

Organic Acids Novel Determination in Wines Aged in Oak Wood Barrels by HPLC

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Keywords

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In this project we describe a reversed-phased HPLC method that allows the simultaneous determination of the preservatives benzoic (BA), sorbic (SA) and ascorbic acids (AA) in wine and foodstuffs. The separations were effected by using a C18 varian column and a mobile phase of ammonium acetate buffer (pH 4.4) – methanol (65:35) to elute BA, SA and AA. The flow rate was 1.0 ml/min and the detector wavelength was set at 245nm. However specific parameters for analysis of wines and beverages (with or without alcohol) were optimized. Under these conditions, effective separation of the three components was achieved in less than 8 min. The developed method was applied to the determination of ten brands of industrial wines, all available on the Greek retail market. The range of preservatives found were from not detected (nd)-1294, nd – 400.5, nd – 489.7 mg kg⁻¹ for BA, SA and AA, respectively.

Introduction

Food preservatives are added to stop or delay nutritional losses due to microbiological, enzymatic or chemical changes of foods during its shelf time. They also prevent consumer hazards due to the presence of microbial toxins or pathogenic microorganisms and economic losses due to spoilage. The role of preservatives has become more prominent with the increase in production of processed and convenience foods. Benzoic and sorbic acids are generally effective to control mold and inhibit yeast growth and a wide range of bacterial attack.

Ascorbic acid is a water-soluble vitamin with antioxidant properties. Inside body the nutrient preserves cell integrity by neutralizing free radicals, which are toxic molecules that can damage healthy cells and cause disease. Ascorbic acid neutralizes oxygen when it comes into contact with it. Oxygen allows foods to continue to ripen, an aging process similar to the one people go through that ends in death. Oxygen is also vital for many microorganisms to thrive, some of which cause decay. Ascorbic acid slows or neutralizes these events. Ascorbic acid, more commonly known as vitamin C, can act as a preservative that stops foods from continuing to ripen, an aging process that leads to decay. The vitamin's acidity makes it hard for the enzyme phenolase to act. Phenolase accelerates oxidation, a chemical process in which oxygen level rises, resulting in decay. This is the process that ascorbic acid combats.

Ascorbic acid (E300) is easily oxidized and so is used as preservative. It improves the shelf life of wines and prevents haze development. Through rapid reaction with oxygen it protects the bottled beverage against chemical oxidations; it stabilizes the beverage bouquet and taste, it affects positive on the taste in beverages low in acids and gives high chemical purity.

Sorbic acid (2,4-hexadienoic acid) is a straight chain unsaturated fatty acid with a molecular weight of 112.13. and the formula: CH₃ - CH = CH - CH = CH - COOH. Sorbic acid is commercially produced as a powder or granules, it has a characteristic acid odor and acid taste. The carboxyl (COOH) group in sorbic acid is very reactive and can form salts with calcium, sodium, and potassium. The antimicrobial (preservative) properties of sorbic acid were recognized in the 1940's.

Since then, sorbic acid has been extensively tested and used as a preservative in many foods. Sorbic acid and its potassium salt are now used in many countries in the production of sweet white wines. In the United States, BATF permits the use of sorbic acid and potassium sorbate to preserve wine. The maximum concentration of sorbic acid allowed in finished wine is 300 mg/L, (300 ppm) but it is important to remember that its taste threshold is well below the legal limit. The taste threshold for experienced tasters has been reported to be about 130 ppm. Sorbic acid occurs naturally in fruit, as a preservative, it inhibits fungal growth but allows for bacterial activity, hence is useful for cheese. Obtained from the berries of mountain ash, sorbic acid is used in conjunction with sulphur dioxide in wine making, without SO₂ bacteria cause reduction of sorbic acid to sorbyl alcohol which converts to a foul smelling ether as it seems to the following reaction.

