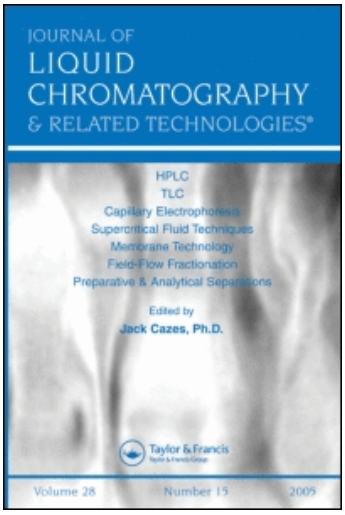

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METHOD DEVELOPMENT FOR THE SIMULTANEOUS DETERMINATION OF CARBOXYLIC ACIDS, PHENOLIC COMPOUNDS, AND SORBIC ACID IN WHITE WINES

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ABSTRACT

A reversed phase liquid chromatographic method was developed for the simultaneous determination of carboxylic acids and phenolics in white wines. The samples, diluted, were injected onto a Spherisorb ODS-2 column with a gradient of sulfuric acid (pH 2.5) / methanol as mobile phase. A diode array detector was used which was set at 210nm for carboxylic acids and altered to 278nm, during the run, for phenolics and sorbic acid. The identification of compounds was based on retention time, co-chromatography and UV spectrum. Some clean-up methods (sep-pak C_{18} and an ion exchange column) were tested and did not improve the results.

The analysis was simple, with no sample preparation. Application of this method was illustrated by analyses of Brazilian Welchriesling wines.

INTRODUCTION

Wines contain a complex mixture of acids which have great significance in their biological stability and sensory properties. The determination of these compounds has importance in enology, in order to control the fermentation process (alcoholic and malolactic). Tartaric and malic acids are originated from the grape and their contents modifies during fermentation and aging. Lactic and acetic acids come from bacterial and yeast metabolism.[1-3] Sorbic acid, a preservative, is permitted in Brazilian wines at levels up to 0.02%.[4] Sensory contributions of phenolic compounds affect mainly flavor (astringency, bitterness), body, and color. The phenolic composition of wine varies with grape variety and ripening.[5-8] It also depends on juice extraction and winemaking techniques, and numerous reactions that take place during aging.[9-10]

High performance liquid chromatography (HPLC) has been used as an efficient technique for identification and quantification of the acids and phenols. Considering that reported methods are costly, time-consuming, and require involved sample preparation to separate every class of compounds, the aim of this work was to develop a method for the simultaneous determination of carboxylic acids, phenolic compounds, and sorbic acid in white wines. The method was applied to various white Brazilian Welchriesling wines.

MATERIALS AND METHODS

Standards and Chemicals

Commercial standards (Sigma Chemical Co) of fructose, acids (tartaric, malic, lactic, acetic, sorbic, galacturonic, glucuronic e shikimic), and phenolics (catechin; gallic, vanillic, p-hydroxybenzoic, protocatechuic, syringic, caffeic, p-coumaric, and ferulic acids) were used. The caftaric acid standard was supplied by Dr. Singleton (Department of Viticulture and Enology - UCLA, Davis). Standards were diluted in the mobile phase. Water was obtained from a Millipore Milli-Q water purification system.

Sample

White Brazilian Welchriesling wines from commercial brands were analyzed. Samples were filtered through a 0.45μm filter (Millipore), adjusted to pH 2.5 and diluted in mobile phase (proportion of 1:9).

Equipment and Chromatographic Conditions

Analyses were carried out in a three-pump gradient HPLC system (Varian Model 9010). The injector was a Rheodyne valve (Mod 7161) with a 20μL sampling loop.

A diode array detector Varian Model 9065 Polychrom connected to a video (300A Amdek) was used. The system was coupled to an integrator (Varian Model 4400) and a recorder (Hewlett-Packard Model 2225 D).

The analysis was performed at room temperature on a reversed phase Spherisorb ODS-2 column (Phase Separations), 4.6 mm x 250 mm i.d., spherical particle (5μm size), preceded by a guard column. Detection was carried out at 210nm (acids) and 278nm (phenolics and sorbic acid). Solutions of sulfuric, phosphoric and formic acids (Merck p.a.) modified by methanol and acetonitrile (Lichrosolv Merck) were tested as mobile phase.

Identification

Each compound was identified by its retention time and UV spectrum. Co-chromatography and purity were also applied.

