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Analytica Chimica Acta 458 (2002) 119–130

ANALYTICA CHIMICA ACTA

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Ultrasound-assisted extraction and determination of tartaric and malic acids from grapes and winemaking by-products

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Received 20 June 2001; received in revised form 31 August 2001; accepted 7 November 2001

Abstract

The optimization of an ultrasound-assisted extraction (UAE) method for tartaric and malic acids from grape derived samples is shown. A fractional factorial experimental design allowed for the determination of the effects of seven extraction variables. Relationships between all the variables were examined. By applying graphical analysis, the best extractions conditions were obtained. The most important variables were the extracting liquid and the extraction temperature. Later, a central composite design was applied for optimizing the temperature and the composition of the extracting liquid. The optimized method was applied to grapes and to winemaking by-products. The repeatability of the method was studied and the recovery of tartaric and malic acids was established. Organic acids quantification was done by liquid chromatography (LC) using a post-column buffer and a conductivity detector. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Ultrasound-assisted extraction; Tartaric acid; Malic acid; Grapes; Grape seeds

1. Introduction

Tartaric acid and malic acid are the most abundant organic acids in grapes. In the must obtained from grapes, tartaric acid is found in the range 3–7 g l^{-1}, and malic acid in the range 1–3 g l^{-1} [1]. The levels in which these acids are present is related to the chemical and biological stability of wines, therefore it is of considerable interest to determine their concentrations.

Their levels in grapes are the data frequently used to determine the harvesting date, particularly since each acid presents a different behavior during the grape ripening process. Malic acid shows a continuous decrease during ripening whereas tartaric acid remains almost unchanged. Therefore, different ratios can be obtained during ripening and the optimum harvest date can be established from their ratio [2].

The determination of these compounds can be conducted by enzymatic methods or by liquid chromatography (LC), after an initial juice extraction step from the grapes. The most usual method for extraction is based on applying pressure to the grapes, thus simulating the industrial process for obtaining must. This method is slow and, additionally, only partial: complete extraction of the acids is not guaranteed and the must thus obtained may be dissimilar to the must obtained in the actual winemaking process. Therefore, a more suitable extraction method would be very useful for winemakers.

Another reason for interest is that tartaric acid is the only compound authorized by law for use to increase the total acidity of wines. Nowadays, its chemical synthesis is more expensive than its recovery from

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0003-2670/02/$ – see front matter
PII: S0003-2670(01)01527-6

winemaking by-products. So, usually, tartaric acid is recovered from winemaking by-products, mainly from those by-products containing relatively high concentrations of tartaric acid such as grape seed and red grape skins. Obviously, reliable knowledge of the concentration of tartaric acid in these materials is of considerable use before devoting resources to its recovery on an industrial scale.

Ultrasound-assisted extraction (UAE) can be used for extraction methods with liquid solvents applied to analytes in solid matrices. This extraction process is fast in comparison to the traditional methods, because of the contact surface area between solid and liquid phase is much greater, due to particle disruption taking place [3]. This type of extraction has been applied to biological matrices [4] such as plant materials [3,5,6] and even human hair [7]. In some cases, recoveries similar to those obtained by microwave-assisted extraction have been found [8].

The application of UAE to plants has produced very interesting results [9], to the extent that industrial processing has been proposed for obtaining compounds with pharmacological properties [10]. In many of these cited studies, the optimum extraction conditions were established using experimental design techniques. These techniques were used because several of the extraction variables must be optimized and such techniques allow the most significant variables to be determined easily [10].

In this paper, the optimum extraction conditions based on UAE have been determined as the initial step in the determination of the tartaric and malic acid content of whole grapes and grape seed.

2. Experimental

2.1. Samples

Red grapes of the Napoleon variety and grape seeds from grapes of the white Palomino Fino variety, obtained from winemaking by-products, were used. Around 1 g of solid sample was used in each extraction. All the samples were freeze-dried before the extraction in order to increase the sensitivity of the analysis and because different grape seeds could have different moisture contents.

2.2. Reagents

Tartaric acid and malic acid were obtained from Panreac (Barcelona, Spain) and methanol was purchased from Scharlau (Barcelona), all of them of analytical reagent grade. HPLC grade water was supplied by a Milli-Q water purifier system from Millipore (Bedford, MA).

2.3. Extraction

A high intensity probe ultrasound generation system of 200 W, 24 kHz, was used. The instrument was a model UP 200S from dr.Hielscher GmbH (Teltow, Germany). Its amplitude controller allows the ultrasonic vibrations at the probe microtip to be set at any desired level in the 10–100% range of the nominal power. Also the cycle controller allows the duration of the application of the ultrasound to be set, to a fraction of a second, in the 0.1–1.0 range.

