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Ultrasound-assisted extraction of amino acids from grapes

Ceferino Carrera [a], Ana Ruiz-Rodríguez [b], Miguel Palma [b,*], Carmelo G. Barroso [b]

[a] Andalusian Center for Wine Research, Agrifood Campus of International Excellence (ceiA3), University of Cádiz, Campus de Puerto Real, 11510 Puerto Real, Cádiz, Spain
[b] Department of Analytical Chemistry, Faculty of Sciences, Agrifood Campus of International Excellence (ceiA3), University of Cádiz, Spain

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ABSTRACT

Recent cultivar techniques on vineyards can have a marked influence on the final nitrogen content of grapes, specifically individual amino acid contents. Furthermore, individual amino acid contents in grapes are related to the final aromatic composition of wines.

A new ultrasound-assisted method for the extraction of amino acids from grapes has been developed. Several extraction variables, including solvent (water/ethanol mixtures), solvent pH (2–7), temperature (10–70 °C), ultrasonic power (20–70%) and ultrasonic frequency (0.2–1.0 s^{-1}), were optimized to guarantee full recovery of the amino acids from grapes. An experimental design was employed to optimize the extraction parameters. The surface response methodology was used to evaluate the effects of the extraction variables. The analytical properties of the new method were established, including limit of detection (average value 1.4 mmol kg^{-1}), limit of quantification (average value 2.6 mmol kg^{-1}), repeatability (average RSD = 12.9%) and reproducibility (average RSD = 15.7%). Finally, the new method was applied to three cultivars of white grape throughout the ripening period.

1. Introduction

Total free amino acid contents in grapes and also the amino acid profiles can vary dramatically depending on the grape cultivar [1], cultivar practices [2], ripening degree [3], vine nutrient/water/fungicide management and health status, including fungal infections [4].

Additionally, grape cultivar using different rootstocks usually present significant differences for the final composition of grapes. It has been demonstrated that different rootstocks for the same grape variety, for example, produce different amino acid profiles in the final grapes [3]. The Cabernet Sauvignon grape variety with a high vigor rootstock, produces much higher levels of most amino acids in the grapes than with a low vigor rootstock [1]. Cultivar practices, such as the use of cover crops, have different effects on amino acid levels depending on the variety. For example, changes were not observed for Cabernet Sauvignon [1] but a dramatic reduction in amino acid levels was observed for the Pinot Noir grape variety on using cover crops [2]. Vine health status and fungicide application usually affect the levels of amino acids in grapes. The application of fungicide close to the harvest date leads to lower levels for all amino acids [4] and this makes the alcoholic fermentation process more difficult.

It has been proved that there is a direct relationship between the amino acid levels in grape must and their consumption during the first half of alcoholic fermentation [5]. This means that the starting levels of nitrogen compounds, specifically amino acids, will determine the evolution of the first step in the alcoholic fermentation, therefore affecting the final composition of wines.

It has been demonstrated in several previous studies that there is a correlation between amino acid contents in grapes/must and the aroma of the final wines, specifically for young wines [6].

Free nitrogen levels, including amino acids, are also associated with some problems in the winemaking process. Insufficient nitrogen levels can stop fermentation and this leads to the production of numerous secondary metabolites that are not of interest for wine production [7]. Furthermore, some amino acids are directly related to certain volatile compounds that contribute to the wine aroma, including fusel oils, aldehydes and esters [8]. Wines with very high levels of nitrogen compounds can also have higher microbiological instability, including those processes related to biogenic amines and ethyl carbamate generation [9].

Given the information outlined above, it is advantageous to determine amino acids in grapes during ripening in an effort to identify the effects of cultivar practices and cultivar conditions. It is also of interest to determine amino acid levels in grapes just prior to alcoholic fermentation in order to establish more suitable winemaking practices.

* Corresponding author. Address: Department of Analytical Chemistry, Faculty of Sciences, Agrifood Campus of International Excellence (ceiA3), University of Cádiz, 11510 Puerto Real, Spain. Tel.: +34 956016775; fax: +34 956016590.
E-mail address: miguel.palma@uca.es (M. Palma).

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There are several methods for the determination of free amino nitrogen [10] and amino acids in must. Most of these approaches involve chromatographic techniques [11], but colorimetric methods are also employed [12]. In many cases, the final determination requires some kind of derivatization step to increase the analytical signal, including by fluorescence detection systems [11]. However, the problem of the determination of amino acids in grapes has not yet been solved satisfactorily.

Differences in individual free amino acid profiles and concentrations have been observed between the different sample preparation methods usually applied, i.e. determination in grape juice or chemically extracted grapes, before amino acid determination [1]. There are three main reasons for these differences. Firstly, extraction – rather than grape juice preparation – will include amino acids from grape skins in the final level. Proline, for example, has been reported to be found in grape skins [1]. Secondly, extraction usually leads to dilution of the sample due to the presence of extraction solvents and this means that some levels could go below the limits of quantification. Finally, quantification in grape juice will be expressed as mg per liter whilst quantification in grapes will be expressed as mg per kg. The relationship between kg of grape and liter of grape juice is dependent on the winemaking process.

