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Stability of phenolic compounds during extraction with superheated solvents☆

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Abstract

The stability of nine phenolic compounds in the extraction with superheated methanol at different temperatures (40, 50, 100 and 150°C) has been tested. The evolution of the same compounds in boiling methanol (65°C) in contact with air was also determined. All the assayed phenolic compounds were stable under the extraction conditions with the exception of catechin and epicatechin (recoveries: 87.4% for catechin and 86.0% for epicatechin at 150°C and 94.1% for epicatechin at 100°C). Phenolic compounds kept at the boiling point of methanol (65°C) showed lower recoveries: gentisic acid (85.5%), syringic aldehyde (92.8%), catechin (63.7%) and epicatechin (63.4%). Extraction with superheated solvents was also applied to the extraction of phenolic compounds from solid wastes of the winemaking process. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Extraction methods; Grapes; Pressurized liquid extraction; Superheated solvents; Stability studies; Phenolic compounds

1. Introduction

During the winemaking process several compounds are extracted by the must from the grape pomace [1,2]. Phenolic compounds are extensively extracted, especially in the red wine making process, because the grape solids are in contact with the must during the fermentation process. However, there are phenolic compounds bound to the cell walls that are not extracted during the winemaking process [3]. Some of these phenolic compounds are of considerable interest for their biological properties [4–6]; these include gallic acid and related benzoic acids, the tartaric esters of cinnamic acids and the free cinnamic acids and catechins [7]. In any case, the grape pomace by-product from winemaking is only used as feed for animals due its high fiber content [8].

Currently, industrial recovery methods are being developed to extract the phenolic compounds from this waste material [3]. However, the analytical methods available for determining the phenolic composition of wastes are not adequate, mainly because of difficulties in the extraction stage. The subsequent chromatographic analysis has already been developed by modifying the analogous methods

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for wine analysis. Therefore, it is necessary to find better extraction methods to extract phenolics from waste. The extraction method should be efficient, fast and capable of being automated, if possible.

The extraction methods for simple phenolics (hydroxylated and methoxylated benzoic acids, cinnamic acids and catechins) from solid or semi-solid material have been focused on soaking with organic solvents [9,10]. However, these methods involve long extraction times, giving rise to possible degradation. The process of degradation can be originated by both external and internal factors. Light and oxygen in the air are the two most important factors that facilitate degradation reactions. Enzymes (mainly oxidative enzymes) already present in the pomace which are released during the extraction can promote such degradation reactions.

Recently, the sample preparation methods for analyzing phenolic compounds in fruits have been reviewed [11]. Extraction by soaking with either organic solvents or aqueous mixtures is the most often used method. Supercritical fluid extraction (SFE) has been proposed as a faster choice. However, high percentages of organic modifier are needed when sub- or supercritical carbon dioxide is used, to increase the polarity of the extracting fluid to allow for the extraction of phenolic compounds [12–14]. In this case, selectivity between compounds is substantially reduced.

As with SFE, pressurized liquid extraction (PLE) offers the possibility of performing the extractions under an inert atmosphere and protected from light. Phenolic compounds are very sensitive to these two factors, which represents an attractive advantage. Compounds such as resveratrol can be found in two isomeric forms (i.e., its *cis* and *trans* configurations) but only one of these, *trans*-resveratrol, shows biological activities. Light can catalyze the reaction transforming the compound from the active to the inactive form [15,16]. Moreover, the short extraction times possible (less than 1 h) may reduce the adverse effect of enzyme activity.

For SFE, its main advantage is the high capacity of diffusion offered by the extraction fluid for accessing these compounds in the matrix. For PLE, high temperature and high pressure are used to accelerate the extraction. Pressure is used to increase the contact between the extracting fluid and the sample. Temperature is used to break the analyte–matrix bonds. Moreover, temperature can dramatically modify the relative permittivity of the extracting fluid, increasing selectivity [17].