2.4. Chromatographic analysis

The extracts were filtered before chromatographic analysis using 0.45 μm nylon filters. The chromatographic analysis was carried out as described by Guillén et al. [11]. Two model 2150 pumps and a model 2155 oven for the column, all from LKB (Pharmacia, Sweden): a Model Conductomitor III conductivity detector from Milton Roy (LDC, FL), a model 717 automatic injector and a millenium data treatment system, both from Waters (Milford, MA).

The chromatographic separation was carried out with two ION-300 ion exclusion columns (Interactions Chromatography, San José, CA) installed in series (300 mm length, 4.6 mm i.d.). The oven temperature was set at a constant 60 °C. The mobile phase used was 2.5 mM trifluoroacetic acid (TFA) with a flow rate of 0.4 ml min^{-1}. The sample volume injected was 50 μl. In order to increase the detection sensitivity, a solution consisting of 2.5 mM TFA, 20 mM bis–Tris buffer and 0.1 mM EDTA was added at the outlet of the column, by means of the second pump, at the flow rate of 0.4 ml min^{-1}.

2.5. Software

Experimental design and the statistical treatment of the results were performed using Minitab 10.0

(State College, PA) and Unscrambler 7.5 (CAMO, Oslo).

3. Results and discussion

3.1. Extraction variables

Grape seeds are more stable than grapes because of they cannot suffer biological degradation processes such as sugar fermentation, which produces variations in the amounts of acids found in the samples. Therefore, the experimental design was applied only to grape seeds and the fine tuning of the extraction method was developed for both grape seeds and whole grapes separately.

A fractional factorial experimental design was carried out in order to determine the more significant variables for the extraction process. The variables in the experimental design were: extraction temperature, solvent, solvent volume, extraction time, size of ultrasonic probe, ultrasonic power and cycle time applied. These variables were evaluated at two levels. The experimental conditions and the concentrations of tartaric acid and malic acid found in the extracts are shown in Table 1. All the experiments were done in duplicate.

The experimental design was fractional in order to reduce the number of experiments needed to evaluate the influence of the variables. In this way, 16 experiments were done instead of the 128 (2^7) that are needed for the full evaluation of seven variables. Graphical analysis of the results by comparing the main effects of each variable and the graphs of the interactions of each pair of variables allowed the influence of each variable on the recovery of the acids to be determined. This kind of analysis has previously been applied with good results for developing extraction methods [12].

Table 1 also shows the concentrations of tartaric and malic acids found in the extracts obtained. All the concentrations are shown relative to the amount found using the most effective conditions (100%). This means that recovery was not calculated relative to the total amount of acid present in the samples, but relative to the highest concentration found in the extracts.

Analyzing the main effect plots (Fig. 1), it can be concluded that the more significant variables for the extraction process are temperature and the solvent used as extractant. It can be seen that the higher the

Table 1
Fractional factorial experimental design for the determination of significant variables and relative recovery for tartaric and malic acids ($n = 2$)[a]

Experiment	Temperature (°C)	Solvent	Volume (ml)	Time (min)	Probe (mm)	Amplitude	Cycle	Tartaric acid (%)	Malic acid (%)
1	20	Methanol	25	5	2	30	0.2	3.5	28.7
2	50	Water	25	5	2	30	0.8	75.6	57.0
3	20	Water	100	5	2	70	0.2	53.6	9.8
4	50	Methanol	100	5	2	70	0.8	1.0	38.4
5	20	Water	25	15	2	70	0.8	84.5	51.5
6	50	Methanol	25	15	2	70	0.2	5.0	40.3
7	20	Methanol	100	15	2	30	0.8	0.0	16.0
8	50	Water	100	15	2	30	0.2	100.0	43.2
9	20	Methanol	25	5	7	70	0.8	6.0	30.7
10	50	Water	25	5	7	70	0.2	44.7	26.5
11	20	Water	100	5	7	30	0.8	44.7	3.4
12	50	Methanol	100	5	7	30	0.2	17.8	100.0
13	20	Water	25	15	7	30	0.2	40.3	24.5
14	50	Methanol	25	15	7	30	0.8	1.0	7.4
15	20	Methanol	100	15	7	70	0.2	2.2	1.6
16	50	Water	100	15	7	70	0.8	46.4	1.7

[a] Volume: volume of extracting liquid; probe: diameter of probe used; solvent: extracting solvent; amplitude: amplitude of ultrasounds (percentage of maximum ultrasonic power); cycle: pulse of ultrasound in fractions of second.

