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In-line pressurized-fluid extraction–solid-phase extraction for determining phenolic compounds in grapes

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Abstract

A new method of pressurized-fluid extraction coupled in-line with solid-phase extraction has been used for the extraction of phenolic compounds from grapes. The full extraction method is performed under an inert atmosphere. Five different solvents have been assayed using different extraction pressures and temperatures. Using two extraction stages with two different solvents, water and methanol, quantitative recovery for most of the assayed compounds has been found in the second extract. Only the most polar phenolic compound, gallic acid, was found distributed in both extracts. The application to real samples allows for a clean-up of the extracts. Cinnamic esters like caftaric acid, *cis* and *trans*-coutaric acids were found only in the methanolic extract. The reproducibility for the new method was measured using both an inert solid spiked with standards and grapes. Using between 202 and 424 μg of spiked standards, the resulting relative standard deviations were less than 5%, with the exception of gallic acid (RSD=13%). For grapes, the resulting RSD were 11% for *trans*-coutaric acid, 10% for caftaric acid and 6% for *cis*-coutaric acid.
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1. Introduction

The determination of phenolic compounds in grapes, wines and other foods has been of increasing interest in last years. The reasons for this interest are: first, these compounds play an important role in the flavor and color of the foods [1]; second, they act as natural antioxidants [2]; and third, various interesting biological activities have been found [3], including those related to human health [4].

For beverages and other liquid samples, high-performance liquid chromatography (HPLC) analysis using direct injection is the analytical method usually applied [5,6]. Filtration of the samples is the only pre-treatment needed. Solid-phase extraction (SPE) has been also used to obtain clearer chromatograms [7]. In solid foods, the analytical procedures first need a suitable extraction stage. In most cases, soaking with solvents has been used as the extraction stage [8,9]. Various different solvents such as ethanol, acetone and methanol have been used [10]. For phenolic compounds, special attention must be paid to the extraction stage. Because these compounds are easily oxidizable and rapidly degraded by light, the

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extraction stage must be performed under an inert atmosphere and protected from the light [11].

In the search for an extraction method whereby the phenolics are protected from degradation by both light and air, supercritical fluid extraction (SFE) methods using CO_2 or modified-CO_2 have been applied [12]. Using these techniques, phenolics have been extracted from the solid parts of grapes [13]. However, owing the polarity of some of the more interesting phenolic compounds, large quantities (10–20%) of the organic modifier were needed. Then the supercritical fluid extraction becomes a subcritical fluid extraction and it is better described as a pressurized-fluid extraction (PFE) process. Using different pressurized organic solvents instead of CO_2, a higher selectivity can be achieved in the extraction, because a larger number of solvents can be used.

PFE has been applied to different families of phenolic compounds in several matrices [14]. It has been proved that they are not degraded when 100 °C is used as extracting temperature in PFE [15]. Using a SPE stage subsequent to the first PFE stage, a high degree of selectivity can be achieved. Several extraction methods coupling both PFE or SFE with SPE have been developed [16,17]. However most of them performed the SPE outside the extraction cell. Only an oil clean-up was done in-line inside the extraction chamber during a PFE [18].

For the determination of phenolics in grapes, wines and other beverages, different solid-phases have been tested for SPE. Polymers of styrene–divinylbenzene produced good results, while C_{18} based phases produce less satisfactory results for the more polar phenolics. The main goal of this investigation was developing an in-line clean-up method to obtain an extract with phenolic compounds but with a reduced amount of sugars.

2. Materials and methods

All the standards were obtained from Sigma–Aldrich (St. Louis, MO, USA). A stock standard mixture containing gallic acid (204 ppm), *p*-hydroxybenzoic acid (424 ppm), aesculetin (230 ppm), ferulic acid (221 ppm), scopoletin (202 ppm), sinapic acid (203 ppm) and veratric aldehyde (205 ppm) was prepared in methanol–water (50:50, v/v). The mixture represents all families of phenolics found in grapes and wines.

Sea sand, used as supporting material, was obtained from Panreac (Barcelona, Spain). For PFE, nitrogen was used to purge and to dry the samples during the extractions. LiChrolut EN (2.5 g), from Merck (Darmstad, Germany), was used as solid-phase inside the extraction chamber. White grapes of the Viura variety grown in Jerez (Spain) were used as real samples.

