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Determination of Organic Acids in White Wines by RP-HPLC

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Summary

The chromatographic conditions for the optimal separation of tartaric, malic and citric acids on a LiChrosorb RP-18 column (10 μm, 25 cm x 4.0 mm i.d.) at 210 nm were determined. The optimized RP-HPLC method (mobile phase: $c(H_3PO_4) = 6 \cdot 10^{-3}$ mol/L, pH = 2.1, flow rate 1.0 mL/min) was validated. Calibration curves were linear for all three acids in the concentration range tested; r^2 was better than 0.999. RSDs for tartaric and malic acids were within ± 2 %, and for citric acid ± 10.4 %. The average relative error for tartaric acid was 3.2 %, for malic acid 2.5 % and for citric acid 6.0 %. Ethanol caused an insignificant negative response at t_R = 5.69 min, whereas glucose and fructose eluted in the void volume. According to the validation results, and from analysis of wine samples, the described HPLC method was found adequate for routine determination of tartaric and malic acids and to some extent also of citric acid in dry, semi-dry, semi-sweet and sweet white wines.

Key words: organic acids, RP-HPLC analysis, white wine

Introduction

Quantitative determination of organic acids can be an additional support to sensorial and microbiological quality assessment of wines (*1*). The most widely used HPLC methods for their determination are ion exchange, (*2*,*3*) and ion exclusion (*4*) HPLC techniques. Today, the reversed phase HPLC methods are very popular in general (*5*), but not for organic acids determination in wine and must samples.

Our aim was to introduce LiChrosorb RP-18 as a stationary phase for routine and inexpensive HPLC determination of tartaric and malic acids in wines. These two acids are present in grapes in much higher concentrations than other acids. Their ratio is also an indicator of vintage quality (*6*). We found that with RP-HPLC on LiChrosorb RP-18 six organic acids (*i.e.* galacturonic, tartaric, malic, lactic, succinic and citric acids) can be separated. The chromatographic conditions for the optimal separation of the organic acids were established, and the method was validated. The results of selectivity, linearity, precision and accuracy are presented here. Due to some interferences, the RP-HPLC method described was found adequate only for the routine determination of tartaric and malic acids and to some extent also of citric acid in dry, semi-dry, semi-sweet and sweet white wines. The method was used to determine tartaric and malic acid concentrations in 28 white wine samples (vintage 1995) from three major wine-producing regions in Slovenia. Wine samples were of different quality and also varied in ethanol and sugar contents.

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Materials and Methods

Chemicals

All acids and reagents used were of analytical grade. Organic acids (p.a.) were from Merck, Germany.

Solvent

In preparation of wine samples and standard solutions a mixture of 96 % ethanol and double distilled water (volume ratio 10/90) was used, which is referred to as the solvent. Prior to use, the solvent was sonicated for 5 minutes in an ultrasonic bath to remove air bubbles.

Standard solutions of organic acids

All organic acids used for standards were dissolved in the solvent to simulate the matrix effect of wine samples. The concentrations of organic acids varied from 0.5 to 10.0 g/L for tartaric acid; 0.2 to 15.0 g/L for malic acid; 0.1 to 5.4 g/L for lactic acid, and 0.05 to 1.0 g/L for citric acid. The prepared standard solutions of organic acids were stored at 4 °C.

Standard solutions of sugars

Two standard sugar solutions were prepared. The first one contained γ(glucose) = 100 g/L, and the second one γ(fructose) = 100 g/L of the solvent.

Wine samples

The samples were provided from local wineries. Samples of 28 Slovenian white wines of vintage 1995 (see Table 4) were tested. They differed in quality, sugar concentration and provenience. Ethanol volume fraction varied from 10.1 to 12.6 %, according to the producers. An aliquot of wine sample was diluted (volume ratio 1/1) with solvent and 20 μL of the obtained solution were injected. Before injection, all standards and sample solutions were filtered through Sartorius RC15 membrane filter units.

Spiked wine samples

For precision and accuracy validation, wine samples were spiked with organic acids to such an amount that the final concentration of the added acid varied from 1 to 3 g/L for tartaric and malic acids, and from 0.09 to 0.27 g/L for citric acid. Organic acid standards were accurately weighed in 50 mL volumetric flasks and dissolved in about 10 mL of the solvent. Then 25.0 mL of wine sample was added and the solution obtained was further diluted to 50 mL with the solvent.