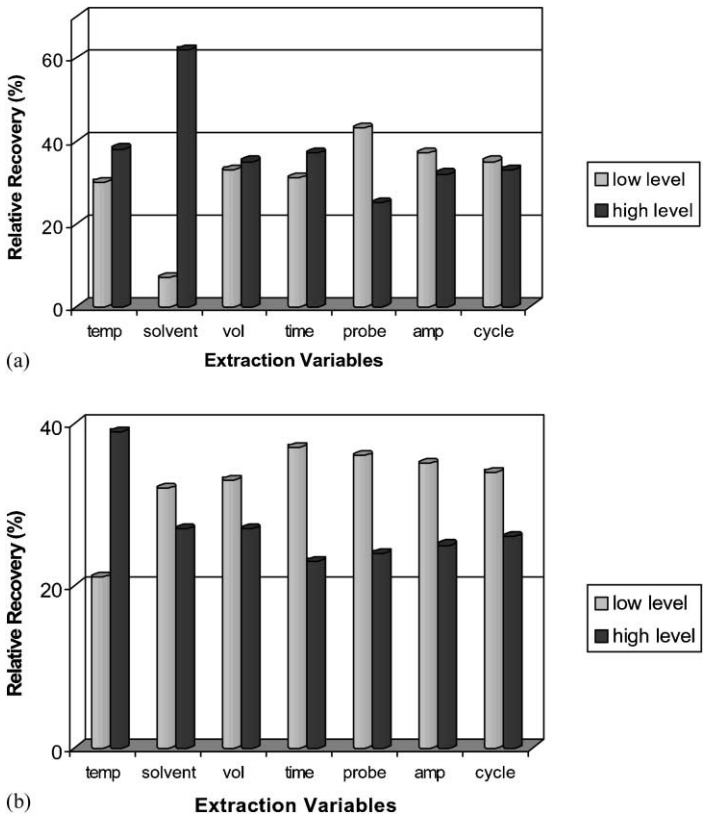


Fig. 1. Main effects plot of variables on the average relative recovery of tartaric acid (a) and malic acid (b). Temp: temperature; solvent: extracting liquid; vol: volume of the extracting liquid; time: extraction time; probe: ultrasonic probe; amp: amplitude of ultrasounds; cycle: cycle of ultrasounds.

temperature, the higher the recovery. This effect is much higher for malic acid than for tartaric acid. For malic acid, a recovery on average 20% higher is obtained by using 50 °C instead of 20 °C, whereas the increase in the average recovery for tartaric acid is less than 10% using 50 °C instead of 20 °C.

A higher temperature for UAE means a higher efficiency in the extraction process due to the increase in the number of cavitation bubbles and in the surface contact area, but this effect is less when the temperature is near the boiling point.

In order to determine possible interactions and their effects on average recoveries, graphs of the following data were constructed: average recovery of experiments with (a) the highest value for two variables, (b) the lowest value for two variables, and (c) the highest and the lowest values for each pair of variables. In this way, interactions graphs for all pairs of variables were obtained. The most important interactions are described in the following paragraphs.

Interaction graphs obtained for the temperature and the amplitude, and for the temperature and the ultrasound cycle time (Fig. 2) showed similar behavior. The lower the amplitude and/or the cycle time, the higher the recovery at high temperature. When large amplitude and/or long cycle time are used, little difference between recoveries is found. Furthermore, recoveries obtained with large amplitude and/or long cycle time are dramatically lower than those obtained using small amplitudes and/or cycle times and high temperatures.

The influence of the solvent selected was highly significant for tartaric acid. Water produces a much higher recovery than methanol for tartaric acid, 62%

compared with 7%. For malic acid, the opposite effect was found, although it was less important. Recovery of malic acid was 32% using methanol and 27% using water as extracting fluid.

It has been suggested that during UAE, dissociation of the solvent can be produced and the resulting radicals can react with the analytes [3]. Thus, water may produce H and OH radicals; their behavior with respect to the acids is not well established.

Due to the opposing effects of the two solvents, it was necessary to optimize the composition of the extracting fluid later.

The volume of extracting liquid has no effect on the extraction of tartaric acid, but it is a significant variable for the recovery of malic acid. The greater the volume used, the lower the recovery of malic acid.

Fig. 3 shows the interactions between volume and temperature variables for tartaric acid and malic acid. For both compounds, it can be seen that when 50 °C was used as the extraction temperature, it is much more productive to use a large rather than a small volume. Thus, even though the graph of the main results suggests that a small volume would be better, since the extracting temperature was to be 50 °C, 100 ml should be used as the extracting liquid volume instead of 25 ml.

