OPTIMIZATION OF ANALYSIS CONDITIONS FOR ORGANIC ACIDS IN WINE BY HPLC METHOD OF ANALYSIS

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INTRODUCTION

This article presents the results of study of eluent composition influence on the efficiency of organic acids separation in the wine analysis by HPLC method. For this purpose was used modified column by *Macherey-Nagel* – Nucleodur C18 Pyramid. All investigations were carried out in the same conditions, except of the eluent composition.

1. METHODS AND MATERIALS

For searching of optimal conditions for HPLC analysis of organic acids in wine there was used immobile phase, such as modified C18 type. Thus NUCLEODUR C18 Pyramid (*Macherey-Nagel*) is a silica phase with hydrophilic endcapping, designed especially for use in eluent systems of up to 100 % water. It has the next technical characteristics: 250x4,0 mm; special phase with polar endcapping;

pore size 100Å; particle sizes 5 μm; pH stability 1–9 [1].

For searching of separating possibilities of this phase was used liquid chromatograph LC-20AD by *Shimadzu* with spectrophotometer SPD-20AV on the wave length 210nm.

All investigations were carried out in the following conditions: oven temperature 35°C, flow rate – 0,5ml/min.

2. RESULTS AND DISCUSSION

For evaluation of studied chromatographic systems effectiveness there were done analysis of standard mixture of the main organic acids (tartaric acid, L-malic, lactic, acetic, citric, succinic acids) [2,3,4] and sample of a real wine five times diluted.

Chromatograms of standard solution obtained in conditions described above are shown on the fig.1. The main characteristics of the peaks are indicated in Tab.1.
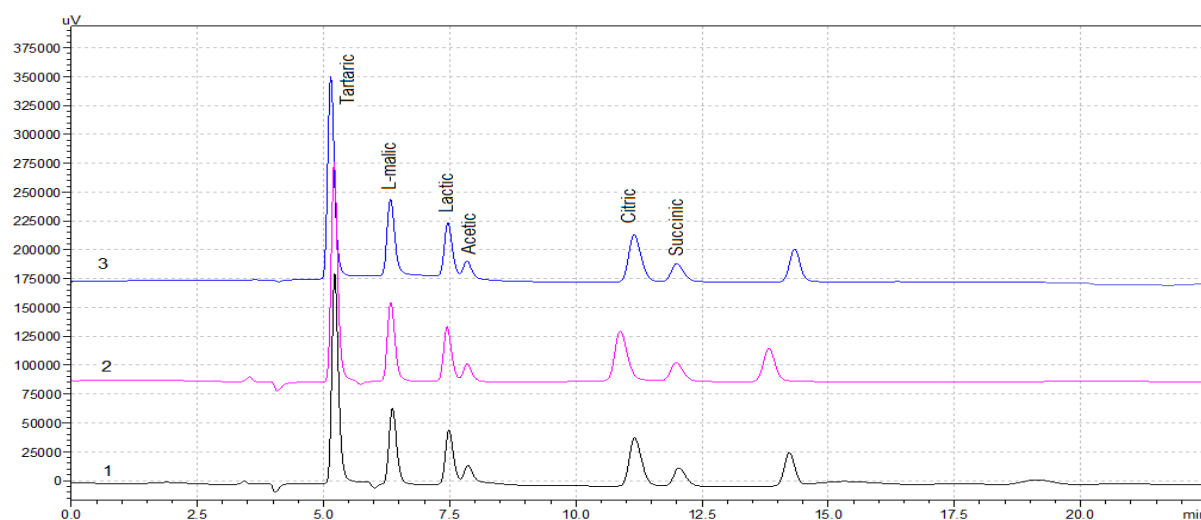
Figure 1. Chromatograms of organic acids standard solution, obtained in the following conditions: chromatographic column Nucleodur C18 Pyramid, 250 x 4,0mm (*Macherey-Nagel*), oven temperature 35°C, flow rate 0,5ml/min, detection on 210nm, eluents: *1* – 0,2% H_3PO_4, *2* – 5mM H_2SO_4, *3* – 12,5mM H_2SO_4.

For the first HPLC system 0.2% solution of H_3PO_4 was used as eluent with flow rate 0.5ml/min and

column oven temperature 35°C with detection on the 210nm wave length. For the second system –

5mM H$_2$SO$_4$ solution with the same other conditions. And the third system had 12,5mM H$_2$SO$_4$ solution as the eluent.

Table 1. Characteristics of the main wine acids peaks obtained by results of separation of organic acids standard solution in the following conditions: chromatographic column Nucleodur C18 Pyramid, 250 x 4,0mm (*Macherey-Nagel*), oven temperature 35°C, flow rate 0,5ml/min, detection on 210nm, eluents: *1* – 0,2% H$_3$PO$_4$, *2* – 5mM H$_2$SO$_4$, *3* – 12,5mM H$_2$SO$_4$.

