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CUANTIFICACIÓN DE ÁCIDOS ORGÁNICOS EN MOSTOS Y VINOS DE OPORTO

CUANTIFICACIÓN DE ÁCIDOS ORGÁNICOS EN MOSTOS E VIÑOS DE PORTO

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Abstract

A simple, rapid and accurate method for derivatization and quantitative HPLC analysis of organic acids, was optimised in order to provide the determination of the most important acids found in Port wines and grape musts used for their elaboration. The method was based on the use of O-(4-nitrobenzyl)-N,N'-diisopropylisourea (NBDI) as derivatizing reagent. Samples were primarily treated 15 min with a strong cation-exchange resin (Dowex 50W-X8) to free the organic acids. HPLC determination was accomplished by using a RP-18 (3 μ m) column, a mobile phase composed of water and acetonitrile (non-linear gradient) at a flow-rate of 1 mL min⁻¹, and a UV detector set at 265 nm. Sensitive and well-resolved peaks were obtained from the main organic acids (lactic, acetic, succinic, tartaric, malic and citric acids) in less than 30 min, either with Port wines or grape musts. Benzylmalonic acid was used as internal standard. Precision and recovery assays were performed with good results for all of 6 acids considered. The major differences between grape musts and Port wines were the greater overall amounts of organic acids in the former and the higher content of lactic, acetic and succinic acids in the latter. © 2002 Altaga. All rights reserved.

Key words: Port wines, organic acids, HPLC.

Resumen

Se optimizó un simple, rápido y exacto método para la derivatización y análisis por HPLC de ácidos orgánicos, con el objeto de permitir la determinación de los principales ácidos encontrados en mostos y vinos de Oporto. El método se basa en el uso de O-(4-nitrobenzil)-N,N'-diisopropilisourea (NBDI) como agente de derivatización. Las muestras se pasaron en primer lugar por una resina de intercambio catiónico fuerte (Dowex 50W-X8) durante 15 min para liberar los ácidos orgánicos. La determinación por HPLC se desarrolló usando una columna RP-18 (3 μ m), una fase móvil compuesta de agua y acetonitrilo (gradiente no-lineal) con un caudal de 1 mL min⁻¹. Se usó un detector UV ajustado a 265 nm. Analizando muestras de mostos y vinos de Oporto se obtuvieron picos resueltos de los principales ácidos orgánicos (ácido láctico, acético, succínico, tartárico, málico y cítrico) en menos de 30 min. Se usó como patrón interno ácido benzilmalónico. Los ensayos de precisión y recuperación se realizaron con buenos resultados para los 6 ácidos considerados. La mayor diferencia entre los mostos y vinos de Oporto fue la mayor cantidad global de ácidos orgánicos en el primero y de ácido láctico, acético y succínico en el segundo. © 2002 Altaga. Todos los derechos reservados.

Palabras clave: Vinos de Oporto, ácidos orgánicos, HPLC.

Resumo

Optimizouse un simple, rápido e exacto método para a derivatización e análise por HPLC de ácidos orgánicos, co obxecto de permiti-la determinación dos principais ácidos atopados nos mostos e viños de Porto. O método basease no uso de O-(4-nitrobenzil)-N,N'-diisopropilisourea (NBDI) como axente de derivatización. As mostrás pasáronse nun primeiro lugar por unha resina de intercambio catiónico forte (Dowex 50W-X8) durante 15 min para libera-los ácidos orgánicos. A determinación por HPLC desenvolveuse usando unha columna RP-18 (3 μ m), unha fase móbil composta de auga e acetonitrilo (gradiente non-lineal) cun caudal de 1 mL min⁻¹. Usouse un detector UV axustado a 265 nm. Analizando mostrás de mostos e viños de Porto obtivéronse picos resoltos dos principais ácidos orgánicos (ácido láctico, acético, succínico, tartárico, málico e cítrico) en menos de 30 min. Usouse como patrón interno ácido benzilmalónico. Os ensaios de precisión e recuperación realizáronse con bos resultados para os 6 ácidos considerados. A maior diferencia entre os mostos e viños de Porto foi a maior cantidade global de ácidos orgánicos nos primeiros e de ácido láctico, acético e succínico nos segundos. © 2002 Altaga. Tódolos dereitos reservados.