Before proposing an extraction method based on extraction with superheated solvents, it is necessary to prove that:

(1) analytes can be extracted with the extracting fluid;

(2) analytes are not degraded under the extraction conditions.

Phenolic compounds are easily oxidized at high temperatures, so it is very important to prove that they will not be oxidized during PLE.

The aim of this work is to study the stability of phenolic compounds during PLE using methanol at different temperatures up to 150°C as extracting fluid. The results have been compared with their stability at 65°C (boiling point of methanol) under normal atmospheric pressure.

Afterwards, the same extraction conditions have been applied to the solid wastes from the winemaking process, i.e., grape seeds and skins, to prove the feasibility of extracting phenolic compounds with PLE.

The same method can be applied to extract the phenolic compounds from any similar solid matrix.

2. Materials and methods

Standards of phenolic compounds have been obtained from Sigma–Aldrich (St. Louis, MO, USA). They were diluted in 3 g/l of tartaric acid and 15% (v/v) of ethanol, as a wine-like media. The standards used were: caffeic acid, catechin, *p*-coumaric acid, epicatechin, gentisic acid, protocatechuic aldehyde, syringic aldehyde, vanillin and veratric acid. The chemical structures are shown in Fig. 1. The internal standard used was 2,5-dihydroxybenzaldehyde, which was added after the extraction.

Sea sand has been used as supporting material. Sea sand was obtained from Panreac (Barcelona, Spain).

Nitrogen was used to purge and to dry the samples during the extractions.

The grape pomace was obtained from white grapes of the Palomino Fino variety grown in Jerez (Spain).

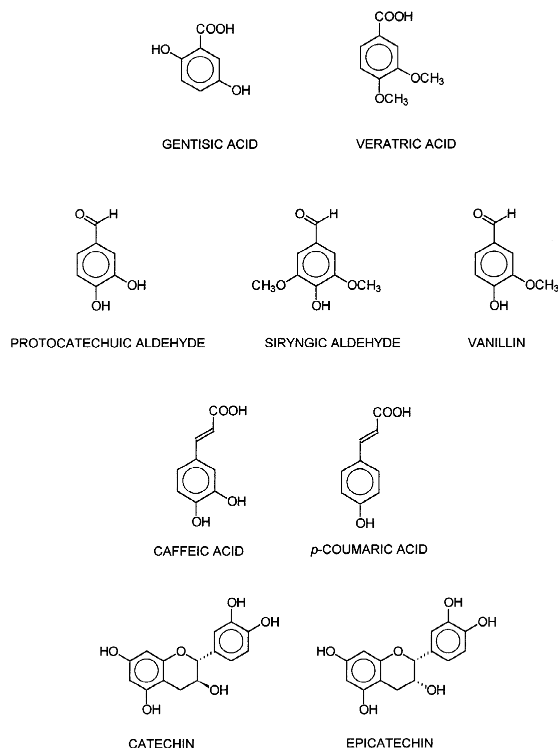

Fig. 1. Chemical structures of the phenolic compounds studied.

Seeds were separated out from the skin and then milled in a coffee grinder for 2 min, in bursts of 15 s in order to avoid sample heating. The ground samples were dried for 24 h at 40°C and kept at −20°C before the extraction.

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 20 ml.

For the extractions, the sea sand inside the cells was spiked with 1 ml of standard solution. Three 10 min cycles were programmed under 100 atm of pressure (1 atm=101 325 Pa). After each extraction cycle, the sample was rinsed with 3 ml of methanol and finally purged with nitrogen for 1 min. Four temperatures were assayed: 40, 50, 100 and 150°C.

For the real samples, the extraction cell was filled with 4 g of samples and sea sand. The same extraction method was used.

