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RESEARCH NOTE / NOTA TÉCNICA

A METHOD TO ANALYSE BOUND AROMA COMPOUNDS IN NON-AROMATIC RED GRAPE JUICES

UM MÉTODO PARA DOSEAR OS COMPONENTES LIGADOS DO AROMA EM SUMOS DE UVAS TINTAS NÃO AROMÁTICAS

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SUMMARY

A method to study the glycosidically bound fractions in red grape juice was developed. Bound fractions were fractionated by solid-liquid chromatography on LiChroprep RP-18. The release of free aglycones was achieved by acid hydrolysis. In a preliminary approach the acid hydrolysis was performed at pH 1.0 and pH 3.0 levels. The pH 3.0 was selected in this study mainly due to its similarity with the pH levels that occur naturally in juices and grapes. Nine extractions of the same juice sample were carried out to study the repeatability of the method at pH 3.0. All the extracts were analysed by GC-MS and GC-FID. The repeatability found in the various quantified components in all extracts of the bound fraction of juice is expressed by their coefficient of variation, which varied in the range between 11.5% and 24.7%. Four aglycones, *trans*-furan linalool oxide, *cis*-furan linalool oxide, vitispirane, and β-damascenone were quantified after pH 3.0 hydrolysis.

RESUMO

Desenvolveu-se um método para estudar as fracções ligadas glicosidicamente em sumos de uva tintas. As fracções ligadas foram fraccionadas por cromatografia sólido-líquido utilizando resina RP-18. A libertação das aglíconas foi realizada por hidrólise ácida. Numa abordagem preliminar a hidrólise ácida foi efectuada a pH 1.0 e pH 3.0. Seleccionou-se o pH 3.0, principalmente, devido à sua similaridade com os valores de pH que ocorrem naturalmente nos sumos e nas uvas. No estudo da repetibilidade do método com hidrólise a pH 3.0 realizaram-se nove extracções de uma amostra de sumo. Todos os extractos foram analisados por GC-MS e GC-FID. A repetibilidade, expressa pelo coeficiente de variação, encontrada nos diversos compostos quantificados, em todos os extractos da fracção ligada do sumo, variou entre 11.5% e 24.7%. Após hidrólise a pH 3.0, foram quantificadas quatro aglíconas: *trans*-óxido furânico de linalol, *cis*-óxido furânico de linalol, vitispirano e β-damascenona.

Key words: glycosidically bound compounds, LiChroprep RP-18, acid hydrolysis, red grape juice

Palavras-chave: compostos ligados glicosidicamente, LiChroprep RP-18, hidrólise ácida, sumo de uva tinta

INTRODUCTION

Increasing interest has been devoted in the past years to the study of non-volatile and flavourless glycoconjugates which correspond to a pool of aroma to be exploited in both plants and fruits (Stahl-Bishop *et al*., 1993; Mateo and Jimenez, 2000; Sarry and Gunata, 2004; Maicas and Mateo, 2005).

Glycoconjugates occur mainly as monoglucosides or disaccharides, with sugar moieties occurring as β-D-glucose, 6-O-(β-L-rhamnopyranosyl)-β-D-glucopyranose, 6-O-(β-L-arabinofuranosyl)-β-D-glucopyranose and 6-O-(β-D-apiofuranosyl)-β-D-glucopyranose (Williams *et al*., 1982a; Williams *et al*., 1983; Voirin *et al.*, 1990). Glycoside hydrolysis liberates aglycones, a complex group of chemical compounds that include monoterpenes, sesquiterpenes, C_{13} norisoprenoids, phenol acids, volatile phenols, vanillin, and aliphatic or cyclic alcohols such as 1-hexanol, 2-phenylethanol, benzyl alcohol (Williams *et al.*; 1982a,b; Strauss *et al.*, 1986; Gunata *et al.*, 1988; Winterhalter *et al.*, 1990; Sefton *et al.*, 1993; Sefton *et al.*, 1994; Sarry and Gunata, 2004).

Naturally, in grape berries, the liberation of free aglycones from glycosides is performed by acid catalyzed reactions or by the action of endogenous β-glucosidases (Bayonove *et al.*, 1984; Aryan *et al.*, 1987; Gunata *et al*., 1985; Gunata *et al.*, 1988; Sarry and Gunata, 2004; Maicas and Mateo, 2005).