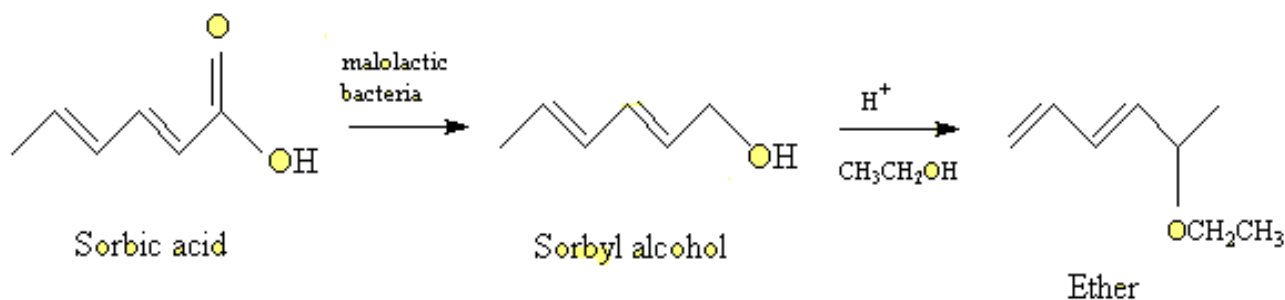


Figure 1. The reaction is taking place when sorbic acid was added without SO₂.

Also, it used as a preservative in cosmetics and pharmaceuticals. The antimicrobial action of sorbic acid is due to its inhibitory influence on various enzymes in the microbial cell. The enzymes inhibited by sorbic acid include the following: 1. Enzymes involved in carbohydrate metabolism such as enolase and lactate dehydrogenase. 2. Enzymes of citric acid cycles such as malate dehydrogenase, isocitrate dehydrogenase, ketoglutarate dehydrogenase, succinate dehydrogenase, and fumerase. 3. Several enzymes containing SH group, and other enzymes such as catalase and peroxidase.

Sorbic acid (E200) and its salts are physiologically harmless, but may still influence the taste of food. They show a strong effect on microorganisms over a wide pH range, including high levels.

Derivatives of benzoic acid are the main phenolic compounds that impart a characteristic flavor to many wine products [1]. However benzoic acid and its salts may have allergic effects on susceptible persons.

The use of BA, SA and AA as preservatives for various kinds of foods is permitted throughout the European Community. According to Directive 95/2/EC dated on 20.02.1995 which by article 1 paragraph 3 points that “the preservatives are substances that increase the food preservation time by protecting them against the damages caused by micro-organisms”, sorbic acid (E200) and benzoic acid (E210) belong to the preservatives allowed conditionally, singly or in combination. However, excessive quantities of these acids cause serious hazards for consumers and they must be strictly controlled [Joint FAO/WHO Expert Committee on Food additives (JECFA), 2004]. High performance liquid chromatography (HPLC) has been widely used for these organic acids in foods and beverages [2, 3, 4, 5]. Many analytical methods have been published [6, 7, 8, 9] including spectrophotometric methods and gas chromatography [10]. The maximum content in wine allowed by European legislation is 200mg/kg for BA when used alone and 300mg/kg for SA. The sum of BA and SA contents cannot exceed 400mg/kg (Directive no.98/72/CE). The legally allowed max AA content in wine or champagne must not exceed 250mg/l(ppm). As the directives of the EU regulate the applicability fields and the maximum quantities for each of these preservatives, it is required that methods should be elaborated and validated for determining their quantities.

The most common analytical method for the determination of BA, SA and AA has been reversed-phase HPLC, although other analytical methods such as TLC, capillary electrophoresis [11] and gas chromatography [12] have also been reported. Such a method is important as there seem to be an increasing trend in using combination of preservatives, not only in the beverage industry but also in pharmaceutical formulations and cosmetic products.

Here, we report on a specific procedure followed by HPLC separation of a mixture of benzoic, sorbic and ascorbic acid. The aim of this study was to develop an analytical method for routine laboratory work for the rapid and efficient analysis of sorbic, ascorbic and benzoic acids in wine and beverages. The developed method was validated to the analysis of these preservatives in ten brands of wine.

Experimental

Instrumentation & Reagents

Sorbic, ascorbic and benzoic acids were purchased from Carlo Erba (Italy). Stock solutions of all acids were prepared at 1000 mg/l with methanol HPLC grade (Chem Lab, www.chemlab.be). Standard curves were done with the concentration of 0, 2, 5 10 ppm for Ascorbic acid, 0, 5, 10, 20, 25 ppm for Benzoic acid and 0, 0.1, 0.5, 1, 1.5 for Sorbic acid. All dilutions were made with HPLC methanol. The correlation coefficient for each standard curve exceeded 0.9999. The detection limit, calculated as the concentration corresponding to three times the background noise, was 1.0 for Ascorbic acid, 1.5 for Benzoic acid, and 0.05 for Sorbic acid.

