual injector valve (10 μl injection loop) and a G1315A diode array UV–vis detection (DAD) system. A Hypersil ODS column (250 mm × 4.6 mm I.D., 5 μm) protected with a Hypersil ODS guard column (10 mm × 4.6 mm I.D., 5 μm) was used.

2.2. Reagents

Caffeic, *p*-coumaric, ferulic, gallic, *p*-hydroxybenzoic, veratric, gentisic, vanillic, chlorogenic, protocatechuic, syringic, syrinic and salicylic acids, syringaldehyde and vanillin were purchased from Sigma–Aldrich (Madrid, Spain). A methanol stock standard solution of 1000 mg/l each analyte was prepared and stored at 4 °C in the dark. By dilution of this stock solution with methanol the solutions to be used were prepared every week. Ultrapure water from Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. Methanol (Panreac, Spain) was of LC grade. Acetic acid and hydrochloric acid (Panreac, Spain) were of analytical grade.

2.3. Sample preparation

Strawberry samples were collected in the field (Huelva, Spain) by people from the Department of Biochemistry, University of Córdoba (Córdoba, Spain). The strawberries were stored for 9 months at −80 °C. The frozen samples were crushed in a food processor and stored at −20 °C until use. In this study, strawberries were frozen in approximately 100 g batches and stored at −20 °C until use. Strawberries used in precision studies were from the same batch and analyzed within 24 h.

Spiked green strawberries were used as the matrix to carry out the optimization study. Approximately 5 g of sample was spiked with the necessary amount of phenolic compounds (0.5 ml of the stock standard solution of 1000 mg/l) to obtain a final total concentration in the food of 100 mg/kg in each analyte (concentration: at the center of the linear range used in the standard addition method). Then, the samples were stored for 2 h at 4 °C before extraction in order to simulate the normal interaction between strawberries and the phenolic compounds. In order to minimize errors in spiking, sampling was done according to the protocol established by legislation [24]. Red strawberries were used in order to demonstrate the suitability of the proposed method for the extraction of the target compounds from strawberries in other maturation stage. These samples were prepared and spiked in the same way as green strawberries.

2.4. Extraction and hydrolysis

Five g of strawberry were placed in a precipitate glass and 10 ml of an aqueous 0.4 M HCl solution were added for simultaneous extraction and hydrolysis. This unit was immersed in a water bath and sonicated for 2 min (duty cycle 0.2 s, output amplitude 20% of the nominal amplitude of the converter, applied power 100 W with the probe placed 1 cm from the bottom of the water bath and 5 cm from the walls of the precipitate glass). After complete extraction, the extract was filtered through a filter paper (ashless filter paper, 12.5 cm) and evaporated to dryness using a rotary evaporator and a water bath at 60 °C.

The residue was dissolved in 5 ml of methanol–water (10:90, v/v) adjusted to pH 3 with acetic acid and filtered through a 0.45 μm filter that was compatible with organic solvents (nylon syringe filter 25 mm/0.45 μm, Análisis Vínicos, Ciudad Real, Spain) prior to injection into the LC–DAD system.

2.5. Chromatography

The LC separation was performed using a methanol–water gradient. The mobile phase consisted of: methanol–water (10:90, v/v) (A) and methanol–water (50:50, v/v) (B), both adjusted to pH 3 with acetic acid. The gradient program was as follows: 0–1 min, 95% A and 5% B, flow rate 0.5–1 ml/min; 1–5 min, 95% A and 5% B, flow rate 1 ml/min; 5–15 min, 95–90% A, flow rate 1–0.8 ml/min; 15–30 min, 90% A and 10% B, flow rate 0.8–0.5 ml/min; 30–40 min, 90–85% A, flow rate 0.5 ml/min; 40–60 min, 85–80% A, flow rate 0.5 ml/min; 60–80 min, 80–60% A, flow rate 0.5 ml/min; 80–100 min, 60–50% A, flow rate 0.5–0.8 ml/min; 100–110 min, 50–45% A, flow rate 0.8 ml/min. The chromatograms were acquired at 254, 275, 280, 310 and 325 nm. Quantification was done by peak area measurement (Fig. 1).