Acid hydrolysis of grape glycosides occurs when

protonated reagents break the glycosyl bound between D-glucose and the aglycone, producing one molecule of water. Experiments on both whole juice and monoterpene glycosides isolated from juice have demonstrated that significantly different patterns of volatile monoterpenes are produced when each one is hydrolysed at different pH values. Furthermore, there appears to be a pH-dependent inter-relationship among several of the grape monoterpenes (Williams *et al*., 1982b; Maicas and Mateo, 2005).

Several methods for the isolation of glycosides of volatiles from grape juices and wines have been described. The great majority of them involve the selective retention of glycosides from aqueous extracts on two hydrophobic adsorbents: the RP-18 reversed-phase resin (Williams *et al.*, 1982a,b; Di Stefano, 1991; Sefton and Williams, 1991) and the Amberlite XAD-2 resin (Gunata *et al.*, 1985; López *et al.*, 2004; Oliveira *et al.*, 2004). Besides, a rapid method to assess the glycoconjugates (Glycosyl-Glucose, (G-G)) was developed by Williams *et al*., (1995) and further modified by Iland *et al*., (1996). Also, Schneider *et al*. (2004) developed a fast method using Fourier-transform infrared spectrometry and chemometric techniques for grape aroma glycoconjugates analysis.

Recently, López *et al.*, (2004) in a study about Tempranillo (syn. Aragonez) and Grenache grapes, confirmed that these two red grapes should be considered neutral cultivars. In global terms, according to these authors, the small number and amount of terpenes found in these grape varieties is in agreement with the non-floral character of those red grapes.

The main aim of this work is to develop a method for the glycosidically bound fractions determination from non-aromatic red grape juices in order to increase the knowledge of the identity of the aglycones and to obtain a reproducible quantification of them. This method allows the quantification without significant changes in the grape juice matrix and it would contribute to the distinction among several clonal grape juices from non-aromatic varieties.

MATERIAL AND METHODS

Juice samples

Grapes of three certified clones of *Vitis vinifera* L. *cv*. Aragonez with code designation of 54 EAN (PT), 57 EAN (PT) and 59 EAN (PT) were collected in one vineyard of Estremadura wine region (Portugal).

About 60 Kg of grapes of each clone from Aragonez variety, in good sanitary conditions at the final stage of ripening, were hand-harvested, crushed and destemmed in the experimental cellar of INIA-Estação Vitivinícola Nacional. Replicate samples of the juices were taken in 500 mL glass bottles for later analysis and stored at -30 ºC.

Reagents

Analytical grade solvents and reagents were used. Water used was deionised (conductivity < 0.1 mS/cm obtained through a Seralpur Pro 90 CN from SERAL (Water Purification Systems, Ransbach-Baumbach, Germany)). LiChroprep RP-18 (40-63 µm), anhydrous sodium sulphate (99%), perchloric acid, ethanol LiChrolsolv, methanol and dichloromethane were purchased from Merck (Darmstadt, Germany). The last one was purified by redistillation before use. The GC standards hexanal, benzyl alcohol and vanillin were purchased from Fluka Chemie (Buchs, Switzerland); trans-2-hexenal and 4-nonanol (IS, internal standard) were purchased from TCI Europe nv (Zwijndrecht, Belgium) and ?-damascenone was kindly supplied by Symrise (Holzminden, Germany).

Extraction of glycosidic bound fractions

The bottles were taken out at a temperature of -30 ºC, and they were thawed at 4 ºC just before analysis. The liquid juice was then centrifuged at 4 ºC (15000 rpm, Sorvall RC-5B, Newtown, USA) for 15 min.

Juice samples were fractionated by solid-liquid chromatography using LiChroprep RP-18 non ionic resin in a glass column (30 x 3 cm i.d.).

The LiChroprep RP-18 (40 g) was purified with methanol for 4 h before poured into the glass column. A peristaltic bomb (Masterflex, Barrington, USA) was used to help the elution of all solvents. The LiChroprep RP-18 on the column was first pre-conditioned with 100 mL of methanol, then with 200 mL of ultrapure water. A sample of 200 mL clarified juice was passed through the column and it was washed with ultrapure water (400 mL) following the adsorption step. During this step, free sugars and other polar constituents are removed while the less polar glycosides are retained (Williams *et al*., 1982b). The free fraction was eluted with dichloromethane (100 mL) to avoid the presence of free volatile compounds in the methanolic extract. This free extract was then discarded. Glycosides were recovered by elution with methanol (100 mL) and this fraction was collected in a separate 150 mL volumetric flask in ice-water bath. Afterwards, the eluate was concentrated to a final volume of 2 mL, in a rotary evaporator (Büchi rotavapor R-200 and Büchi heating bath, B-490, Switzerland) at 32 ºC (±0.5 ºC) under vacuum (Büchi vacuum system, Büchi B-169, Switzerland). The main steps are described in Figure 1.