An ASE-200 extractor (Dionex, Sunnyvale, CA, USA) was used for the extraction. The extraction cell volume was 11 ml and the collection vial volume was 60 ml. For the extractions, sea sand inside the extraction cells was spiked with 1 ml of stock standard mixture. Three 10 min cycles were programmed under pressures ranging from 40 to 150 atm. (1 atm=101 325 Pa). After each extraction cycle, the sample was rinsed with 3 ml of methanol and finally purged with nitrogen for 5 min. For real samples, the extraction cell was filled with around 0.5 g of samples. All the extractions were performed using nitrogen as pressurizing gas. The solid-phase was place into the extraction chamber just below the sea sand or the sample. Two cellulose filters were used at the top and bottom of the solid-phase to avoid losses.

The chromatographic analysis was performed by HPLC with a Waters (Milford, MA, USA) chromatographic system (M-45 and 510 pumps, 717 automatic injector, 996 photodiode array detector, Millennium 2.10 software) using a RP-18 LiChrospher column (Merck). The standards were measured at their own maximum absorbance wavelength. An elution gradient was used according to the method proposed by Guillén et al. [19]. Briefly, two solvents were used: solvent A (5% methanol, 2% acetic acid in water) and solvent B (90% methanol, 2% acetic acid in water). The initial conditions were flow-rate: 1 ml/min and 100% A, reaching 85/15 (A:B) in 15 min and 50/50 in 35 min, both changes were done by using a convex gradient.

3. Results and discussion

3.1. Solvents for the in-line PFE–SPE

Recovery using in-line PFE–SPE was checked

applying five different extracting solvents: water, ethanol, diethyl ether, ethyl acetate and methanol. For the extractions 1 ml of stock standard mixture was added into the extraction chamber, just over the sea sand. All the extracts were analyzed directly by HPLC with the exception of extracts obtained with ethyl acetate and diethyl ether. They were evaporated to dryness with a nitrogen gas stream. The dry residue was dissolved in methanol–water (1:1, v/v). So, higher deviations should be expected for the compounds in these extracts.

Four different extraction conditions were checked. Temperatures of 40 and 100 °C and pressures of 40 and 100 atm were used. The recoveries of the spiked phenolics were measured. The resulting recoveries are shown in Table 1. At 40 °C and 40 atm., water produced the lowest recoveries; only 51% of gallic acid was recovered. No other compounds were detected in the PFE extracts. Ethanol and methanol produced good recoveries for all the assayed compounds. Diethyl ether and ethyl acetate produced worse recoveries. At 100 °C and 40 atm., water showed the lowest recoveries. Only gallic acid was detected in the extracts. Methanol is the only solvent that produced good recoveries for all the assayed compounds. Ethanol, diethyl ether and ethyl acetate showed lower recoveries. Therefore, only methanol, and in some cases ethyl acetate, can be considered good solvents for extraction at 100 °C and 40 atm.

In order to determine the effect of the pressure on the extraction process, extractions were run at higher pressures than those needed to maintain the solvent in the liquid state. Water was able to extract several compounds, but only with very low recoveries. Using methanol, ethanol or diethyl ether, the recoveries were lower than when ethyl acetate was used (Table 1).

Comparing these results with those obtained at 40 °C and 40 atm, only ethyl acetate produced better recoveries at higher pressure. Pressure should not influence the first step of the extraction process, i.e. the dissolution by the solvent of spiked phenolics from the sand. Therefore the effect of the pressure should be on the second step, i.e. the process of rinsing from the solid-phase. There are no data about changes of the retention properties of the solid-phase at different pressures. It is most likely that the problem occurs during the de-pressurizing step of the extraction. At this moment, the pressure of the system drops from 150 to 1 atm. This depressurizing step is so fast that it could result in the compounds retained on the solid-phase not being completely rinsed from it. To check this hypothesis, re-extractions of the solid-phase were done using a lower pressure. The compounds not recovered at 40 °C and 150 atm, were then found in the re-extracts, confirming that they were not rinsed from the solid-phase during the first extraction.

Lastly, 100 °C and 150 atm were used as extraction conditions. Ethyl acetate and methanol produced the best recoveries. Ethanol produced an 81% average recovery. Diethyl ether produced poor results. Water produced the recovery of 37% of gallic acid; other compounds were detected but at recovery levels lower than 10% (Table 1).