Hydrolysis was used for caftaric and coutaric acids (caffeic and coumaric acid tartrate), which have no commercial standards.[5,11] A phenolic extract (wine phenolics eluted from sep-pak with methanol and concentrated in a rotavapor evaporator) was treated in different ways in order to hydrolyze tartrates: boiling $H_2SO_4$2N and HCl 2N; NaOH 2N at room temperature. The hydrolyzate pH was adjusted to 2.5 before injection.

Clean-up Procedures

Different methods were applied to separate the sample into neutral and acid fractions.

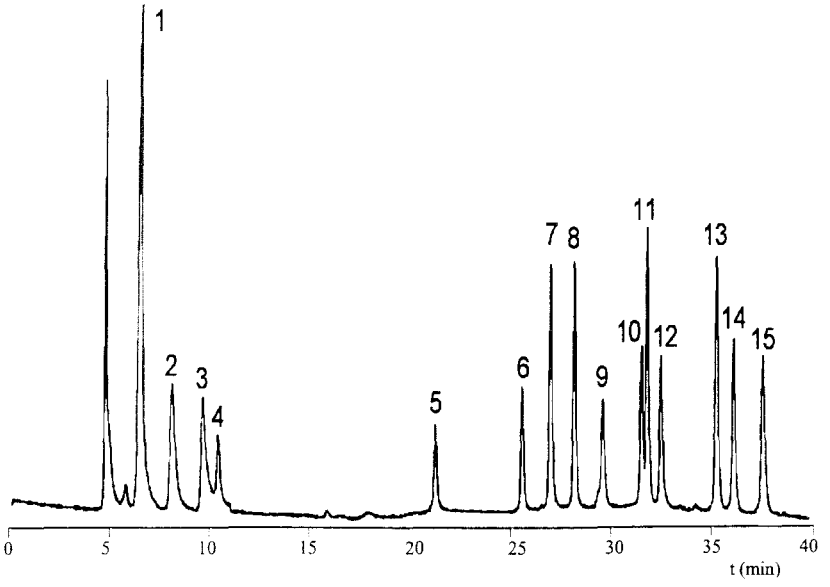

Figure 1. Chromatogram of the Standard Mixture. Peaks: 1= Tartaric acid, 2= Malic acid, 3= Lactic acid, 4= Acetic acid, 5= Gallic acid, 6= Protocatechuic acid, 7= Caftaric acid, 8= Catechin, 9= p-Hydroxybenzoic acid, 10= Vanillic acid, 11= Caffeic acid, 12= Syringic acid, 13= Coumaric acid, 14= Ferulic acid, 15= Sorbic acid. Conditions:Column Spherisorb ODS-2. Detection UV 210nm (up to 10 min) and 278nm. Mobile phase: sulfuric acid (A) / methanol (B). Gradient from B to A: 0 min, 0%, 10 min,; 5%, 20 min, 30%, 30 min, 50%. Flow rate: 0.5mL/min (up to 10 min) and 0.7mL/min.

Fractionation in Sep-Pak C18

Two kinds of fractionation using Sep-Pak C_{18} (Millipore)[12] were applied:

- Absorption of neutral compounds: 1mL of wine previously neutralized (pH adjusted to 8 with NaOH) was passed through the sep-pak (preconditioned by passing 2mL dropwise of methanol and 2mL of deionized water).
- Absorption of acid compounds: 1mL of wine with the pH adjusted to 2.5 with H_2SO_4 was passed through the sep-pak (preconditioned by passing 2 mL dropwise of methanol and 2mL of sulfuric acid solution pH 2.5).

The absorbed fractions were eluted with methanol from their respective cartridges and the first 2 mL were collected for HPLC analysis. In order to test the efficiency this was repeated three times. The effluent fraction of each fractionation was also analyzed.

Fractionation on an ion exchange resin

An ion exchange resin, in the chloride form, was used to fractionate neutral and acid compounds.[13] The resin was activated with methanol before use. A 25 cm mini-column (6mm i.d.) was stoppered with glass wool just above the tap and filled with resin (10cm). The packed column was washed with deionized water.

A 2 mL wine aliquot (neutralized to a pH between 8 and 9), was pipetted onto the resin bed and allowed to run through freely, followed by deionized water to a final volume of 20mL. This effluent might contain a neutral fraction and was collected for HPLC analysis. The acid fraction was desorbed from the resin with sulfuric acid solution (pH 2.5) to a final volume of 15mL and analyzed.

RESULTS AND DISCUSSION

Separation

Figure 1 shows a chromatogram of a standard mixture of organic acids and phenols. The analysis was completed within 40 minutes, requiring an extra 10 to 15 minutes for column re-equilibration. The best resolution was obtained with sulfuric acid solution pH 2.5(A) and methanol (B) and the following gradient from B to A: 0 min, 0%; 10 min, 5%; 20 min, 30%; 30 min, 50%. Flow-rate and detection (UV) were established as 0.5 mL/min and 210 nm (up to 10 min) and 0.7 mL/min and 278 nm (up to the end).