HPLC system

This comprised an X-act 4-channel degassing unit, (Jour Research, Sweden), a Maxi Star, K1000 HPLC pump (Knauer, Germany), a Marathon-XT autosampler (Spark-Holland, Holland), a UV/VIS detector (Knauer, Germany), a K-2301 RI detector (Knauer, Germany) and a ValueChrom data acquisition system (Bio-Rad, USA).

Chromatographic conditions for determination of organic acids

A LiChrosorb RP-18 column (10 μm, 25 cm x 4.0 mm i.d.), (Merck, Germany), with an injection volume of 20 μL, wavelength 210 nm, and a mobile phase as below, flow rate 1.0 mL/min was employed.

For optimization of the separation of organic acids, aqueous solutions of H_3PO_4 in three different concentrations were tested: mobile phase 1 = $3.0 \cdot 10^{-4}$ mol/L (pH = 3.0), mobile phase 2 = $1.5 \cdot 10^{-3}$ mol/L (pH = 2.5), mobile phase 3 = $6 \cdot 10^{-3}$ mol/L (pH = 2.1).

Chromatographic conditions for glucose and fructose determination

Before determination of organic acids, glucose and fructose were determined on a Bio-Rad Aminex HPX--87C (30 cm x 7.8 mm i.d.) column at 80 °C using an RI detector. Double distilled water was used as the mobile phase, with an injection volume of 20 μL, and a flow rate of 0.6 mL/min (7).

Results

Influence of pH of the mobile phase

Separation of the organic acids on an HPLC LiChrosorb RP-18 (10 μm, 25 cm x 4.0 mm i.d.) column was tested with three H_3PO_4 solutions. Mobile phase 3 $c(H_3PO_4) = 6 \cdot 10^{-3}$ mol/L was the best mobile phase for HPLC separation of the organic acids tested as shown for 4 of them in Fig. 1. Although the pH of the mobile phase was 2.1, no column deterioration was observed even after prolonged use.

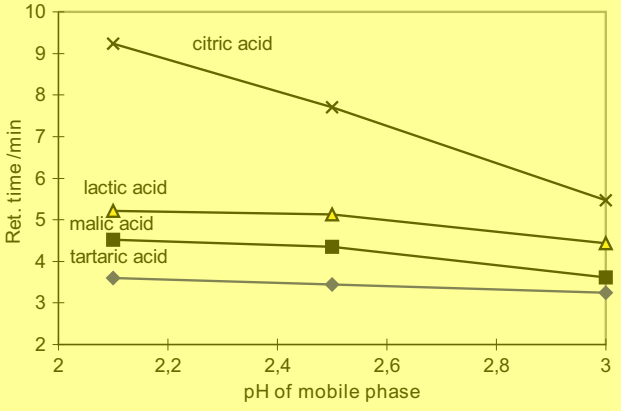

Fig. 1. Influence of the mobile phase pH on the separation of organic acids, LiChrosorb RP-18 column (10 μm, 25 cm x 4.0 mm i.d.), UV detection at 210 nm

Selectivity of the method

Under the conditions described galacturonic, tartaric, malic, lactic, succinic and citric acids could be separated on a LiChrosorb RP-18 (10 μm, 25 cm x 4.0 mm i.d.) column (Fig. 2). The peaks of all acids were symmetrical and well separated, but the chromatogram in Fig. 2 shows 7 peaks. It was found that two peaks (t_R = 9.35 and 10.63 min) belong to succinic acid. We cannot

explain the reason for such behaviour. Succinic acid has $pK_1 = 4.16$ and $pK_2 = 5.61$ in aqueous solution, but these do not explain the occurrence of two peaks in a mobile phase with pH = 2.1. It is unclear why two peaks appear only in the case of succinic and not in the case of any other polycarboxylic acid. When the same standard solution of succinic acid was injected on a Bio-Rad Aminex HPX-87H column, only one peak was observed (unpublished results).

With the optimal mobile phase we were able to separate 6 organic acids (Fig. 2), but when validating the method, we found it suitable only for the 3 most representative (tartaric, malic and citric) acids in white wines.