Acid	Retention time, min			Height of the peak			Area of the peak			Width at a half height of the peak		
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
Tartaric	5,21	5,21	5,14	179140	188543	172936	1641805	1682365	1595811	0,137	0,134	0,145
L-malic	6,36	6,33	6,32	64341	67423	64634	665231	686724	668622	0,162	0,154	0,156
Lactic	7,51	7,44	7,46	45977	46393	46423	521052	505326	515175	0,162	0,173	0,177
Acetic	7,89	7,84	7,83	14800	13767	156677	172323	148015	14255	0,162	0,154	0,167
Citric	11,23	10,87	11,15	40419	41848	39756	655063	689254	655115	0,263	0,261	0,260
Succinic	12,14	11,98	11,99	14670	15318	15226	231841	245371	245649	0,250	0,216	0,260

For study of influence of a complex organic matrix on the chromatographic signal was analyzed a real sample of a white dry wine.

Chromatograms of the real sample of wine are shown on the fig.2.
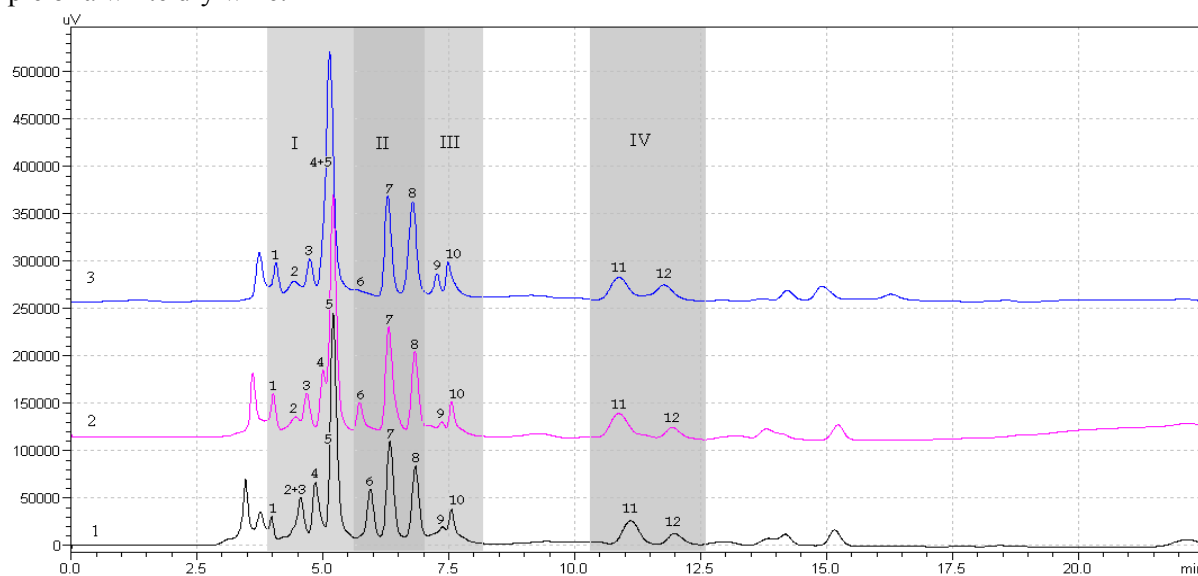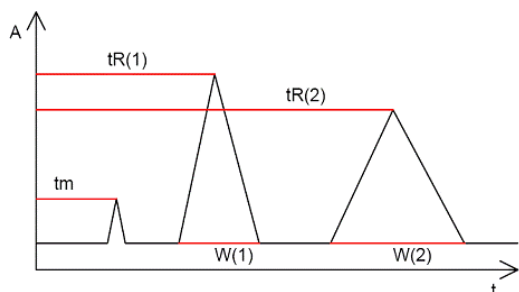

Figure 2. Chromatograms of white dry wine (with dilution factor equal to five), obtained in the following conditions: chromatographic column Nucleodur C18 Pyramid, 250 x 4,0mm (*Macherey-Nagel*), oven temperature 35°C, flow rate 0,5ml/min, detection on 210nm, eluents: *1* – 0,2% H$_3$PO$_4$, *2* – 5mM H$_2$SO$_4$, *3* – 12,5mM H$_2$SO$_4$.

Dividing obtained chromatograms, shown on the fig.2, into the four sectors we get zones with twelve important peaks, which will be discuss more detail. Thus, considering the first sector it can be notice that, moving from the first to the third chromatogram – the fifth peak "absorbed" the forth one, transforming into a fronting peak. When the fifth peak is the significant for analysis – it's a signal of tartaric acid.

In the second sector there can be sighted decreasing of the sixth peak height moving from the first to the third chromatogram. Obviously, this peak contributes to tailing fifth peak (tartaric acid). It can be concluded that the peak of the tartaric acid can not be correct quantified in the chromatographic conditions using 12,5mM H$_2$SO$_4$ solution as eluent. An important signal in the second sector is the seventh one – the signal of L-malic acid.

The pair of the ninth and tenth peaks (lactic and acetic acids respectively) have close retention times in all of the described chromatographic conditions (third sector, fig.2). Calculated resolution factors Rs (*1*) (fig.3) for this couple of peaks obtained in each of the studied conditions are indicated in Tab.2.