Extraction time was a variable with opposite effects on the recovery of the two acids. When tartaric acid was extracted for 15 min, the recovery was slightly higher than when extracted for 5 min. However, for malic acid, extraction for 5 min produced a 14% higher recovery than 15 min. This may be related to the interactions recorded in the corresponding graph for the

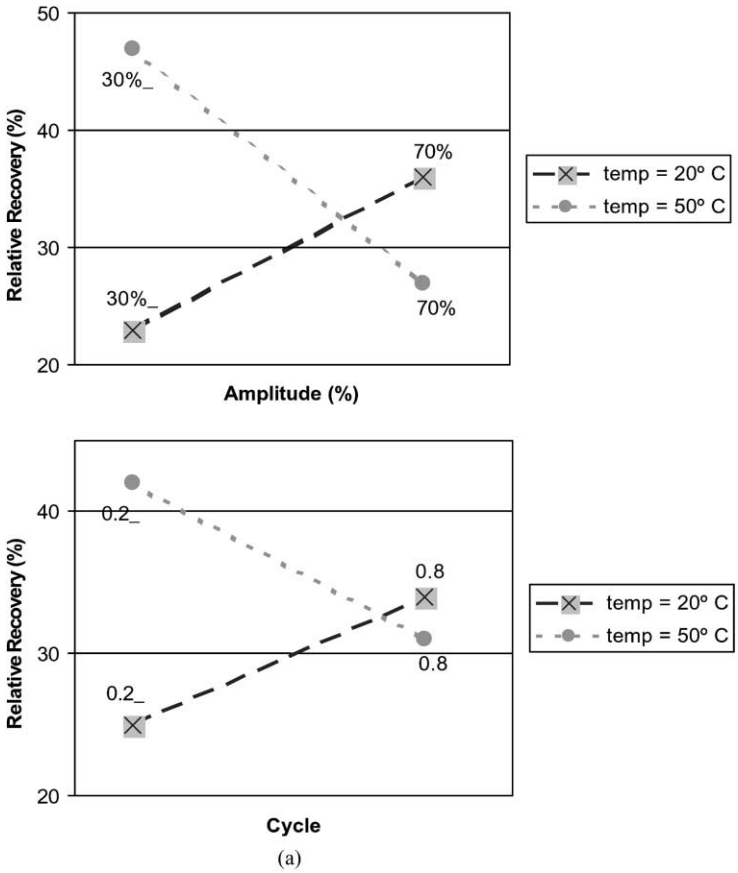
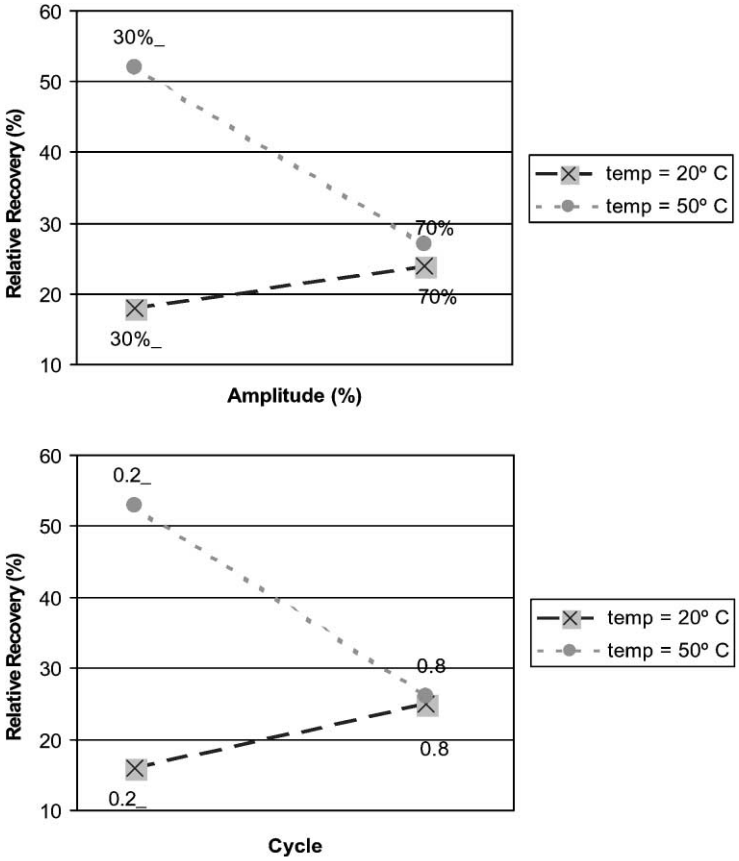


Fig. 2. Effects of interactions between temperature and amplitude and cycle over the average recovery of tartaric acid (a) and malic acid (b).



(b)

Fig. 2. (*Continued*).

variables of time and size of probe (Fig. 4). If the thinner probe (2 mm diameter) is used, the recoveries from 5 min extraction are very similar to those obtained from 15 min. However, recoveries are considerably different when the thicker probe (7 mm diameter) is used. So, the influence of the extracting time must be determined later, after the size of probe has been selected.