3. Results and discussion

3.1. LC–DAD

No single wavelength is appropriate to monitor all phenolics since they display absorbance maxima at different wavelengths. Five wavelengths were used as a compromise solution. Most benzoic acid derivatives, such as protocatechuic, *p*-hydroxybenzoic, vanillic and veratric acid show maximum absorbance close to 254 nm, but for gallic, salicylic and syringic acid which have their maxima absorbance at 275,

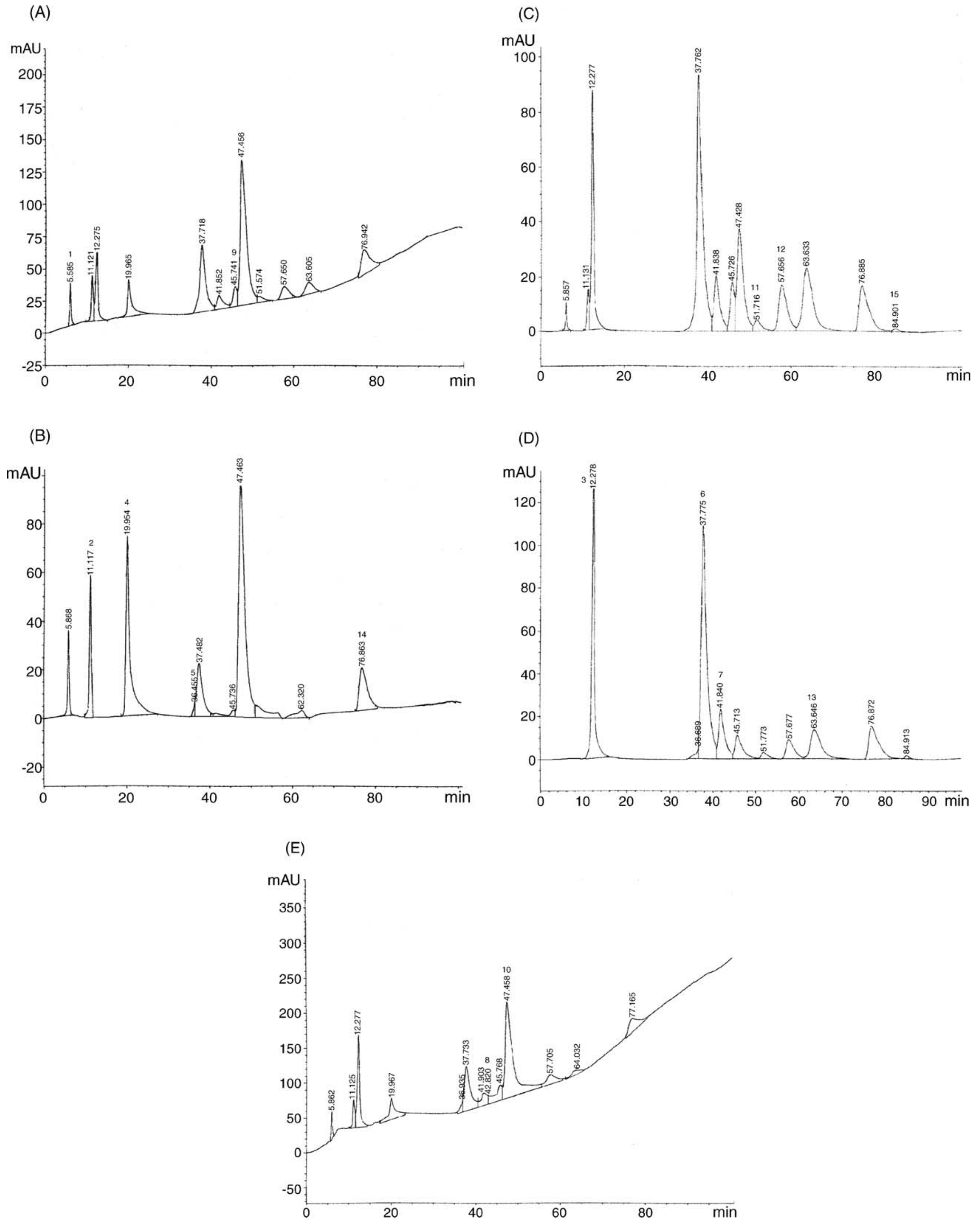

Fig. 1. LC chromatogram of the standard mixture recorded at 5 wavelengths: (A) at 275 nm: 1, gallic acid; 9, vanillin; (B) at 254 nm: 2, protocatechuic acid; 4, *p*-hydroxybenzoic acid; 6, vanillic acid; 15, veratric acid; (C) at 310 nm: 12, syringaldehyde; 11, salicylic acid; 14, ferulic acid; (D) at 325 nm: 3, gentisic acid; 5, chlorogenic acid; 8, caffeic acid; 13, *p*-coumaric acid; and (E) at 280 nm: 7, syrinic acid; 10, syringic acid.

Table 1
Comparison of the proposed method with other methods: working conditions

Solid–liquid method	Subcritical water extraction	Microwave-assisted extraction	Ultrasound-assisted extraction
Temperature: 35–37 °C	Temperature: 130 °C		
Time: 15–16 h	Time: 10 min	Time: 3 min	Time: 3 min
HCl: 1.2 M	HCl: 1.2 M	HCl: 1.2 M	HCl: 1.2 M
Extract volume: 35 ml	Extract volume: 12 ml	Extract volumel: 35 ml	Extract volume: 35 ml
		Power: 150 W	Amplitude: 70–80%
			Duty cycle: 50%
			Probe distance: 5 cm
			Probe heigth: 1 cm

310 and 280 nm, respectively. Gentisic, chlorogenic, caffeic and *p*-coumaric acid have their maximum absorbance close to 325 nm, syringaldehyde and ferulic acid close to 310 nm and vanillin at 275 nm.

The composition and flow rate of the mobile phase were optimized. Different water–methanol mixtures (90:10, 75:25 and 50:50) and different gradients were tested on the Hypersil ODS column. The best separation was achieved using the gradient program given in Section 2.5. The influence of the flow rate was studied in the range 0.3–1.2 ml/min. Flow rates given in Section 2.5 were selected as the values providing separation in a shorter time.