Methods

This study was performed on ten brands of industrial wines, available on the Greek retail market. All wine samples were aged in oak wood barrels for at least twelve months. One wine was prepared at home without the addition of preservatives (Table I: Sample number 9). The sample was prepared with precipitation of proteins and fat by addition of methanol, followed by centrifugation and/or filtration that provided an extract suitable for chromatographic analysis. Methanol extraction is simple and appropriate for routine analysis.

Chromatography was carried out with a Perkin Elmer instrument, using a UV- visible detector 785A model at wavelength 245nm. Column was C18 varian column type XRs 5 - C18S 250 x 4.6 Col. This isocratic method employed (65:35, 0.01M ammonium acetate buffer :Methanol (HPLC grade, Carlo Erba Reagent, UK) as mobile phase at the rate of 1 mL/min. Total volume of injection was 20µl. Total running time was 8 min for each sample after the injection. Pressure was fixated at 214 Bar.

Data Analysis

Primary data analyses and statistical analyses were performed using IBM SPSS Statistics 20.

Results

The new HPLC method was compared with the reference method [AOAC (Association of Official Analytical Chemists)] procedures 963.19 and 971.15. The results of duplicate analyses were equivalent. All results are presented in table I.

Values of organic acids were determined by two methods for each different oak barrel, commercially sold in Greek market.

Table I. Benzoic, sorbic and ascorbic acids in commercial available wines, aged in oak wood barrels.

Samples	Benzoic acid(mg/Kg)		Sorbic acid(mg/Kg)		Ascorbic acid(mg/Kg)	
	HPLC method	Reference method	HPLC method	Reference method	HPLC method	Reference method
1.	455.9	457.0	310.2	312.8	410.5	415.5
2.	1294	1281.0	n.d	n.d	n.d	n.d
3.	413.9	411.8	n.d	n.d	n.d	n.d
4.	439.3	432.1	n.d	n.d	n.d	n.d
5.	750.5	760.5	n.d	n.d	n.d	n.d
6.	489.7	481.3	n.d	n.d	489.7	481.3
7.	509.1	506.2	400.5	405.5	310.1	315.5
8.	n.d	n.d	290.8	295.6	n.d	n.d
9.	n.d	n.d	n.d	n.d	n.d	n.d
10.	650.5	660.4	250.5	260.6	n.d	n.d

Discussion

A good correlation between the two methods: the present method (C) and reference method(R), was obtained for the quantification of benzoic, sorbic and ascorbic acids as apparent from inspection of Table I. For example, a linear relationship between the two methods (C and R) was established for benzoic acid as reflected by regression equation $C=0,993R + 2,693, r^2=0.999$. No significant statistical difference between the two methods was observed (Figure2).