Palabras chave: Viños de Porto, ácidos orgánicos, HPLC.

INTRODUCTION

Port is a fortified dessert wine traditionally manufactured in the Douro Valley in Northern Portugal, using a mixture of grape varieties. In spite of its great economical significance for Portugal, little information is available on the organic acid contents of grape musts and Port wines, mainly tartaric (TA), malic (MA), citric (CA), succinic (SA), lactic (LA) and acetic (AA) acids. Their origins are diverse, the most important being biosynthesised by the wine, metabolic pathways related to sugar fermentation and malolactic fermentation. Conjointly, they are of critical importance for the chemical and microbiological stabilisation and they deeply affect the colour and taste of the final product.

Attending their importance, an accurate and reproducible method, involving simple sample preparation and suitable for the routine analysis, is therefore desirable. The official method actually adopted by the International Office of Wine (OIV) does not fulfil these requirements: it makes use of two C_8 columns for the separation of TA, MA and SA and implies the use of a different column (Aminex®, HPX 87 H) for separating the LA, AA and CA acids. The detection is achieved at 214 nm, a wavelength at which many other compounds (sugars, e.g.) absorb and can interfere in the separation of the acids (OIV, 1990). However, other HPLC/UV methods also without derivatization, but with complex purification techniques to eliminate matrix interferences, are described for the determination of organic acids in grape juices and wines, namely by Herrera *et al.* (1993), Romero *et al.* (1993), Radin *et al.* (1994) Escobal *et al.* (1997) and Castellari *et al.* (2000)

A simple, rapid and accurate method for derivatization and quantitative HPLC analysis of organic acids in samples of roasted coffee was proposed a few years ago by Badoud and Pratz (1986). The method was based on the use of O-(4-nitrobenzyl)-N,N'-diisopropylisourea (NBDI) as derivatizing reagent. This is a strong chromophoric labelling agent that readily reacts with free carboxylic acids to form the corresponding p-nitrobenzyl esters in high yields and offers better sensitivity and selectivity.

Taking into account the simplicity and the excellent chromatographic performance obtained when applied to samples of Port wine and grape musts (characterised by their high sugar contents) we decided to undertake a study in order to validate the method for determination of organic acids in this kind of samples. The final objective was to apply the optimised method to the determination of quantitative profiles of organic acids in grape musts and Port wines.

MATERIAL AND METHODS

Apparatus and operating conditions

The separation of the 6 organic acids PNB ester derivatives was achieved on a Waters Spherisorb 3 μ m, C_{18} (4.6 x 150 mm) with a Nucleosil guard-column C_{18} (4 x 30 mm). The mobile phase was composed of water (A)

and acetonitrile (B). The gradient was as follows: 30 to 45% B within 10 min, 45 to 55% B within 10 min, 55 to 80% B within 10 min and returning to initial conditions within 2 min. The flow rate was 1 mL min⁻¹. Detection was accomplished with UV detector set at 265 nm.

Reagents and solutions

All reagents used were (p.a) from Merck. Derivatizing reagent O-(4-nitrobenzyl)-N,N'-diisopropylisourea (NBDI) was from Sigma Chemicals Co.

Sample Preparation and Derivatization Procedure

A 5 mL aliquot of the samples or standard solutions were added with 100 μ L of aqueous benzylmalonic acid solution 2 gL⁻¹ (I.S.) and 0.5 g of activated strong cation-exchange resin (Dowex 50W-X8). The mixture was shaking for 15 min. Afterwards, a 50 μ L aliquot was mixed with 500 μ L of a solution of NBDI in dioxane (10 mgmL⁻¹). The mixture was heated at 80 °C for 60 min. After cooling, the solution was diluted by addition of 2 mL of acetonitrile and cleaned-up with another portion of the same ion-exchange resin, to remove the excess of reagent. Finally, the mixture was filtered through 0.22 μ m filters and an aliquot of 10 μ L was injected into the chromatograph.

Sampling

Seven samples of Port wines from Portuguese market and five samples of grape musts obtained directly from the producer were analysed.