The main effects plot showed that the probe size has the same influence for both tartaric and malic acids. The thicker probe produced lower average recoveries than the thinner probe. Hence, it was decided that the thinner probe should be used.

Variation of amplitude and cycle time produced the same effect on the recoveries of the two acids. The larger the amplitude or the longer the cycle time, the lower the recovery. This effect is shown more clearly for malic acid than for tartaric acid.

Therefore, from the graphical analysis, it can be concluded that the best conditions for extracting the two acids are: 100 ml of extracting liquid, rather than 25 ml; a thin probe (2 mm) rather than a thick probe (7 mm); 30% amplitude and 0.2 s of cycle time, rather than 70% and 0.8 s, respectively.

3.2. Fine tuning for temperature and solvent

Using the graphical analysis, it was not possible to determine the best temperature and extracting solvent, so these had to be studied separately from the other variables. A central composite design was used to optimize them. The range used for temperature was from

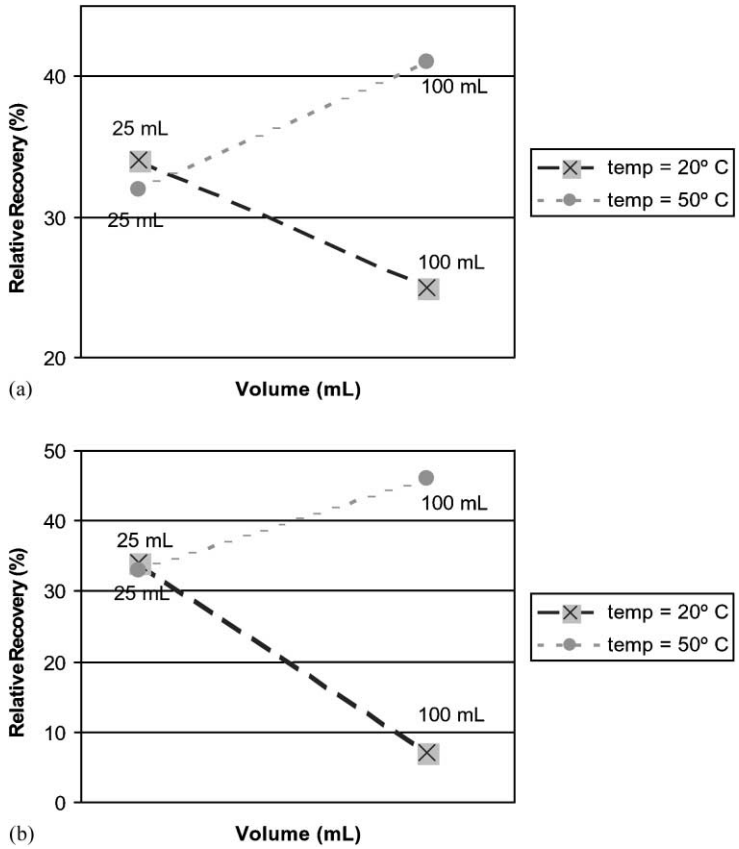


Fig. 3. Effects of interactions between temperature and volume over the average recovery of tartaric acid (a) and malic acid (b).

30 to 70 °C and different solvent solutions ranging from 0 to 60% methanol in water were used. Experimental conditions and recoveries obtained for tartaric acid and malic acid are shown in Table 2. All experiments were done in duplicate.

With the results obtained, the response surfaces for both acids were drawn. These are shown in Fig. 5. The response surface fits the behavior of both variables on the recovery of tartaric acid (multiple correlation coefficient = 0.925). For the recovery of malic acid, the obtained model was worse (multiple correlation coefficient = 0.822).

For both acids, the highest recoveries were obtained at the highest temperature (70 °C). So, additional experiments were needed to determine if temperatures higher than 70 °C could produce even higher recoveries. The effect of temperature on the recovery was greater for tartaric acid than for malic acid. Before investigating the effect of higher temperatures, the effect of solvent composition was studied.

The percentage of methanol in the extracting fluid also had a great effect on the recovery, mainly for tartaric acid. Recovery for tartaric acid using 60% methanol was about one-half of the recovery obtained using 100% water. For malic acid, a recovery of only 2% lower was obtained using a 60% methanol/water solution instead of 100% water. Therefore, it was decided that 100% water should be used as the extracting liquid.