Gradient elution with methanol was applied to several wines. The sample diluted in the mobile phase (1:1) was injected, and the following results were observed:

- elution too close among the organic acids,
- co-elution of other compounds with tartaric acid, probably uronic acids and fructose;
- poor separation between lactic acid, acetic acid and ethanol.
- appearance of unidentified peaks, some of considerable area.

Clean-up Procedures

Clean-up methods for the separation of acid and neutral fractions were tried. The aim was to improve the chromatogram removing neutral compounds (fructose and ethanol), increasing the retention time of the acids and obtaining better separation of tartaric and acetic acids. As a disadvantage, catechin (a neutral phenol), which might be interesting to determine, would be lost. The fractionation on sep-pak and an ion exchange column was not selective for the compounds of interest, according to the described below.

Fractionation on Sep-Pak C18

Two tests employing different Sep-Pak conditioning were carried out:

- To elute the acid compounds (organic and phenolic acids) and absorb neutral ones (neutral phenols, ethanol and sugars): The organic acids eluted, but caftaric and sorbic acids were partially absorbed on the cartridges.
- To elute neutral compounds and absorb acids: The organic acids were not absorbed on sep-pak eluting with the neutral fraction. Acid phenols, sorbic acid and a part of the neutral phenols were absorbed on the cartridges.

Fractionation on an ion exchange column

Organic acids were absorbed onto the resin, but caftaric and sorbic acids were partially eluted with the neutral fraction. In addition, the applied procedure diluted the sample excessively.

Other Tests

Other acid mobile phases (phosphoric and formic acids) were applied and presented lower efficiency than sulfuric acid. Changes of sample pH were tested to improve efficiency of the acid compounds separation, as the literature usually described the use of buffered mobile phases. We prefer not to use buffers to avoid equipment maintenance problems. The best chromatogram was obtained by correcting the pH to 2.5 and adding enough acid mobile phase to dilute the sample ten times.

Gradient elution using acetonitrile, which presented a lower pressure and a better base line, was also tested. No significant improvement in the chromatogram was observed, so it was decided to use methanol because of its

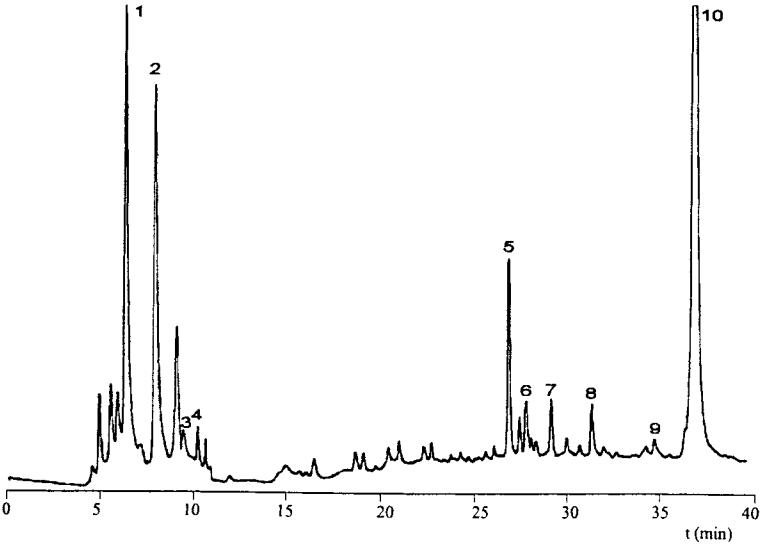

Figure 2. Chromatogram of a White Brazilian Welchriesling wine. Peaks: 1= Tartaric acid, 2= Malic acid, 3= Lactic acid, 4= Acetic acid, 5= Caftaric acid, 6= Catechin, 7= Coutaric acid, 8= Caffeic acid, 9= Coumaric acid, 10= Sorbic acid. Conditions: Column Spherisorb ODS-2. Detection UV 210nm (up to 10 min) and 278nm. Mobile phase: sulfuric acid (A) / methanol (B). Gradient from B to A: 0 min, 0%, 10 min, 5%, 20 min, 30%, 30 min, 50%. Flow rate: 0.5mL/min (up to 10 min) and 0.7mL/min.

lower cost and toxicity. The chromatogram of a typical white Brazilian Welchriesling wine, obtained under the optimum conditions, can be seen in Figure 2. Compounds which appear in greater concentration are identified on the chromatogram.