The chromatographic analysis was performed by high-performance liquid chromatography (HPLC) with a Waters (Milford, MA, USA) chromatographic system (M-45 and 510 pumps, 717 automatic injector, UV-440 detector, Millenium 2.10 software) using a LiChrospher column (Merck, Darmstadt, Germany). UV detection by a diode array detection (DAD) system and fluorescence detection were used. For the fluorescence detection, 351 nm was used for excitation and 453 nm for emission. In UV detection, the standards were measured at their own maximum absorbance wavelength. An elution gradient was used according to the method proposed by Guillén et al. [18]. Briefly, two solvents were used: solvent A (10% 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 A–B (85:15) in 15 min and A–B (50:50) in 35 min, both changes were done by using a convex gradient.

Peak areas were measured automatically and corrected by reference to the internal standard.

Table 1
Recoveries for PLE at 40, 50, 100 and 150°C and stability at 65°C in methanol of nine phenolic standards[a]

Sample	*p*-Coumaric acid	Vanillin	Veratric acid	Protocatechuic aldehyde	Gentisic acid	Caffeic acid	Syringic aldehyde	Catechin	Epicatechin
PLE, 40°C	101.2±6.8	96.4±6.5	97.1±6.5	100.3±0.4	103.0±4.3	101.4±1.9	101.8±4.2	99.4±1.2	101.8±1.6
PLE, 50°C	99.2±2.4	95.8±2.5	97.5±2.0	98.9±2.1	98.6±2.3	99.4±2.6	94.4±1.5	98.9±3.1	98.6±1.5
PLE, 100°C	102.3±1.3	98.1±1.7	99.9±1.4	103.6±1.0	97.6±0.5	102.8±1.4	101.8±2.6	92.6±4.9	94.1±1.7[b]
PLE, 150°C	98.8±8.6	100.7±2.1	103.8±4.9	103.6±1.4	100.6±2.9	101.9±2.9	101.6±0.8	87.4±3.0[b]	86.0±2.0[b]
Reference	100.3±0.3	99.3±1.4	100.2±0.3	100.9±1.4	100.8±1.5	99.7±1.4	101.1±2.8	96.6±4.9	99.5±1.7
65°C	109.1±9.3	99.2±1.1	100.8±0.3	96.2±4.8	85.5±1.6[b]	92.5±6.8	92.8±2.2[b]	63.7±8.7[b]	63.4±7.9[b]

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

3. Results and discussion

The standards were extracted at 40, 50, 100 and 150°C. The lowest temperature was used as it is the minimum temperature allowed by the ASE-200 system. All the extractions were run in triplicate. Table 1 shows the results for the recovery related to the standards solution prepared daily using the same dilution. All the samples were analyzed by HPLC.

As shown in Table 1, the average recovery for all the extracted compounds was over 90%, with the exception of catechin and epicatechin at 150°C. Statistical methods were used to determine if there are significant differences between the extracted samples and the reference solutions. Differences were found for catechin extracted at 150°C and for epicatechin extracted at 100 and 150°C. Box and whisker plots are shown in Fig. 2 for caffeic acid, catechin and epicatechin. It can be seen that for catechin and epicatechin, higher extraction temperatures mean lower recoveries, whereas for caffeic acid there is no decrease.

It should be noted that catechins are the most oxidizable compounds assayed [19,20]. Furthermore, it has been reported that catechins show the lowest recovery rates in solid–fluid based extraction processes [12].

The same solutions of phenolic compounds were kept at the boiling point of methanol (65°C) as long a time as used by PLE, i.e., 45 min. The vials were kept open in contact with the air. Afterwards, the solutions were analyzed by HPLC and compared with the reference. Table 1 shows the results of the chromatographic analyses. The averages for all the analyzed compounds were lower than the reference, except for *p*-coumaric acid. There were significant statistical differences for the compounds: syringic aldehyde, veratric acid, catechin and epicatechin. Their concentrations were significantly lower than the references. Specifically, in the case of catechin, the concentration was 40% lower than the reference.

Thus, it has been proved that several phenolic compounds react easily at high temperature (65°C) when they are in contact with the air. However, when higher temperatures are applied under nitrogen atmosphere, there were no degradations, since the degradation process for phenolic compounds is an oxidative process requiring the presence of oxygen.