Additional experiments were carried out to determine if a temperature of >70 °C would produce higher recoveries. Extractions by water at 70, 80 and 90 °C were done in triplicate. The recoveries obtained are shown in Fig. 6. Extracting at 80 and 90 °C, gave

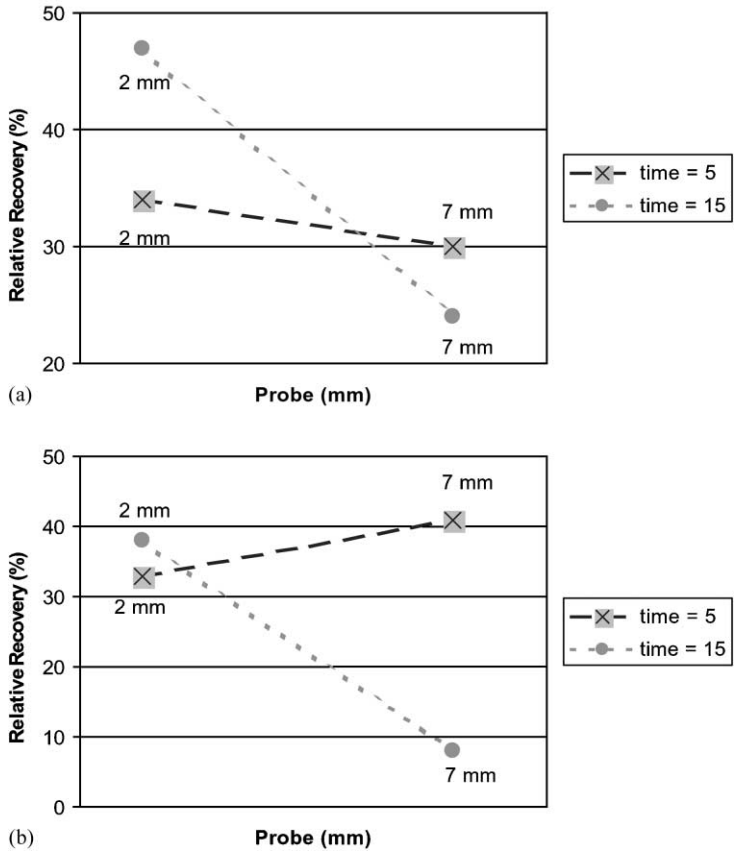


Fig. 4. Effects of interactions between time and probe over the average recovery of tartaric acid (a) and malic acid (b).

Table 2
Central composite experimental design for the determination of optimal values for temperature and solvent and concentration of tartaric and malic acids in the resulting extracts ($n = 2$)

Experiment	Temperature (°C)	Methanol (%)	Tartaric acid (ppm)	Malic acid (ppm)
1	30	30	146.4	34.7
2	70	30	193.4	38.8
3	50	0	199.2	39.0
4	50	60	119.9	34.6
5	30	0	188.7	33.9
6	70	0	194.3	37.4
7	30	60	130.3	33.0
8	70	60	163.0	40.8
9	50	30	168.2	36.7
10	50	30	164.0	34.9
11	50	30	163.1	35.7

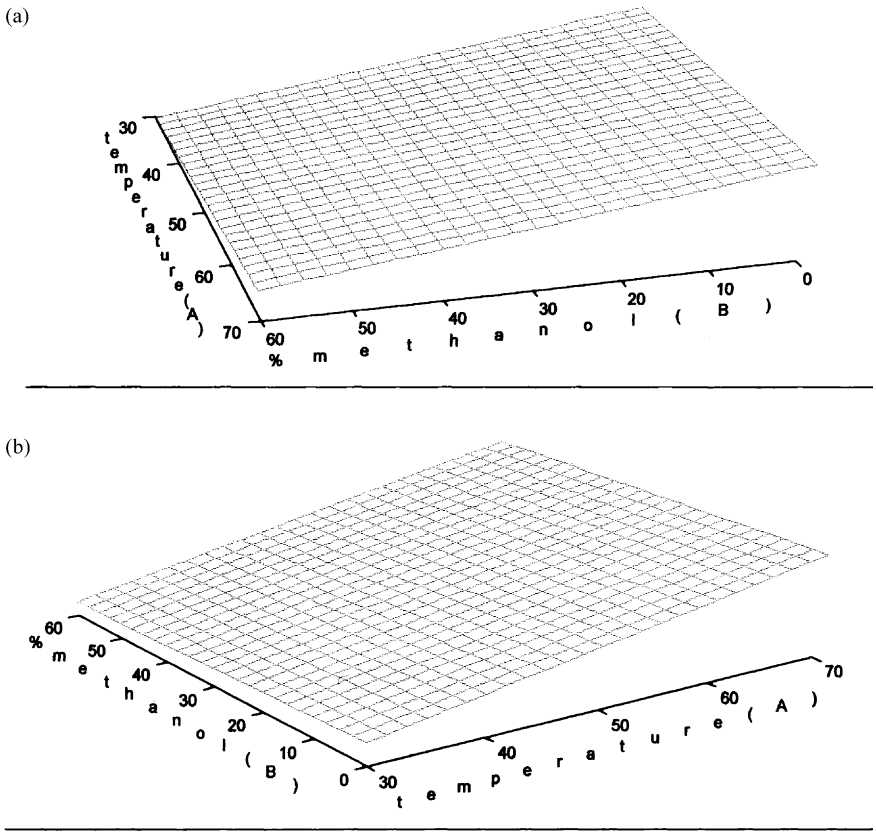


Fig. 5. Response surface for recovery of tartaric acid (a) and malic acid (b) obtained from the central composite experimental design.