Identification

The peak, with a retention time of about 9 minutes (Figure 2), was identified as shikimic acid by co-chromatography, UV spectrum and purity. The literature relates that this acid, despite being present in wine in very low concentrations, can appear on chromatograms due to its high UV absorption, about 20 times greater than tartaric acid. By the use of spiking, it was observed that galacturonic and glucuronic acids and fructose eluted very close to each other and to tartaric acid (Figure 2, retention times between 4 and 6 minutes).

Coumaric acid tartrate, which has no commercial standard, was identified by its UV spectrum as the peak with a retention time of about 29 minutes (Figure 2). Hydrolysis was applied to confirm its identity. Acid treatment did not produce good results. Several concentrations and time reactions were carried out but it was not possible to hydrolyze the esters and also by-products appeared. Basic treatment (for 0, 15, 30, 60 e 120 minutes) allowed one to observe a constant decrease in peak ester areas and a proportional increase in the coumaric acid peak, which confirmed its identity.

Hydrolysis also allowed the confirmation of caftaric acid previously identified by spiking with a non commercial standard.

Repeatibility

Table 1 shows the retention time of the compounds identified in Figure 2. For samples of Welchriesling wine it was observed that the retention times were kept very constants for the compounds analyzed (CV between 0.7 and 1.7%) and there was no interaction among then. Similar variations were reported in the literature for organic acids (0.6 to 1.4%) and phenols (catechin, caftaric and coutaric acids) (1 to 2%) (7).

Considering the great number of compounds observed and the relative small differences between their retention times, we recommend the application of this method with the use of a diode array detector or other confirmation method.

In order to verify the repeatibility of the method, analyses were done using standards during a day (n=5) and in alternated days (n=20). The values of CV observed were acceptable and close to the reported in the literature. Studding the repeatibility between runs during a day, it was noticed CV lower than 2% for all phenols and sorbic, tartaric and malic acids. Lactic and acetic acids, which have the worst separation in the chromatogram, presented higher CV (close to 2.5%). Considering the repeatibility between days, it was determinated CV about 4% for lactic and acetic acids, and lower than 3% for the other compounds.

The literature relates better repeatibility (CV about 1%) for standards of tartaric, malic, and lactic acids.[14] Other authors reported higher values for analyses of tartaric acid (CV of 5%),[15] and catechin (CV of 2.9%).[16] Some workers using samples (red wines) reported CV, during a day (n=6), of 3.7 and 5.3% for malic and lactic acids, respectively. The variation between days (n=10) was about 3%, for malic, lactic, and acetic, and 7% for tartaric acid.[17]

Table 1

Retention Time of the Compounds in White Brazilian Welchriesling Wine

Compounds	Retention Time (Min)* Average ± SD	Range
Tartaric Acid	6.26 ± 0.11	6.60 - 6.43
Malic Acid	7.83 ± 0.12	7.61 - 8.01
Lactic Acid	9.32 ± 0.14	9.08 - 9.55
Acetic Acid	10.06 ± 0.14	9.84 - 10.28
Caftaric Acid	26.40 ± 0.29	26.10 - 26.97
Catechin	27.49 ± 0.23	27.07 - 27.90
Coutaric Acid	28.74 ± 0.26	28.38 - 29.23
Caffeic Acid	31.14 ± 0.28	30.62 - 31.53
Coumaric Acid	34.55 ± 0.26	34.05 - 35.07
Sorbic Acid	36.75 ± 0.27	36.30 - 37.20

* For 90 runs, except for catechin (84) and sorbic acid (62) which were not detected in all samples.

The literature usually restricts the use of an acid mobile phase with reversed phase, but just a few authors discuss this. Changes in a column Hypersil SAS using a pH lower than 3.0 were observed, but were not noticed with a LiChrosorb RP-8 and a Spherisorb Hexyl even pH 2.0.[18] Other authors[2,19] using low pH (2.1 and 1.5), related that there was no column alteration after several analyses.

During the experimental work, no problem on column and equipment by the use of an acid mobile phase occured. After all the analyses, the column was compared with a similar unused one (same model and brand) and there was no observed difference in the efficiency.

CONCLUSIONS

The method proposed is simpler and faster than those described in the literature, with no sample preparation, so it may be used routinely. Some clean-up procedures (sep-pak C18 and an ion exchange column) were tested to remove interferents but did not improve the results, under the conditions used.

The developed method allowed one to analyze, simultaneously, organic acids and phenols, decreasing the time and cost of the analysis. The results indicated the potential of this HPLC method for use in quality control and as a research tool in the wine industry.

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