RESULTS AND DISCUSSION

Figure 1 and Figure 2 show chromatograms of a typical grape juice and a Port wine, respectively.

Linearity

Calibration curves (6 points) were constructed by analysing aqueous standard solutions of the 6 organic acids studied with increasing amounts of each acid, treated in exactly the same way as the samples. The range of concentrations was 0.1-6 g.L⁻¹ for MA and TA, 0.1-2 g.L⁻¹ for LA and AA, and 0.1-2g.L⁻¹ for SA and CA. Correlation coefficients higher than 0.998 were obtained for all the 6 acids (Table 1).

Limits of detection

The detection limits for each acid, based on a signal-to-noise ratio of 3, were 0.005 g.L⁻¹ for TA, 0.038 g.L⁻¹ for MA, 0.046 g.L⁻¹ for SA, 0.041 g.L⁻¹ for AA, 0.056 g.L⁻¹ for LA and 0.098 g.L⁻¹ for CA.

Precision

The validity of the method was verified by evaluation of precision and accuracy. Precision was evaluated by assaying six times a Port wine sample and a grape must sample. Good results were obtained with coefficients of variation being less than 2.9 % for all 6 acids studied (Table 2).

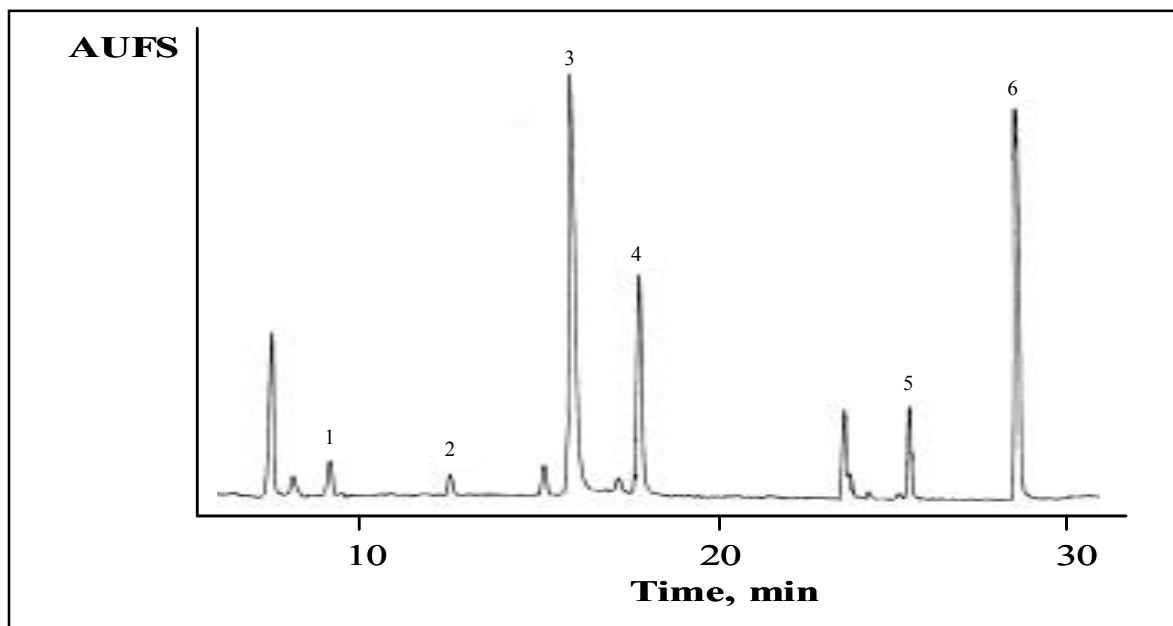


Figure 1. Chromatogram of a grape must sample with: 1-lactic acid (0.201 g L⁻¹), 2-acetic acid (0.070 g L⁻¹), 3-tartaric acid (3.034 g L⁻¹), 4-malic acid (1.971 g L⁻¹), 5-citric acid (0.500 g L⁻¹) and 6-I.S (2 g L⁻¹).