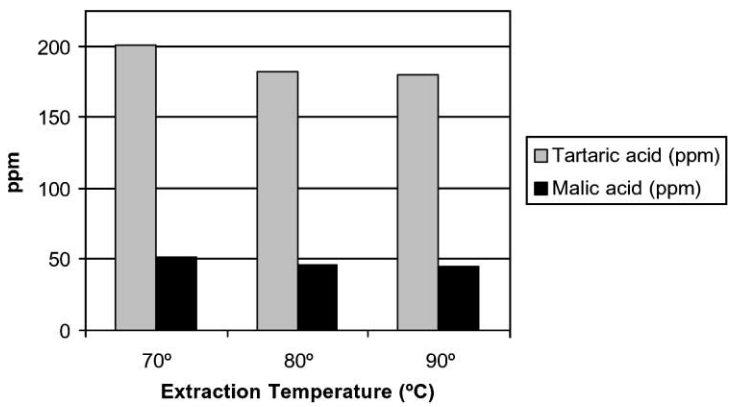


Fig. 6. Concentrations of acid in the extracts obtained at different extraction temperatures.

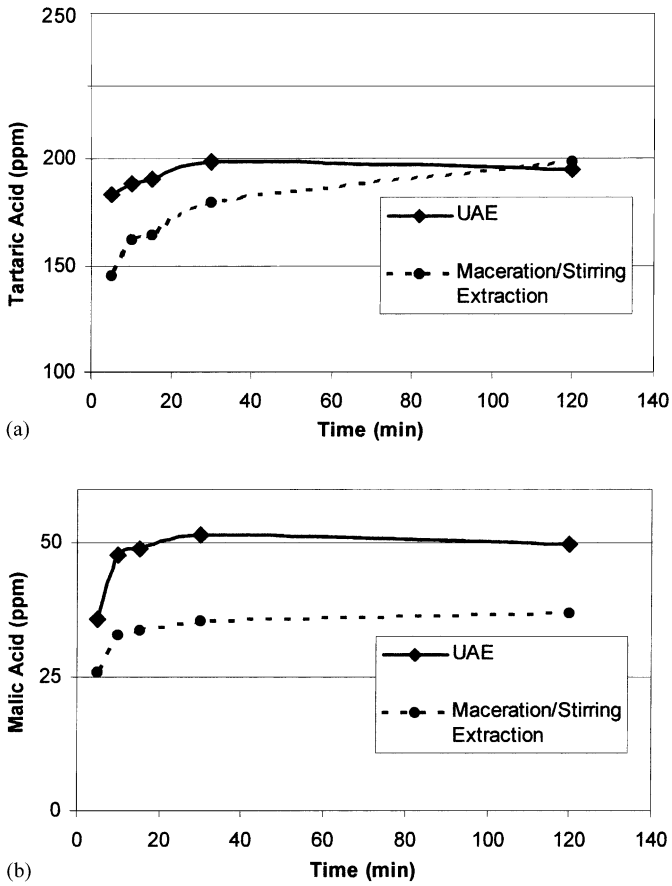


Fig. 7. Kinetics of extraction obtained for tartaric acid (a) and mailc acid (b) from grape seeds.

slightly lower recoveries, hence 70 °C is the optimum temperature for the extraction of these two acids. The effect of temperature on the recovery was greater for tartaric acid than for malic acid.

3.3. Optimization of extraction time

The extraction time must be adjusted to obtain quantitative recoveries of both acids. To determine the time needed, different extractions were done using increasing extraction times to establish the kinetics of the extraction. All the extractions were done in duplicate.

Both grape seed and whole grapes were used to determine separately the best extraction time. The rate was compared with the rate of the extraction method of maceration and continuous magnetic stirring (at 1000 rpm), to determine the influence of ultrasound on the recoveries. The resulting graphs are shown in Fig. 7.

For grape seeds, tartaric acid showed a maximum recovery with extraction for 30 min. Longer extraction times produced a lower recovery, which could be explained by reactions between the radicals generated by the ultrasound and tartaric acid, since such reduction in recovery for times >30 min does not occur with the maceration/stirring extraction method. It is notable that ultrasound produces a much faster extraction of tartaric acid than the maceration/stirring method. UAE produces the same recovery after 30 min of extraction as the other method does after 120 min.