Possible interference of ethanol, glucose and fructose on the determination of the acids was checked by separate injection of 20 μL of ethanol, glucose and fructose standard solutions. Both sugars were dissolved in ethanol. Ethanol did not interfere with the determination of organic acids. Its elution at 5.69 min caused a very small, but negative response under the chromatographic conditions described. Glucose and fructose eluted in the void volume with $t_R = 3.10$ min.

The influence of shikimic and acetic acids on the determination of the main organic acids in wine was checked too. When a mixture of shikimic, lactic and acetic acids was injected, the separation of shikimic acid

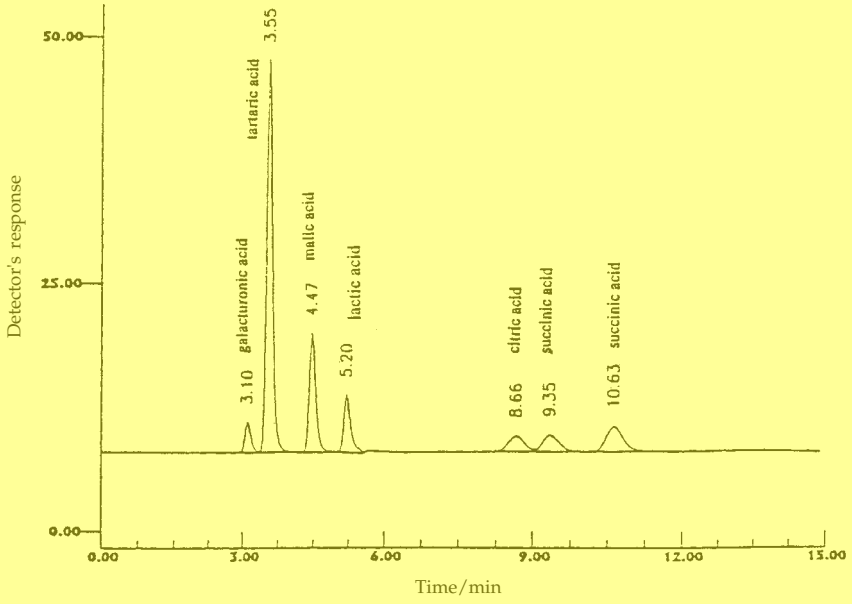

Fig. 2. HPLC separation of galacturonic, tartaric, malic, lactic, succinic and citric acids on LiChrosorb RP-18 (10 μm, 25 cm x 4.0 mm i.d.) with $c(H_3PO_4) = 6 \cdot 10^{-3}$ mol/L (pH=2.1), UV detection at 210 nm

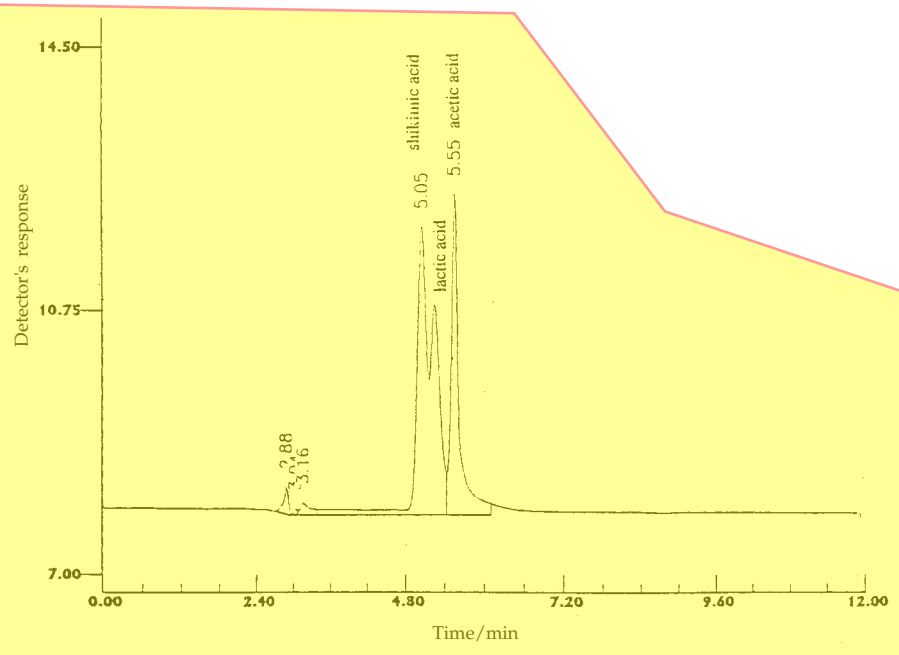

Fig. 3. Separation of shikimic, lactic and acetic acids on RP-HPLC column under the same conditions as in Fig. 2