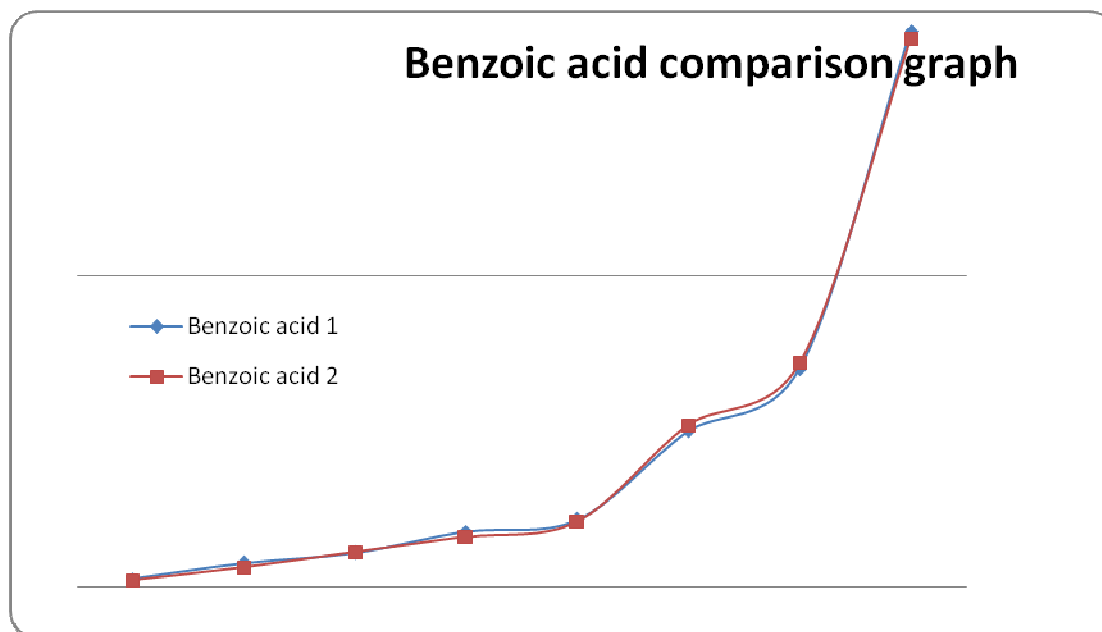


Figure 2. Benzoic acid graph with the values determined of the two methods (benzoic acid 1 by HPLC and benzoic acid 2 by reference method).

We used the non-parametric Wilcoxon-test for related samples to evaluate the organic acids values between the two methods. The p-value is greater than 0.05, so the values of benzoic acid as calculated by HPLC method are not statistically different to the values of benzoic acid were measured by the reference method (Table II).

Table II. Statistical tests.

Test statistics ^a	
	Benzoic acid 2 – Benzoic Acid 1
Z	-,560 ^b
Asymp. Sig. (2-tailed)	,575

a. Wilcoxon Signed Ranks Test; b. Based on positive ranks

The HPLC chromatogram of wine is shown in Figure 3 with the clear peak of benzoic acid at 5.664 min, sorbic acid at 7.862 min and ascorbic acid at 2.62 min.

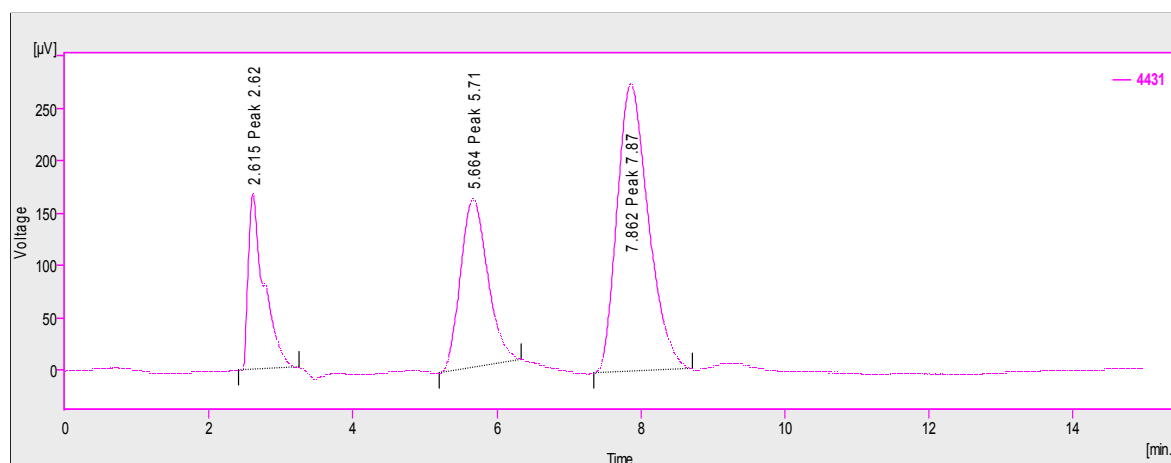


Figure 3. Ascorbic, benzoic and sorbic acids chromatogram.

Also the extraction of phenolic acids, gallotannins, ellagic tannins and volatile substances from oak barrels did not affect the clearness of the chromatogram.

Conclusions

Determining the value of organic acids by method HPLC, we observe that there is no definite trend to increase or decrease the value of organic acids compared with the reference method. The rates of organic acids as determined by both methods are very close. We can conclude that the new proposed here method can be employed without any problems.

Abbreviations Used

HPLC, High pressure liquid chromatography; SA, Sorbic acid; BA benzoic acid; SA, sorbic acid and AA, ascorbic acid.

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Research Interests:

- Artificial aging red wines in different wood types
- Mark spirits applications
- Identification of tree species with genetic-molecular techniques(DNA barcoding)
- Wood extracts (essential oils etc) applications
- Usage - applications of High Pressure Liquid Chromatography and GC-MS

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