Two injection volume were tested (10 and 20 μl). As the number of phenolic compounds present in strawberries is high, some coelution was observed and an injection loop of 10 μl was selected in order to minimise peak overlapping.

3.2. Extraction/hydrolysis

Preliminary studies were carried out in order to decide the type of energy to assist analyte extraction and the extractant to be used and also to study the effect of freeze-drying on the target phenolic compounds.

The stability of the analytes was studied with different types of energy and different times. An evaluation of the decomposition of the phenolic compounds when subject to solid–liquid, subcritical water or microwave-assisted extraction and sonication was carried out in order to assess the type of energy that produces a lower degradation of the analytes. The working conditions used are shown in Table 1 [12,19,25]. After observing the results obtained and selecting the ultrasound-assisted extraction as the best, the stability of the phenolic compounds at different times was studied. Between 1 and 30 min were tested. Similar degradation (close 100%) was observed by applying ultrasounds between 10 and 30 min. Degradation decreased by reducing ultrasound exposure time to less than 10 min. For 1–5 min degradation of the analytes was close to the degradation percent of ultrasound-assisted extraction in Table 2.

Two extractants were studied in order to select the most suitable in this case: a water–methanol mixture (0:100 and 50:50) containing hydrochloric acid (1.2 M) and an aqueous solution containing hydrochloric acid (1.2 M) were tested. Maximum efficiency was observed when the latter extractant was used.

The effect of freeze-drying on the strawberries was studied and the results were compared with those obtained by extraction of the analytes in non freeze-dried samples. The strawberries were spiked after being frozen. Differences between results were not observed.