After each sample extraction, the resin was thoroughly rinsed with methanol acidified with perchloric acid (0.1%) and was left in methanol between each use.

Acidic hydrolysis of bound fractions

Considering the acidic hydrolysis of bound fractions,

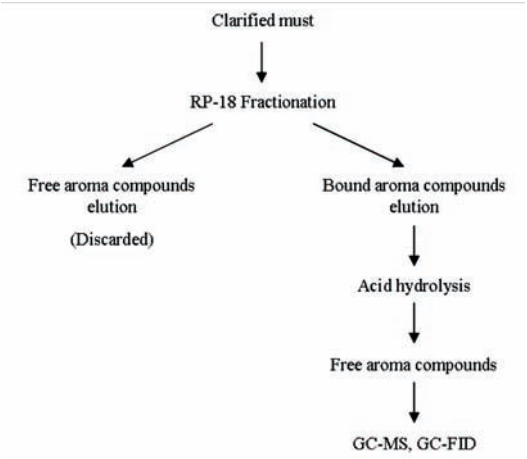

Fig. 1 - Experimental scheme of the bound fractions extraction and analysis
Esquema experimental da extracção e da análise das fracções ligadas

experiments were done at pH 1.0 (perchloric acid 10%) and pH 3.0 (perchloric acid 1%).

Hydrolysis was carried out by heating the acidic extract into a glass vial, at 100 ºC for 20 min. Then the extract was cooled to room temperature (20 ºC). An aliquot of 100 µL of 4-nonanol (IS, 82.7 mg/L, 50% ethanol solution) was added for quantification.

The free volatile compounds generated were extracted with the successive addition of 15, 5 and 5 mL of dichloromethane by ultrasonification (P Selecta, model 3000515, 40 KHz, Barcelone, Spain) for 10 min, for each extraction. Between extractions, centrifugation (10000 rpm, 4 ºC, 5 min Sorvall RC-5B, Newtown, USA) was done to help the phase separation process. The organic phases obtained were pooled, dried over anhydrous sodium sulphate and concentrated to approximately 100 µL, on a rotary evaporator at 42 ± 0.5 ºC (Büchi rotavapor R-114 and Büchi heating bath, B-480, Switzerland), without vacuum. The extracts were stored at -20 ºC until analysis by GC-MS and GC-FID.

Analysis by GC-MS

A Finnigan MAT (San Jose, CA, USA) GC-MS equipment (Magnum) was used. An aliquot of 1.2 µL was injected and volatile compounds were separated using a fused silica capillary column of polyethylene glycol (DB-WAX, 30 m length x 0.25 mm i.d. x 0.25 µm film thickness, J&W Scientific, Agilent Technologies, USA). Operating conditions were as follows: injector and interface temperature, 250 ºC; carrier gas helium, inlet pressure 12 psi and split ratio 1:40; the temperature gradient used began at 50 ºC for 2 min, and was raised to 180 ºC at 3.5 ºC min^{-1} and was held at this temperature for 25 min. The mass spectrometer was operated in the electron impact mode at 70 eV, scanning the range m/z 39-340.

Identification of volatile compounds was systematically confirmed with the retention indices of the available pure standard compounds (determined in the same analysis conditions) and with the comparison between the mass spectra of the volatile compounds and of the pure standard compounds. All mass spectra were also compared with those of data system libraries (NIST and Wiley).

Analysis by GC-FID

The GC analysis was carried out in an Agilent Technologies 6890N series chromatograph equipped with a flame ionization detector (FID) and a fused silica capillary column of polyethylene glycol (INNOWAX, J&W Scientific, Agilent Technologies, USA) of 30 m, 0.32 mm i.d., and 0.25 µm film thickness. The injection volume was approximately 1.2 µL for juice extracts. Operating conditions were as follows: injector and detector at 250 ºC; carrier gas hydrogen at a flow rate of 2.0 mL min^{-1} and split ratio 1:3; the temperature gradient used began at 45 ºC for 5 min, and was raised to 210 ºC at 3.5 ºC min^{-1} and was held at this temperature for 20 min. The compounds were quantified as nonan-4-ol equivalents.

Statistical analysis

The statistical analysis was performed with Microsoft Office Excel 2003 (Microsoft Corporation, USA).