Additionally, during the red winemaking process, grape skins are in contact with grape must for several days and amino acids will therefore be extracted from the skins to some extent. As a consequence, determination of the total amount of amino acids in grapes would be more useful than the amount in the grape juice alone.

The aim of the work described here was to develop and validate a new ultrasound-assisted extraction method for amino acids in grapes. The overall goal was to provide a rapid and reliable method for winemakers to determine amino acids in grapes during grape ripening and harvest. Ultrasound assisted extraction was used because of this technique usually produce faster extraction methods, lower solvent volumes and higher extraction yields [13].

2. Materials and methods

2.1. Chemicals and solvents

Ethanol and methanol (Panreac, Barcelona, Spain) were HPLC grade. Ultra pure water was supplied by a Milli-Q water purification system from Millipore (Bedford, MA, USA).

2.2. Grape samples

The white grape (*var. Verdejo*) was employed in the development of the ultrasound-assisted extraction method. Samples were obtained from local vineyards in the Jerez region (Spain). The full berry, i.e. skin, pulp and seeds, was studied. The berries were triturated with a conventional beater until a homogeneous sample was obtained for analysis. The triturated sample was stored in a freezer at −20 °C prior to analysis.

2.3. Extraction procedure

The extraction of amino acids from grapes by means of ultrasound was performed by employing water/ethanol mixtures as solvent. The effects of the extraction solvent (0–25% EtOH in water), temperature (10–70 °C), output amplitude of the nominal amplitude of the transducer (30–70%), duty cycle (0.2–0.7 s), pH of the extraction solvent (2–7) and the sample/solvent ratio (1 g 10 mL^{-1}–1 g 20 mL^{-1}) were studied.

Ultrasonic irradiation was carried out using a UP200S sonifier (200 W, 24 kHz) (Hielscher Ultrasonics, Teltow, Germany), with the sample immersed in a water bath coupled to a temperature controller (Frigiterm, J.P. Selecta, Barcelona, Spain).

2.4. Experimental design for the evaluation of the effects of extraction variables

Optimization of extraction variables was performed using the Box–Behnken statistical methodology by considering the total amount of amino acids extracted. The results for the 54 extractions carried out in duplicate for the seven extraction variables (each variable has three levels: low, medium and high) including 6 center points are shown in Table 1 along with the respective responses. The results for the total amount of amino acids (mmol kg^{-1} of sample) determined by HPLC were used as the response variable.

The responses obtained from the various extractions were entered into to a second-order polynomial equation into which each of the various parameters was introduced. The polynomial equation is as follows:

$$\begin{aligned} Y = {} & \beta 0 + \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 4X4 + \beta 5X5 + \beta 6X6 + \beta 7X7 \\ & + \beta 12X1X2 + \beta 13X1X3 + \beta 14X1X4 + \beta 15X1X5 + \beta 16X1X6 \\ & + \beta 23X2X3 + \beta 24X2X4 + \beta 25X2X5 + \beta 26X2X6 + \beta 34X3X4 \\ & + \beta 35X3X5 + \beta 36X3X6 + \beta 45X4X5 + \beta 46X4X6 + \beta 56X5X6 \\ & + \beta 11X1^2 + \beta 22X2^2 + \beta 33X3^2 + \beta 44X4^2 + \beta 55X5^2 + \beta 66X6^2 \end{aligned}$$

In this equation Y is the aforementioned response, $\beta 0$ is the ordinate at the origin; $X1$ [percentage of EtOH in the extraction solvent], $X2$ [temperature (°C)], $X3$ [ultrasound amplitude], $X4$ [duty cycle], $X5$ [pH], $X6$ [ratio solid sample (g)/extraction volume (mL)] are the independent variables; βi are the linear coefficients; βij are the cross product coefficients and βii are the quadratic coefficients.

The analysis of data for the Box–Behnken design was carried out using Unscrambler X (Camo, No). This software was used to estimate the effects of the variables on the final response, the variance analysis, the second order mathematical model, the optimum levels of the significant variables and the surface graphs.

2.5. Amino acids determination

The AccQ-Tag method was used for derivatization and chromatographic determination. The AccQ•Tag Reagent Kit was purchased from Waters (Milford, Massachusetts, USA). The reagent kit consists of Waters AccQ•Fluor Borate Buffer, Waters AccQ•Fluor Reagent Powder (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate – AQC), Waters AccQ•Fluor Reagent Diluent, Waters AccQ•Tag Amino Acid Analysing Column (Nova-Pak C18, 4 μm, 150 × 3.9 mm) and Waters Amino Acid Hydrolysate Standard (each ampoule contains a 2.5 mM mixture of the 17 amino acids with the exception of cystine – 1.25 mM.

The following amino acids were found and quantified in the samples: aspartic acid, serine, glutamic acid, glycine, histidine, arginine, threonine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine and leucine.

3. Results and discussion

3.1. Optimization of the extraction method

A Box–Behnken design was used to optimize the extraction conditions for amino acids from homogenized grape samples. Six different extraction variables were studied in the following ranges: ethanol in water between 0% and 50%, temperature values between