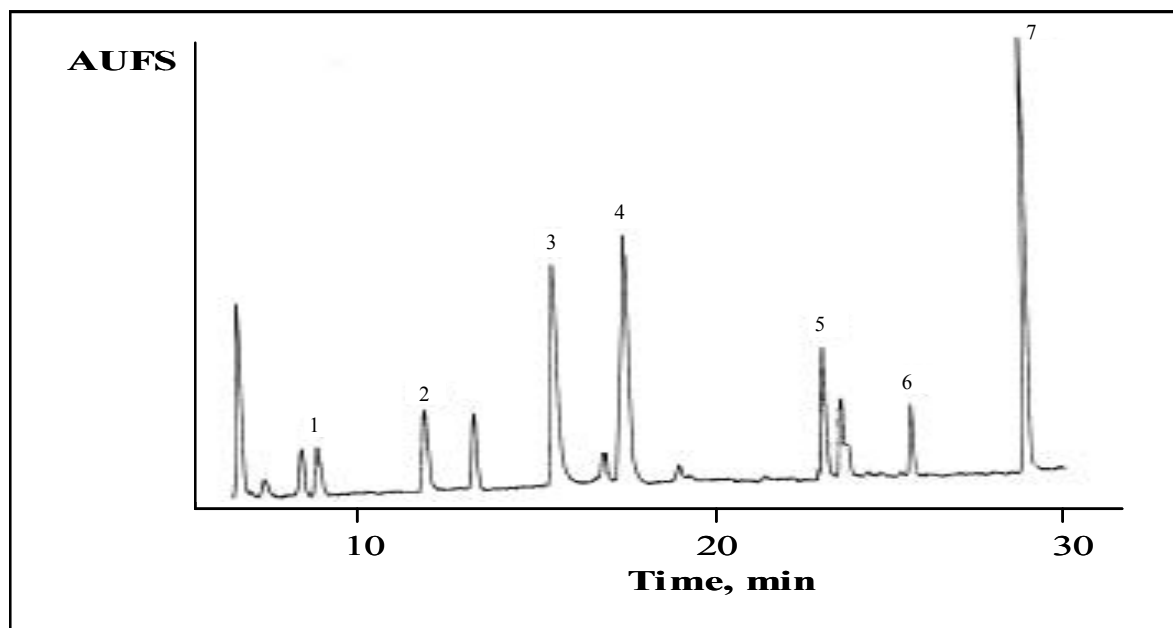


Figure 2. Chromatogram of a Port wine sample with: 1-lactic acid (0.294 g L⁻¹), 2-acetic acid (0.231 g L⁻¹), 3-tartaric acid (1.204 g L⁻¹), 4-malic acid (1.082 g L⁻¹), 5- succinic acid (0.348 g L⁻¹), 6-citric acid (0.270 g L⁻¹) and 7-I.S (2 g.L⁻¹).

Table 1.- Calibration curve parameters ($y = ax + b$), correlation coefficients (r) of calibration plots and retention time for the organic acids under study.

Carboxylic acids	a mean	b mean	r	RT (min)
Lactic	0.287	0.005	0.9997	7.37
Acetic	0.429	0.034	0.9994	12.53
Tartaric	0.367	0.034	0.9983	14.97
Malic	0.382	0.065	0.9982	17.30
Succinic	0.502	-0.002	0.9998	24.00
Citric	0.402	-0.029	0.9992	26.76

Table 2.- Evaluation of precision of six replicate analyses of grape musts and Port wine. Legend: n.d.= not detected.

Carboxylic acids	Grape must		Port wine	
	Mean ±S.D. (g L ⁻¹)	C.V. (%)	Mean ±S.D. (g L ⁻¹)	C.V. (%)
Lactic	0.337±0.010	2.9	0.301±0.007	2.3
Acetic	0.308±0.005	1.7	0.260±0.005	1.9
Tartaric	2.503±0.039	1.6	0.941±0.014	1.5
Malic	1.446±0.014	0.9	1.046±0.007	0.6
Succinic	n.d.	-	0.303±0.001	0.4
Citric	0.543±0.009	1.6	0.164±0.004	2.3

Table 3.- Recovery of organic acids from spiked grape musts and Port wine samples.