RESULTS AND DISCUSSION

Preliminary study of the extraction method

The LiChroprep RP-18 proved to be suitable for free and bound compounds fractionation from red grape juices. The efficiency of the free compounds extraction by dichloromethane in the adsorbent column was carefully confirmed. The presence of possible free residual compounds in the methanolic fraction, before acid hydrolysis, was checked by dichloromethane extraction as previously described. Furthermore, we confirmed the absence of free residual volatile components by GC-FID analysis.

Acid hydrolysis at two different pH levels

The acid hydrolysis of bound fractions of grape juice was performed at pH 1.0 and pH 3.0. The three clonal Aragonez grape juices were used in this study. The proposed method was applied identically to the three clonal grape juices and each sample was analysed twice.

In Figure 2 the released aglycones obtained by acid hydrolysis of bound fractions of grape juice of the clone 59, at pH 1.0 and pH 3.0, are presented in two put upon chromatograms. In both chromatograms, there are four main aglycones identified by GC-MS analysis.

Table I shows the quantification results of four main precursors of aroma identified in grape juice extracts after acid hydrolysis at pH 1.0 and pH 3.0.

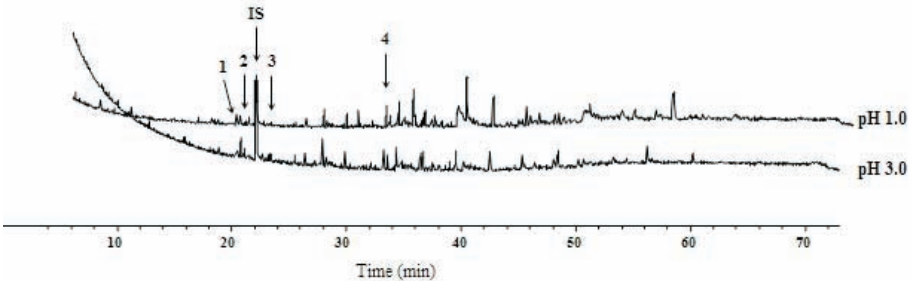

Fig. 2 - Chromatograms of bound fraction extracts at pH 1.0 and pH 3.0 from grape juice of Aragonez clone 59
1: *trans*-furan linalool oxide; **2:** *cis*-furan linalool oxide; **IS**: internal standard; **3:** vitispirane I and II; **4:** â-damascenone
Cromatogramas de extractos da fracção ligada a pH 1.0 e pH 3.0 de sumo de uva do clone 59 de Aragonez
***1**: trans-óxido furânico de linalol; **2**: cis-óxido furânico de linalol; **IS**: padrão interno; **3**: vitispirano I e II; **4**: β–damascenona*

TABLE I

Concentration of four main aglycones found in Aragonez clonal juices (µg of 4-nonanol/L)
Concentração das quatro principais aglíconas identificadas nos sumos dos clones de Aragonez (µg de 4-nonanol/L)

		Clones					
		pH 1.0			pH 3.0		
N.	Compound	54	57	59	54	57	59
1	*trans*-Furan linalool oxide	5.7	8.1	6.3	n.q.	n.q.	n.q.
2	*cis*-Furan linalool oxide	n.q.	6.3	n.q.	n.q.	3.4	n.q.
3	Vitispirane I, II	n.q.	n.q.	n.q.	n.q.	4.0	n.q.
4	β-Damascenone	n.q.	9.0	12.0	13.7	16.4	16.8

n.q. not quantified

Two of them are monoterpenic aglycones and the other two are C_{13} norisoprenoids.
The *trans*-furan linalool oxide was only quantified in the clones at pH 1.0, which can indicate that this low level of pH during hydrolysis reaction induces the appearance of this molecule in the medium. It is very interesting to verify that isomeric vitispiranes were only quantified in clone 57 at pH 3.0 hydrolysis. Winterhalter *et al*. (1990) and Waldmann and Winterhalter (1992) have found vitispirane-yielding precursors in Riesling wine. Recently, López *et al*. (2004) referred to the presence of an unknown compound with a mass spectra similar to vitispirane in their olfactometric experiments with Tempranillo and Grenache juice and skin grapes.