A multivariate optimization approach was used for the extraction–hydrolysis step due to the interrelation between the

Table 2
Comparison of the proposed method with other methods: degradation of the analytes

Analyte	Degradation (%)			
	Solid–liquid method	Subcritical water extraction	Microwave-assisted extraction	Ultrasound-assisted extraction
Gallic acid	61	100	54	45
Protocatechuic acid	36	100	33	36
Gentisic acid	0.5	100	55	16
p-Hydroxy-benzoic acid	54	100	58	17
Vanillic acid	45	100	56	29
Chlorogenic acid	63	100	60	49
Caffeic acid	58	100	55	47
Syrinic acid	65	96	87	55
Vanillin	55	58	38	29
Syringic acid	14	99	7	0
Syringaldehyde	37	55	24	37
Salicylic acid	59	49	63	0
p-Coumaric acid	27	76	28	43
Veratric acid	19	82	19	28
Ferulic acid	67	68	75	29

Table 3
Ranges assessed and optimum values for the variables affecting the leaching-hydrolysis step

Experimental design	Variable	Tested range	Optimum value
First	Probe height (cm)	1–3	1
	Probe distance (cm)	1–5	5
	Duty cycle (%)	20–80	–
	Sonication time (min)	2–10	–
	Radiation amplitude (%)	20–80	20
	Hydrochloric acid (M)	0.6–1.2	–
	Extractant volume (ml)	10–40	10
Second	Duty cycle (%)	10–20	20
	Sonication time (s)	30–120	120
	Hydrochloric acid (M)	0.2–0.6	0.4

variables influencing them. The variables optimized in this step were the probe position (distance to the glass container and height from the bottom of the water bath), radiation amplitude, percent of duty cycle of ultrasound exposure, sonication time, volume and concentration of hydrochloric acid in the extractant (Table 3).

A Plackett–Burman design allowing three degrees of freedom and involving 12 randomised runs plus three center points was built for a screening study of the behaviour of the main factors affecting the extraction step. The conclusions of this screening were that the ultrasound radiation amplitude, extractant volume, distance between the tip horn of the ultrasonic probe and the precipitate glass and height of the probe were not significant factors in the ranges under study. However, the results showed better recoveries with minimum extractant volume (10 ml), height of the probe (1 cm from bottom of the water bath) and ultrasound radiation amplitude (20%) and a maximum distance between the probe and the precipitate glass (5 cm). Thus, the lower values tested for the extractant volume, height of the probe and radiation amplitude were selected for subsequent experiments due to their negative effects. Likewise, the upper values tested for the distance between the probe and the glass container were selected for subsequent experiments due to their positive effect. Lower values for the duty cycle, sonication time and acid concentration in the extractant were tested using a two-level full factor design involving eight randomised runs plus three center points. In this case, the duty cycle was not a significant factor in the range studied for all analytes. However, the upper value tested (0.2 s) was selected due to their positive effect. The sonication time and the acid concentration of the extractant were significant factors in the ranges studied for some analytes. The upper value tested for the sonication time (2 min) and the intermediate value tested for the acid concentration of the extractant (0.4 M HCl) in the range studied were selected for subsequent experiments. Statistical software was used to analyze the data from the experiments [26].

The recoveries of the added analytes were 84–99% (Table 4).

Five milliliters of a 10:90 (v/v) methanol–water solution adjusted to pH 3 with acetic acid was required to dissolve the analytes from the dry residue.