After studying the results, a two-stage clean-up method was designed. The first stage will be run using water at 40 °C and 150 atm. During this stage,

Table 1
Recoveries[a] of spiked phenolics[b] from sea sand/solid-phase material using different extracting solvents at 40 and 100 °C (in *italics*)

	Water		Ethanol		Diethyl ether		Ethyl acetate		Methanol	
	40 atm	150 atm	40 atm	150 atm	40 atm	150 atm	40 atm	150 atm	40 atm	150 atm
Gallic acid	51 (*116*)	26 (*37*)	93 (*53*)	92 (*46*)	77 (*56*)	63 (*48*)	67 (*47*)	80 (*63*)	93 (*81*)	85 (*45*)
p-Hydroxybenzoic acid	n.d. (*n.d.*)	n.d. (*3*)	103 (*79*)	100 (*84*)	93 (*78*)	96 (*68*)	85 (*88*)	89 (*103*)	104 (*111*)	88 (*105*)
Aesculetin	n.d. (*n.d.*)	n.d. (*6*)	105 (*125*)	84 (*97*)	81 (*38*)	32 (*24*)	54 (*82*)	97 (*94*)	112 (*94*)	72 (*76*)
Ferulic acid	n.d. (*n.d.*)	4 (*n.d.*)	92 (*83*)	56 (*80*)	104 (*77*)	34 (*43*)	114 (*120*)	124 (*143*)	107 (*92*)	46 (*108*)
Scopoletin	n.d. (*n.d.*)	3 (*n.d.*)	99 (*88*)	67 (*83*)	94 (*56*)	52 (*41*)	71 (*104*)	98 (*132*)	103 (*109*)	70 (*109*)
Sinapic acid	n.d. (*n.d.*)	2 (*n.d.*)	95 (*89*)	62 (*79*)	95 (*46*)	40 (*32*)	89 (*101*)	110 (*120*)	112 (*111*)	66 (*107*)
Veratric aldehyde	n.d. (*n.d.*)	4 (*n.d.*)	67 (*101*)	30 (*97*)	103 (*90*)	83 (*86*)	118 (*95*)	98 (*94*)	116 (*106*)	27 (*103*)

[a] Average of two replicates.
[b] Spiking levels (μg): gallic acid, 204; *p*-hidroxibenzoic acid, 424; aesculetin, 230; ferulic acid, 221; scopoletin, 202; sinapic acid, 203; and veratric aldehyde, 205.

the phenolic compounds should be transferred from the sand to the solid-phase. All phenolic compounds should be retained on the solid-phase, with the exception of gallic acid, which could be partially rinsed (26%). Subsequently, a second stage using methanol as extracting fluid, at 40 atm, will be performed. At this pressure, few differences were found between the recoveries obtained working at 40 or 100 °C (Table 1). When extracting real samples, it could be advantageous to perform the extraction at 100 °C instead of 40 °C, in order to get a faster extraction. So, the second stage will be run at 100 °C and 40 atm. In this process, the compounds retained in the first stage on the solid-phase should be rinsed out. Moreover, if any compound is not fully transferred to the extracting liquid in the first stage, during the second step additional dissolution/extraction of that compound could be produced. Therefore, the first extract would contain the most polar compounds, mainly sugars, whereas the less polar compounds should be recovered in the second extract, including most of the phenolic compounds.

3.2. Repeatability of the two stages method

Using the method devised, the repeatability ($n=4$) was measured with the same standard solution. Recoveries in the methanolic extract were higher than 90% for all assayed compounds, with the exception of gallic acid (64%). The results are shown in Table 2.

After each extraction, the sample was removed from the extraction chamber. A re-extraction of the solid-phase was performed to determine any possible memory effect. The same extractions conditions were used. None of the compounds were detected in the extracts. A clean-up of the solid-phase inside the extraction chamber was done after each extraction by rinsing the system three times with methanol (5 ml). The same solid-phase was used for all the extractions carried out for this work.

Table 2
Average recoveries ($n=4$) of phenolics obtained in the methanolic extract using the in-line two stages PFE–SPE method

	Recovery	RSD (%)
Gallic acid	64.3	13
p-Hydroxybenzoic acid	99.2	2
Aesculetin	99.1	4
Ferulic acid	95.5	2
Scopoletin	93.6	1
Sinapic acid	94.2	2
Veratric aldehyde	92.2	1

3.3. Application to real samples

Grapes from the Viura variety were extracted in order to determine the phenolic composition. Before the extraction, the grapes were freeze-dried to remove the water (around 80% of initial weight).