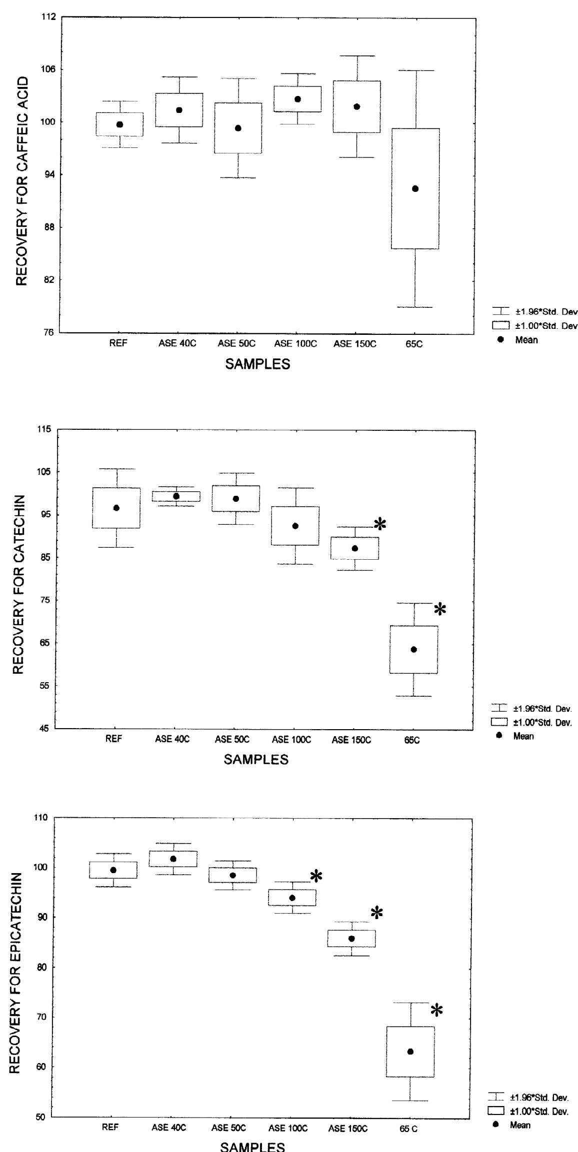

Fig. 2. Box and whisker plots of recoveries for caffeic acid, catechin and epicatechin, *Significant difference at 95% level of confidence.

After studying the stability for standard phenolic compounds, the same PLE was applied to real samples. Grape seeds and skins from winemaking wastes were extracted separately by PLE using the same conditions as those applied to the standards.

For all the extractions, the amount of used sample in the extraction process was intentionally greater

than the amount that can be extracted by the used solvent. In this way, it is possible to compare the extraction capability of the used solvents for the extractions at different temperatures. The extracts were analyzed by HPLC and the resulting chromatograms are shown in Fig. 3. All the chromatograms refer to the internal standard added after the extraction.

Few differences were obtained for grape skin extracted at different temperatures, *cis*-coutaric acid recovery increased by 1% from 50 to 100°C extraction temperature, and decreased by 2% from 100 to 150°C. Caftaric acid recovery increased by 3% from 50 to 100°C but decreased by 7% from 100 to 150°C, and epicatechin increased by 3% from 50 to 100°C and decreased by 2% from 100 to 150°C.

For grape seeds, the results at the temperatures assayed are quite different. Using 50, 100 or 150°C there were big differences in both the identity and recovery rate of the phenolic compounds extracted. For example, catechin increased by 30% and epicatechin increased by 44% from 50 to 100°C extraction temperature. For the 150°C extraction temperature, catechin increased by 32% and epicatechin increased by 99% over the recovery from the 50°C extraction. There are also some compounds not detected at all in the extractions run at 50 and 100°C but which are detected in the extractions obtained at 150°C. In conclusion, for grape seeds, 150°C must be used for PLE.