from lactic acid was poor (Fig. 3). The resolution between shikimic and lactic acid was only 0.5, and the resolution between lactic and acetic acid was 1.3. Shikimic acid eluted at 5.05 min under the chromatographic conditions used. Usually, the concentration of shikimic acid in wines is low, but this acid has a much higher extinction coefficient (*8*) than the other organic acids present in wine. Therefore, this RP-HPLC method is not selective for the determination of lactic acid.

Linearity of the method

The linearity of the method was validated at six to eight concentrations of each acid (tartaric, malic and citric acids). The concentrations of the standard solutions of organic acids were chosen in such a way that the whole expected concentration range of each acid in the samples was covered. A calibration curve for each organic acid was constructed by linear regression of the observed average peak area versus concentration. The coefficients of the regression curves (the slope and the intercept on the y axis) and the squares of the correlation coefficients (r^2) were calculated by the least squares method. Calibration curves were linear for all the organic acids investigated (Table 1).

Table 1. Coefficients of the regression curve and the square of the correlation coefficient for each organic acid; HPLC analysis: LiChrosorb RP-18 column (10 μm, 25 cm x 4.0 mm i.d.), mobile phase $c(H_3PO_4) = 6 \cdot 10^{-3}$ mol/L, UV detection at 210 nm

Organic acid	γ(acid)range g/L	Slope	Intercept	r^2
Tartaric	0.500– 7.508	236.35	14.074	0.9998
Malic	0.200–15.000	128.62	4.621	0.9998
Citric	0.049– 0.987	1685.6	–3.124	1.0000

Precision of the method

The precision of the method was determined by consecutive injections of blank wine samples and wine samples spiked with different concentrations of tartaric, malic and citric acids. For each concentration, the average area of the detector response, the standard deviation and the relative standard deviation (RSD) were calculated (Table 2).

The precision validation indicated that this HPLC method is suitable for tartaric and malic acid determination in white wines under the chromatographic conditions described (Table 2). The precision of the citric acid determination, on the contrary, shows that this method is not suitable for its quantitative determination in white wines. The main reason for such low precision of the citric acid determination is the low concentration of this acid in wines. Citric acid in wine can be quantified by this RP-HPLC method with a precision of only about 10 %.

Table 2. Precision of tartaric, malic and citric acid determination in wine samples on LiChrosorb RP-18 column; chromatographic conditions as in Table 1

Tartaric acid		
γ(spiked) g/L	γ(total) g/L	RSD (N=6) %
0	1.9	1.04
1.0	2.9	0.41
2.0	3.9	0.33
3.0	4.9	0.25
	average	0.51
Malic acid		
γ(spiked) g/L	γ(total) g/L	RSD (N=6) %
0	2.9	2.16
1.0	3.9	1.25
2.0	4.9	0.36
3.0	5.9	0.37
	average	1.03
Citric acid		
γ(spiked) g/L	γ(total) g/L)	RSD (N=6) %
	0.24	15.04
0	0.33	12.91
0.09	0.42	7.30
0.18	0.51	6.41
0.27	average	10.41

Table 3. Accuracy of tartaric, malic and citric acid determination in wine samples on LiChrosorb RP-18 column; chromatographic conditions as in Table 1

Accuracy of tartaric acid determination		
γ(added) (true value) g/L	γ(found) (measured value) g/L	Average RE (N=6) %
1.017	0.966	–5.0
2.012	1.961	–2.6
3.019	2.954	–2.1
	average RE for tartaric acid (%)	–3.2
Accuracy of malic acid determination		
γ(added) (true value) g/L	γ(found) (measured value) g/L	Average RE (N=6) %
1.009	0.988	–2.1
2.013	1.967	–2.3
3.022	2.930	–3.0
	average RE for malic acid (%)	–2.5
Accuracy of citric acid determination		
γ(added) (true value) g/L	γ(found) (measured value) g/L	Average RE (N=6) %
0.093	0.088	–5.4
0.185	0.182	–1.6
0.275	0.245	–10.9
	average RE for citric acid (%)	–6.0