Malic acid showed a different behavior. At the beginning of the extraction process, malic acid

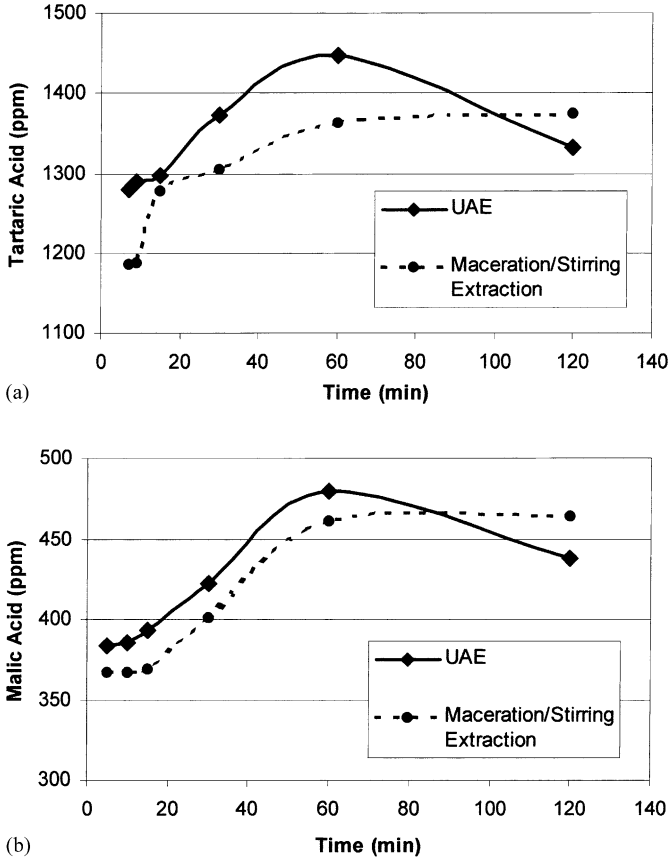


Fig. 8. Kinetics of extraction obtained for tartaric acid (a) and malic acid (b) from grapes.

showed a very similar behavior to tartaric acid, i.e. faster extraction by UAE. Nevertheless, the extraction by the maceration/stirring method never reached the recovery obtained by UAE. After 120 min of maceration/stirring, a 45% lower recovery was obtained compared with the UAE method after 30 min. Therefore, the ultrasound method is clearly superior for extracting malic acid quantitatively from grape seeds.

The observed effect of ultrasound on the recovery of malic acid and the interactions noted above suggest that malic acid is located in a less accessible position than tartaric acid within the structure of the grape seed.

Therefore, to extract quantitatively both tartaric acid and malic acid from grape seeds, the optimum extraction conditions were: 70 °C as extracting temperature, 100 ml of water as extracting liquid, a thin probe (2 mm), 30% amplitude, 0.2 s cycle time and 30 min of extraction time.

For grapes, Fig. 8 shows the resulting rate for the extraction of both acids by the UAE and by the continuous maceration/stirring methods. They are both very similar. The main difference is recorded after 60 min. UAE showed a decrease in recovery after 60 min of extraction, whereas the other method does not show any decrease. Up to 60 min extraction both rates are parallel, though UAE has slightly higher recoveries. After 120 min, the maceration/stirring method produced similar recoveries from grapes as the UAE method after 60 min.

Therefore, for grapes, it can be concluded that 30 min is sufficient for quantitative extraction, since 90% of maximum recovery is obtained in this time.

For grapes, ultrasound would seem to be less necessary than for grape seeds. This is probably because the two acids are more accessible to the solvent in grapes than in grape seeds, and the finding that the extraction from grapes is faster than that from grape seeds at the beginning of the extraction tends to confirm this.

4. Repeatability

The repeatability of the developed UAE method was determined using only grape seeds as samples. Six samples were analyzed. The repeatability of the chromatographic method (six repeated injections) was also established, in order to determine the errors due to the extraction process.

Repeatability obtained for the determination of both tartaric acid and malic was $<5\%$ (R.S.D. $= 4.5$ and 4.7%, respectively). The R.S.D. of the chromatographic method were 1.0% for tartaric acid and 1.9% for malic acid. Therefore, the repeatability of the extraction step is acceptable for the analysis of these compounds.

5. Conclusions

Under the optimized extraction conditions, quantitative recovery is obtained for both acids after 30 min extraction and the method has high repeatability. For grape seeds, UAE offers considerable advantages over the conventional maceration/stirring extraction method, but for grapes the differences are less marked.

Acknowledgements

Support of the Spanish Inter-Ministerial Commission for Science and Technology under the framework of the project 1FD1997-0683 is gratefully acknowledged.

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