Considering that the most important effect of higher temperature is related to the breaking of bonds between the analytes and the matrix, it seems that the analytes in grape seeds are more strongly bonded to the matrix than in grape skin. Therefore, for grape skins it would be interesting to determine whether, with shorter extraction times, such significant differences can be detected in the extractions of the various phenolic compounds using these different temperatures.

Other variables like pressure, extraction time and number of cycles should be optimized and their effects on the recovery have to be explained. But it can be concluded that using PLE at high temperatures (100°C) the maximum degree of degradation suffered by phenolic compounds is 10%, even for the

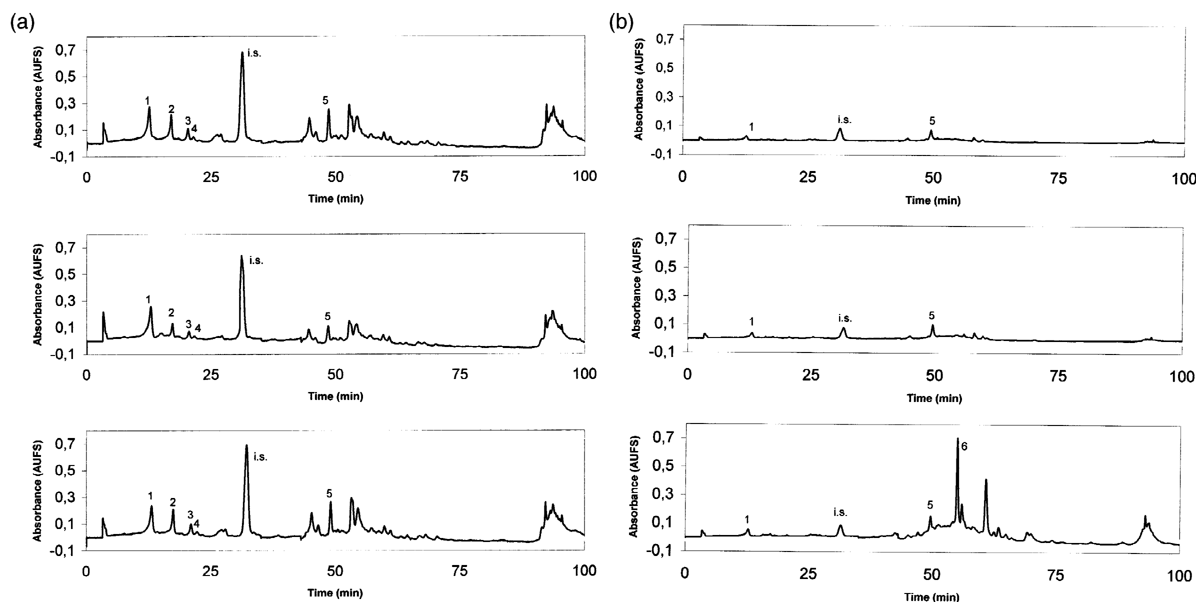

Fig. 3. (a) Chromatograms (280 nm) of extracts at (top) 50, (middle) 100 and (bottom) 150°C of grape skins. Chromatographic conditions: flow, 1.0 ml/min; mobile phase, solvent A: methanol–acetic acid–water (10:2:88); solvent B: methanol–acetic acid–water (90:2:8). Continuous gradient: time (min), B (%): 0, 0; 15, 15; 50, 50. 1=Gallic acid, 2=*cis*-coutaric acid, 3=caftaric acid, 4=catechin, I.S.=internal standard (2,5-dihydroxybenzaldehyde), 5=epicatechin, 6=epigallocatechin gallate. (b) Chromatograms (280 nm) of extracts at (top) 50, (middle) 100 and (bottom) 150°C of grape seeds.

most oxidizable compounds. Also for some samples it is necessary to use higher temperatures in order to increase recovery.

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