Table 4. Mass concentration of glucose, fructose, tartaric acid and malic acid in white wines (1995 vintage, different wine-producing regions in Slovenia); chromatographic conditions as in Table 1

Wine sample	Wine-producing region	γ(glucose)	γ(fructose)	γ(tartaric acid)	γ(malic acid)
		g/L	g/L	g/L	g/L
Belokranjec	Posavje	0.6	0.6	2.58	3.68
Chardonnay – a	Podravje	0.3	4.2	1.67	2.88
Chardonnay – b	Podravje	n.d.	n.d.	0.95	6.07
Furmint	Podravje	1.0	1.1	1.04	2.99
Golden Ribolla	Primorje	0.2	1.3	1.89	2.88
Malvasia	Primorje	<0.1	0.5	1.41	1.77
Mueller-Thurgau	Podravje	3.9	4.2	1.22	4.06
Muškat otonel	Podravje	1.2	12.4	1.10	3.59
Pinela	Primorje	<0.1	2.3	1.54	3.04
Pinot blanc – c	Podravje	3.9	4.3	0.95	5.52
Pinot blanc – d	Podravje	2.8	14.1	1.43	2.72
Pinot gris	Podravje	10.4	10.3	0.95	5.68
Radgonska ranina	Podravje	8.5	9.0	1.57	3.81
Rhine Riesling – e	Podravje	5.8	6.1	1.61	3.15
Rhine Riesling – c	Podravje	6.4	6.4	1.39	3.27
Rhine Riesling – b	Podravje	n.d.	n.d.	2.25	3.71
Ribolla	Primorje	1.2	1.9	1.48	3.18
Sauvignon – f	Podravje	4.7	4.1	2.10	4.67
Sauvignon – c	Podravje	4.0	5.6	1.40	4.85
Sauvignon	Posavje	4.2	4.7	1.78	3.63
Sylvaner verde	Podravje	4.7	4.7	1.77	3.61
Tokay	Primorje	0.3	0.5	2.07	2.47
Traminer	Podravje	28.8	72.6	1.55	2.72
Vrtovčan	Primorje	0.8	2.0	1.52	2.47
Welsch Riesling – a	Podravje	<0.1	14.5	2.52	2.56
Welsch Riesling – c	Podravje	6.7	5.9	1.20	2.72
White Muskat	Podravje	8.0	5.5	1.90	5.37
Zelen	Primorje	1.4	8.5	1.71	3.15

a-f = different microlocation, n.d. = not determined

Accuracy of the method

The accuracy of the method was measured as the agreement between the measured and the true value (found concentration and added concentration). Since for wine samples, the true value was not known, an approximation was obtained based on spiking a wine sample with known amounts of tartaric, malic and citric acids. A wine sample was spiked with three different concentrations of tartaric, malic and citric acids (added concentrations). The found concentration, γ(g/L), (measured value) of each acid at each concentration was calculated by the method of external standards as follows:

$$\gamma = (A_{spiked} - A_{blank}) \cdot \gamma_{std} / A_{std}$$

A_{spiked} – detector's response of spiked sample

A_{blank} – detector's response of blank sample

γ_{std} – concentration of standard solution, g/L

A_{std} – detector's response of standard

By comparing the found concentrations to the added concentrations, the relative error (RE, %) was calculated for the determination of each acid (Table 3).

Similarly as in the case of precision, the best accuracy was found for tartaric and malic acids, the average relative errors being 3.2 % and 2.5 %, respectively, while the average relative error in the determination of citric acid was 6.0 %.

Wine analysis

The described HPLC method was finally used on 28 white wines (Fig. 4) from different wine-producing regions in Slovenia (different sugar contents, all 1995 vintage). The results of the glucose and fructose determinations (analyses were performed as described under Materials and Methods), as well as those of tartaric and malic acid determinations are presented in Table 4.