Carboxylic acids	Grape juice			Port wine		
	Initial amount (g L ⁻¹)	Added (g L ⁻¹)	Recovery (%)	Initial amount (g L ⁻¹)	Added (g L ⁻¹)	Recovery (%)
Lactic	0.337	0.250	102.3	0.301	0.250	106.6
		0.500	102.3		0.500	94.9
		1.000	113.5		1.000	98.6
Acetic	0.308	0.250	117.3	0.260	0.250	109.9
		0.500	113.2		0.500	118.9
		1.000	106.1		1.000	108.5
Tartaric	2.503	1.250	112.9	0.941	1.250	108.8
		2.500	104.4		2.500	106.4
		3.750	100.1		3.750	105.9
Malic	1.446	1.250	101.3	1.046	1.250	101.1
		2.500	98.2		2.500	102.6
		3.750	104.1		3.750	105.7
Succinic	n.d.	0.250	100.9	0.303	0.250	103.1
		0.500	104.2		0.500	106.7
		1.000	114.4		1.000	105.4
Citric	0.543	0.250	101.9	0.164	0.250	105.6
		0.500	113.2		0.500	105.2
		1.000	99.7		1.000	105.5

Recovery

The reliability of the method was confirmed by two recovery experiments. A Port wine and of a grape must were analysed before and after the addition of known amounts of mixtures of the organic acids and analysed in the same way as the samples. Table 3 presents the results for recovery studies. Recoveries varied between 94.9-118.9% for Port wine and between 98.2-117.3% for grape must.

Analytical Results for grape musts and Port wines

The method was used for determining the organic acid contents of some grape musts and Port wines. Results obtained there of, are listed in Table 4.

As apparent from Table 4, there are significant differences between organic acid contents from grape musts and Port wines. As expected Port wines contain lower amounts of organic acids when compared with grape musts, as a result of fermentation and metabolic processes. However, all Port wines contain larger amounts of succinic, lactic and acetic acids, whereas in grape musts the former is lacking in all samples, only traces of acetic acid were detected in most samples and a few amount of lactic acid was present. This, results from the fact that

these acids are products of alcoholic fermentation. Significant variation between samples was observed ($p < 0.05$).

On the other hand, tartaric acid, the most abundant organic acid of grape musts, decreases during alcoholic fermentation owing to the precipitation of calcium tartrate and potassium bitartrate. Thus, significantly lower amounts of this organic acid were observed in Port wine samples. Malic and citric acids were also lower in Port wines owing to their decrease during fermentation process.

The results obtained were compared with those refereed in literature for other types of wines, it is interesting to note that similar profiles were obtained (Ryan and Dupont, 1973).

CONCLUSIONS

Analysis of organic acids as their *p*-nitrobenzyl esters seems to be a valuable alternative to other current available methods for a rapid and very accurate quantification of this kind of compounds in Port wines and grape musts. The method is simple and allows for well-resolved peaks of the main carboxylic acids in less

Table 4.- Organic acid contents in grape musts and Port wine samples. Legend: n.d.= not detected.

Samples	Carboxylic Acids (g L ⁻¹)					
	Lactic	Acetic	Tartaric	Malic	Succinic	Citric
Port Wines	0.414	0.282	1.310	1.352	0.751	0.288
	0.219	0.320	1.178	1.442	0.238	0.260
	0.286	0.221	1.195	1.084	0.978	0.312
	0.497	0.222	1.077	0.764	0.245	0.235
	0.421	0.251	1.133	1.158	0.288	0.260
	0.294	0.231	1.204	1.082	0.348	0.270
	0.350	0.283	1.105	1.180	0.284	0.278
Grape juices	0.256	0.095	4.278	0.361	n.d.	0.581
	0.201	n.d.	3.034	1.971	n.d.	0.500
	0.184	0.301	4.752	2.957	n.d.	0.510
	0.191	n.d.	2.918	3.696	n.d.	0.512
	0.137	0.209	2.176	1.428	n.d.	0.284

than 30 min, either with Port wines or grape juices, with very good precision and enough sensitivity. Thus, it makes possible to study the evolution of this compounds during fermentation and metabolic processes that occur on Port wines production.

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