Regarding β-damascenone, high levels of concentration at pH 3.0 hydrolysis of the three clonal grape juices were found comparatively with those at pH 1.0. It seems to indicate that pH 3.0 is more suitable to release this aglycone. Mechanisms for the formation of ?-damascenone from the carotenoid neoxantin were proposed by Skouroumounis and Sefton (2002). It is well established that this aglycone has an important sensorial impact on wine aroma as it has a very low olfactory perception threshold (Buttery *et al*., 1990; Ferreira and Guedes de Pinho, 2004). Moreover, ?-damascenone has been identified as a key-odourant in several grape juices and wines (Lopez *et al*., 1999; Kotseridis and Baumes, 2000). Recently, Botelho *et al.* (2007) have identified this odourant compound in the evaluation of key-odourants in Aragonez clonal red wines by GC-Olfactometry.

Low differences were found between the number and the amount of aglycones released at pH 1.0 and pH 3.0. As Williams *et al*. (1982b) have suggested, it is possible to establish a pH-dependent inter-relationship among the grape aglycones.

The pH 3.0 acid hydrolysis was selected to evaluate the aglycones of clonal Aragonez juices considering that it is the closest pH value to which naturally occurs in grape juice. Besides, Ibarz *et al*. (2006) underlined that the acid hydrolysis generates a variety of aromas comparable to that of wine.

Repeatability

The repeatability was determined by nine replicates analysis of the same Aragonez juice. The aglycones

released by acidic hydrolysis at pH 3.0 were identified and quantified by GC-MS and GC-FID, respectively. The GC-FID analyses were done in triplicate. For each assay the average value and the standard deviation were calculated for all the volatile compounds that were quantified in all extracts.

Table II presents the compounds' identity, the average relative retention times (ARRT), the average relative areas (ARA), the standard deviation (SD) and the coefficient of variation (CV). The vanillin wasn't quantified in all repetitions probably due to its weak volatility and consequently the SD and CV weren't calculated. Vanillin was found by López *et al.* (2004) in both juice and skin samples of Tempranillo. Moreover, the bound form of vanillin was found in musts, skins and pulps of different grape varieties (Vázquez *et. al*., 2002; Lamorte *et al*., 2007).

TABLE II

Repeatability of the quantification method of bound fraction of Aragonez grape juice
Repetibilidade do método de doseamento da fracção ligada do sumo de uva de Aragonez

Compound	ARRT[a]	ARA[b]	SD[c]	CV (%)[d]
Hexanal	0.270	3.160	0.782	24.7
trans-2-Hexenal	0.491	0.478	0.055	11.5
Vitispirane I, II	1.062	0.243	0.152	20.4
β-Damascenone	1.560	1.006	0.176	17.5
Benzyl alcohol	1.653	0.662	0.125	18.9
Vanillin	2.555	0.199	[e]	[e]

[a]ARRT: average relative retention time, [b]ARA: average relative area, [c]SD: standard deviation, [d]CV: coefficient of variation, [e]Not calculated.

The variability found in the quantification of the various components in all extracts of the bound fraction of the grape juice is expressed mainly in the range between 11.5% and 24.7%.

The C_6 aldehydes, hexanal and trans-2-hexenal, were both quantified in the nine extracts. These six-carbon compounds have been reported to contribute to the "grassy" odour in grape juice as free volatile compounds (Hardy, 1970). Recently, Lamorte *et al.* (2007) found that hexanal and trans-2-hexenal exist as aglycones in grape skins and pulp.

The glycosidically bound aroma composition found confirms the relatively non-aromatic aroma profile of Aragonez compared to the profile of Muscat, Riesling and Gewurtztraminer varieties (Marais 1983, Gunata *et al.*, 1985). Furthermore, the results of this study demonstrate that Aragonez juices are very poor in diversity as well as in amount of aroma precursors.

CONCLUSIONS

It should be emphasized that the current study contributes to the characterisation and development of a method using LiChroprep RP-18 for glycosidically bound compounds isolation in red grape juices. In the suggested method, release of glycosidic compounds from their glycosidically bound forms is achieved through acid hydrolysis, which occurs naturally in grape juices.

According to these results, it is possible to conclude that this method is suitable for identification and quantification of aroma precursors from red juices. The acidification of the juice extracts should be conducted at pH 3.0 in further investigations, since this pH is very similar to the pH that naturally occurs in grape juices. Moreover, the proposed method is sensitive and revealed an acceptable repeatability with the exception of low volatility compounds like vanillin. Given the set of quantified aglycones, the variation coefficients values were considered quite satisfactory taking into account the number of steps that this isolation method involves.

The proposed method can be extensively used as a routine procedure for the quality assessment of grapes and for possible differentiation among non-aromatic grape clones of grape varieties.

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