Conclusions

The described RP-HPLC method using LiChrosorb RP-18 (10 μm, 25 cm x 4.0 mm i.d.) with $c(H_3PO_4) = 6 \cdot 10^{-3}$ mol/L (pH=2.1) as mobile phase and UV detection at 210 nm is fast, all acids eluting in less than 9 min. When analysing different types of white wine no additional unknown interference appeared. According

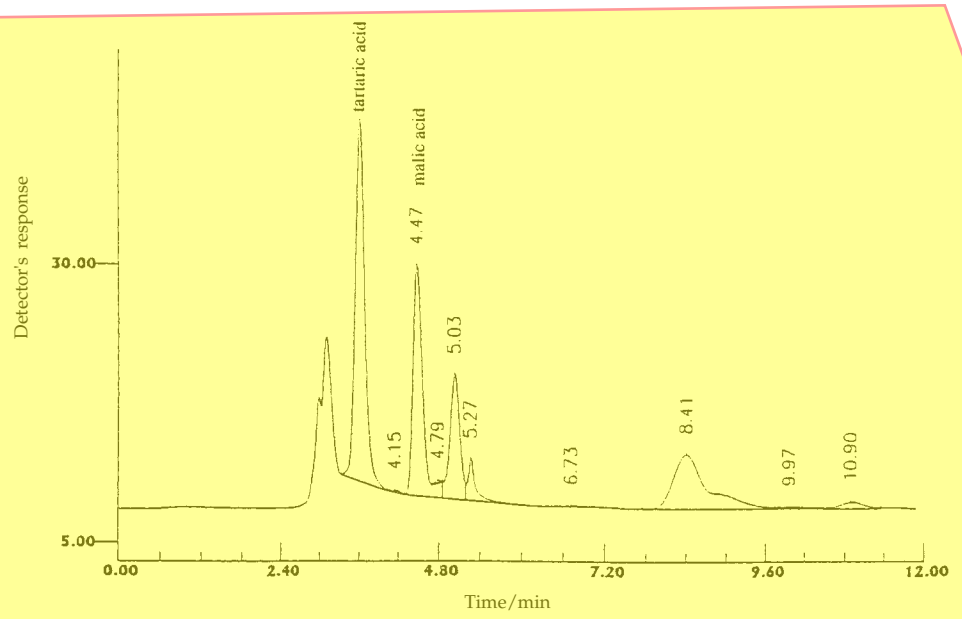

Fig. 4. Separation of organic acids in a sample of sweet wine (Traminer, 1995, Podravje) on LiChrosorb RP-18 under the same conditions as in Fig. 2

Table 5. Comparison of different HPLC methods

Substance analysed	HPLC method used	Reference cited	Linearity r	Recovery %	Precision RSD/%
tartaric acid	Cation exchange HPLC	Frayne, 1986 *(2)*	1.00	101.3 – 103.7	1.1
tartaric acid	Ion-exclusion HPLC	Lopez-Tamames *et al.*, 1996 *(1)*	0.9999	101.4 ± 1.3	2.64
tartaric acid	RP-HPLC on LiChrosorb RP-18	this work	0.9999	96.8	0.51
malic acid	Cation exchange HPLC	Frayne, 1986 *(2)*	1.00	100.5 – 101.4	0.7
malic acid	Ion-exclusion HPLC	Lopez-Tamames *et al.*, 1996	0.9999	99.8 ± 3.3	1.50
malic acid	RP-HPLC on LiChrosorb RP-18	this work	0.9999	97.5	1.03
citric acid	Cation exchange HPLC	Frayne, 1986 *(2)*	0.98		1.5
citric acid	Ion-exclusion HPLC	Lopez-Tamames *et al.*, 1996 *(1)*	0.9996	99.8 ± 5.8	2.85
citric acid	RP-HPLC on LiChrosorb RP-18	this work	1.0000	94.0	10.41

to the validation results and from the analysis of different wine samples, the HPLC method described was found adequate for routine determination of tartaric and malic acids in dry, semi-dry, semi-sweet and sweet white wines. To a limited extent the method can also be considered adequate for routine determination of citric acid in various white wines.

The results of the validation were compared to the results of other authors (Table 5, *1,2*). The linearity of the HPLC methods compared is similar and adequate. Accuracy expressed as recovery shows that on LiChrosorb RP-18 a lower amount of the acids is determined, but the found values are still within the reliability interval of the methods to which our results are compared. The precision of our method for tartaric and malic acid determination is better or comparable to the precision of other HPLC method, while the precision for the citric acid is rather worse.

The main disadvantage of the method presented is the fact that not all organic acids of potential interest can be determined and that succinic acid has two peaks. On the other hand, it offers good routine quantitative determination of the two most important organic acids in white wines, as well as fast and simple isocratic separation on an inexpensive stationary phase with an unsophisticated HPLC system.

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