In this variety of grape, caftaric acid (caffeoyl tartaric acid), *trans*-coutaric acid (*trans*-coumaroyl tartaric acid) and *cis*-coutaric acid (*cis*-coumaroyl tartaric acid) were identified and quantified. Fig. 1 shows the structures of these compounds. Standards are not available for these compounds. The identification was achieved by matching the retention time and their spectral characteristics against those recorded for the compounds previously isolated from grapes in our laboratory. The full identification of these compounds has been previously carried out by spectroscopic techniques (NMR, MS) after isolation by preparative HPLC from grapes and musts [20]. As it is usual for these compounds, quantification was expressed in mg/kg of the corresponding free cinnamic acid (i.e. caftaric acid as caffeic acid, *cis*-coutaric acid and *trans*-coutaric acid as coumaric acid). External calibration was used for the free cinnamic acids in the HPLC method. For caffeic acid the limit of quantification was 0.125 mg/l ($R^2=0.9991$) and for coumaric acid it was 0.170 mg/l ($R^2=0.9986$). Fig. 1 shows a chromatogram obtained from the HPLC analysis of the extract from Viura variety grapes.

The determination of these compounds in grapes is very important because they are directly related to the oxidative degradation of the must obtained from grapes. Usually they are analyzed in grape musts, but their analysis in grapes is not fully resolved yet, since they are easily oxidized during the extraction process.

The results obtained with grapes are shown in Table 3. The repeatability ($n=4$) of the extraction method with grapes was determined for these three compounds. Aliquots of the same freeze-dried grapes were used for the analyses. The first fraction of the

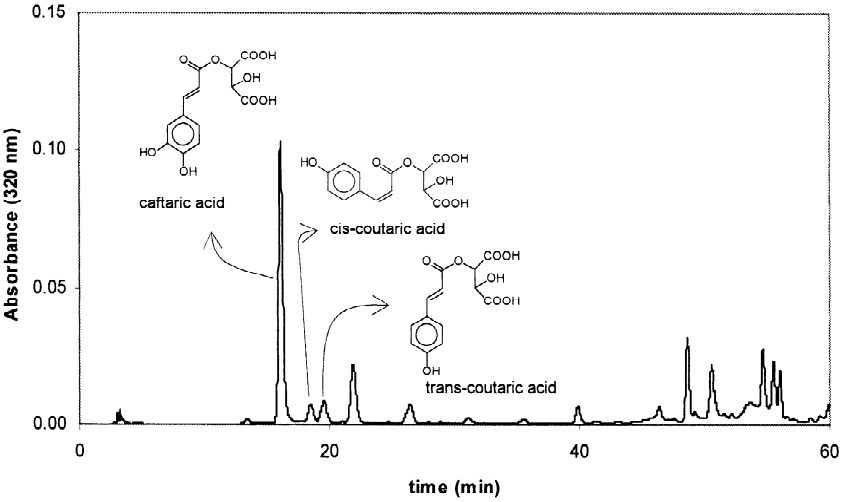

Fig. 1. HPLC chromatogram (320 nm) of extract of whole grapes (*Viura* variety) obtained using the in-line method. Chromatographic conditions: flow, 1.0 ml/min; mobile phase, solvent A: methanol–acetic acid–water (5:2:93); solvent B: methanol–acetic acid–water (90:2:8). Continuous gradient: time (min), B(%): 0, 0; 15,15; 50,50.

analyses (the aqueous extract) was also analyzed and no phenolic compounds were found. After the second extract (the methanolic extract), a re-extraction of the sample was performed using the same conditions. Again no phenolic compounds were detected in the re-extracts.

4. Conclusions

The in-line coupling of PFE and SPE enables the successful extraction of phenolic compounds from grapes. The in-line clean-up can be applied automatically. The full extraction method is performed under an inert atmosphere. The in-line process reduces the sample handling and therefore the possibility of degradation of the extracted compounds. Using similar methods with other samples, extraction methods with increased selectivity could be developed.

Table 3
Amounts of cinnamic esters[a] in grapes obtained using the in-line two stages PFE–SPE method and HPLC analysis ($n=4$)

	Amount	RSD (%)
trans-Coutaric acid	3.7	11
Caftaric acid	10.0	10
cis-Coutaric acid	3.2	6

[a] Quantified as mg/kg of the corresponding free cinnamic acid.

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