Table 4
Recoveries of phenolics obtained in the extraction from green strawberries by the proposed method

Analyte	Recovery (%)	RSD (%, $n=3$)
Gallic acid	91	15
Protocatechuic acid	84	3
Gentisic acid	89	3
p-Hydroxybenzoic acid	87	2
Vanillic acid	93	4
Chlorogenic acid	93	2
Caffeic acid	94	2
Syrinic acid	97	3
Vanillin	90	4
Syringic acid	94	3
Syringaldehyde	89	3
Salicylic acid	86	6
p-Coumaric acid	87	4
Veratric acid	97	5
Ferulic acid	99	4

3.3. Characterization of the overall method

Calibration curves were obtained by plotting the peak area as a function of standard concentrations (Table 4). The linear dynamic ranges (between 2 and 300 mg/l) revealed good linearity with R^2 values exceeding 0.996 in all cases. Quantification was based on the standard addition method with samples spiked with a multistandard mixture of known concentrations (0–300 mg/l for each analyte). Matrix effects with a similar trend were detected for syrinic acid, vanillin (Fig. 2b and c, respectively), syringic and syringaldehyde acids. No matrix effects were detected for gallic acid as the slope was the same for standards and standard addition (Fig. 2).

For the analytes that do not appear in strawberries, calibration curves were run using standards of known concentrations to which the overall process (from extraction to detection) was applied (Table 5). The limits of detection calculated for real-life samples were 1–2 mg/kg for all test analytes and relative standard deviations (RSDs) were 7–12% ($n=3$) for all analytes.

In order to evaluate the precision of the proposed method, within-laboratory reproducibility and repeatability were estimated in a single experimental set-up with duplicates for each method [27]. The experiments were carried out using the optimal conditions involving seven replicates on different days and two replicates on the same day, morning and afternoon, for seven days. The range of within-day relative standard deviations was 4.6–7.7%; the between-day study yielded RSDs within 5.3–8.6%.

The aim of this work was to develop a method for the quantitative analysis phenolics content of strawberries at different stages. The phenolic compounds were therefore determined in green and red strawberries. Significant amounts of gallic acid (4.6 mg/kg), syrinic acid (1.6 mg/kg), vanillin (1.6 mg/kg), syringic acid (1.9 mg/kg) and syringaldehyde (5.8 mg/kg) were found in green strawberries. Only gallic acid (566 mg/kg) and syringic acid (0.12 mg/kg) were found in red strawberries. These differents of the analytes and concentrations in green and red

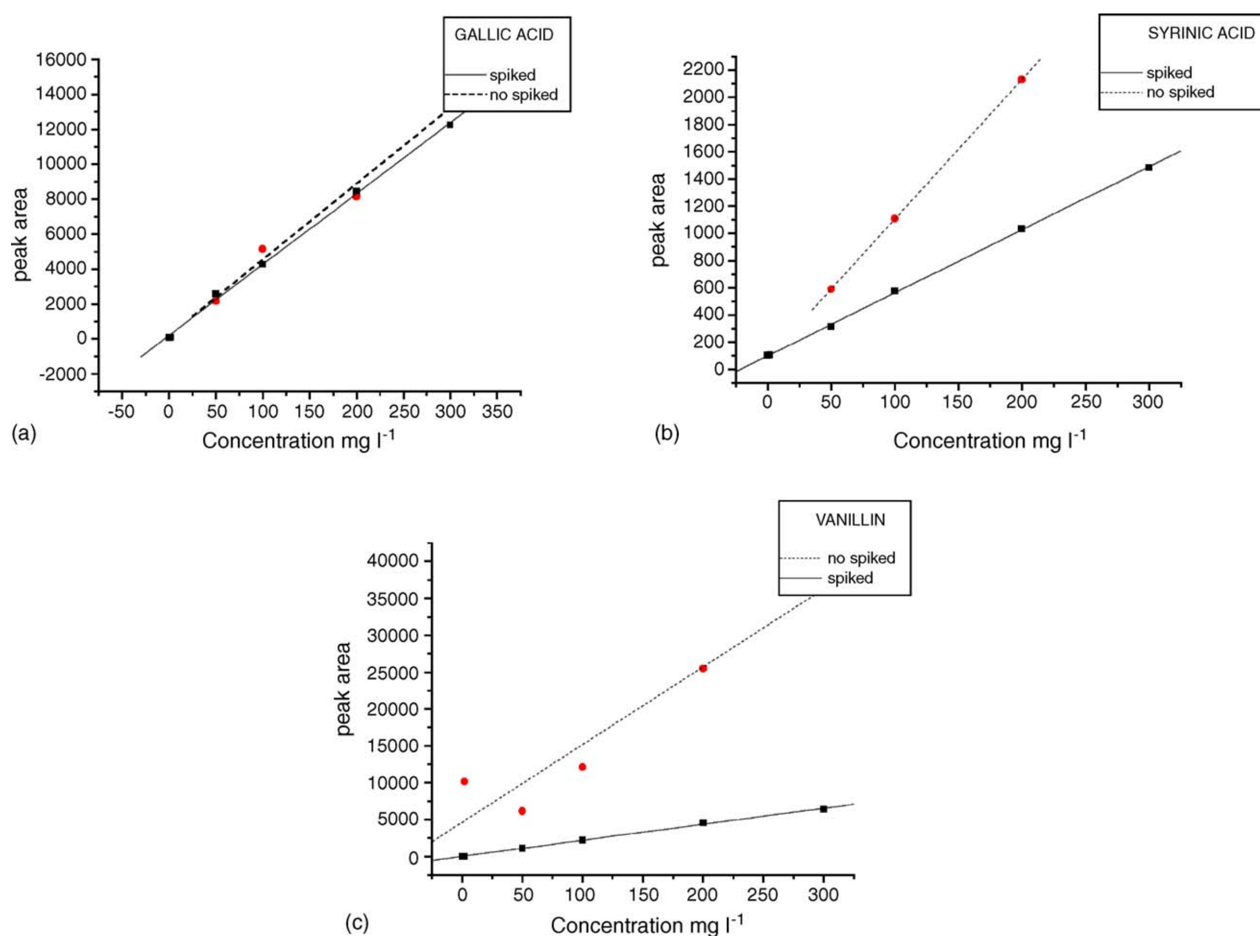

Fig. 2. Calibration curves obtained with spiked and non-spiked samples: (a) gallic acid; (b) syrinic acid; and (c) vanillin.

Table 5
Standard addition and standard calibration data for phenolic compounds

Compound	Calibration equation
Gallic acid	$Y = 40X + 267$
	$Y = 43X + 267$
Protocatechuic acid	$Y = 8X + 13$
p-Hydroxybenzoic acid	$Y = 62X + 10$
Gentisic acid	$Y = 0.6X + 5$
Vanillic acid	$Y = 17X - 94$
Chlorogenic acid	$Y = 2X - 9$
Caffeic acid	$Y = 2X + 3$
Syrinic acid	$Y = 5X + 96$
	$Y = 16X + 385$
Vanillin	$Y = 22X + 75$
	$Y = 112X + 1256$
Syringic acid	$Y = 69X + 142$
	$Y = 464X + 149$
Syringaldehyde	$Y = 34X + 17308$
	$Y = 189X + 2358$
Salicylic acid	$Y = 5X + 33$
p-Coumaric acid	$Y = X - 7$
Veratric acid	$Y = 36X - 47$
Ferulic acid	$Y = 0.8X + 7$

strawberries were found probably due to the different maturity stage of the fruits. This behaviour has been reported in the literature [28].

4. Conclusions

The method here reported describes a fast ultrasonic extraction with simultaneous hydrolysis of a variety of phenolic compounds from spiked strawberries compared with other methods. Two to 20 h, depending on the kind of the analyte to be extracted, were required by Häkkinen et al. [12] for extraction and hydrolysis by refluxing 85 °C; meanwhile the method proposed by Murga et al. needs 3 h extraction using supercritical CO_2 as extractant [29]. Two min are sufficient for extraction and hydrolysis by the method here proposed.

No additional clean-up but filtration of the extracts before and after the necessary solvent change step was required.

The feasibility of the finally proposed method for the determination of selected phenolic compound in strawberries with a different degree of ripeness (green and red strawberries) has been tested.

Results proved that, except for the gallic acid, proper quantitation of the analytes could only be achieved by using the standard addition method due to matrix effects.

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