Figure 3. Calculation scheme of resolution factor for two vicinal chromatographic peaks.

$$Rs = \frac{2 * (t_{R1} - t_{R2})}{w_1 + w_2} \qquad (1)$$

If the difference in retention times of two peaks is relatively large, and the width of the bases ($\omega_1 + \omega_2$) is small, then the resolution of the peaks is good. The two substances can be identified if their Rs=0,5. For a satisfactory separation the Rs factor should be equal to 1,0 [5].

Table 2. Resolution factors for peaks of lactic and acetic acids, obtained in the following conditions: column – Nucleodur C18 Pyramid, 250 x 4,0mm (*Macherey-Nagel*), oven temperature 35ºC, flow rate 0,5ml/min, detection on 210nm.

Eluent type	Rs
0,2% H_3PO_4	1,06
5mM H_2SO_4	1,05
12,5mM H_2SO_4	1,02

In spite of the bigger value of Rs factor in the first case (with eluent 0,2% H_3PO_4) signals of mentioned indexes (lactic and acetic acids) are more significant in the case with less Rs (with eluent 12,5mM H_2SO_4).

In the fourth sector important signals of citric and succinic acids (eleventh and twelfth peaks) not differ significantly in all three cases of chromatographic conditions.

Not less important for analysis is the index of Relative Standard Deviation (in our case %RSD). In probability theory and statistics, the relative standard deviation (RSD or %RSD) is the absolute value of the coefficient of variation. It is often expressed as a percentage. It is useful for comparing the uncertainty between different measurements of varying absolute magnitude.

Relative Standard Deviation can be calculated with the following formulas [6]:

$$s - \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2} \qquad (2)$$

$$RSD = \frac{s}{\bar{x}} \qquad (3)$$

where:
s – sample standard deviation;
RSD – relative standard deviation;
$x_1, ..., x_N$ – the sample data set;
$\bar{x}$, – mean value of the sample data set;
N – size of the sample data set.

Relative standard deviation of ten measures of analyzed white dry wine with every eluent is shown in Table 3, 4. The less value of %RSD of tartaric acid peak's area in the third case (when 12.5mM H_2SO_4 is used as eluent) probably is a result of fusion of chromatographic peaks presented in complex matrix of the wine (fig.2) and consequently increasing of the peak's area. Thus choice of the "best" eleuent by %RSD$_{peak's\ area}$ is false in this case.

Table 3. Relative Standard Deviation (%RSD) of organic acids peaks' areas (obtained by HPLC method of analysis) contained in real sample of wine. Chromatographic conditions: column – Nucleodur C18 Pyramid, 250 x 4,0mm (*Macherey-Nagel*), oven temperature 35ºC, flow rate 0,5ml/min, detection on 210nm. Number of measures is ten.

Organic acid	%RSD (peak's area)		
	0,2% H_3PO_4	5mM H_2SO_4	12,5mM H_2SO_4
Tartaric	2,16	1,27	1,22
L-malic	2,25	4,35	0,39
Lactic	6,35	2,95	5,29
Acetic	7,88	4,60	12,85
Citric	3,66	2,68	5,83
Succinic	4,36	3,72	12,72

In addition % RSD$_{peak's\ area}$ of acetic acid is the biggest when 12.5mM H_2SO_4 solution was used as eluent, what says about error of measure more than in other cases in spite of the more intensive signal. %RSD$_{peak's\ area}$ of succinic acid in the same conditions is more than 10% probably due to the peak's broadening. The cause of this fact can be poor column efficiency in relation to this index, or retention time is too long, thus increasing of the sampling rate can be a decision.

Table 4. Relative Standard Deviation (%RSD) of peaks' retention times of organic acids contained in real sample of wine. Chromatographic conditions: column – Nucleodur C18 Pyramid, 250 x 4,0mm (*Macherey-Nagel*), oven temperature 35°C, flow rate 0,5ml/min, detection on 210nm. Number of measures is ten.

Organic acid	%RSD (peak's retention time)		
	0,2% H_3PO_4	5mM H_2SO_4	12,5mM H_2SO_4
Tartaric	0,23	0,11	0,08
L-malic	0,31	0,10	0,13
Lactic	0,37	0,09	0,66
Acetic	0,30	0,11	0,38
Citric	0,50	0,07	0,94
Succinic	0,61	0,07	0,69

3. CONCLUSIONS

For determination the most effective chromatographic conditions for analysis of ogranic acids in wine there were caried out a number of analysis. Chromatographic column with modified C18 phase was used for this purpose, which was recommended by producer of immobile phase and OIV. Analysis were made in different conditions, in particular, with different eluents. As the eluents there were taken aqueous solutions of inorganic acids – phosphoric and sulfuric acids. Nitric acid didn't demonstrate satisfactory results.

After chromatographic analysis of standard solution of organic acids in mentioned conditions (chromatographic column Nucleodur C18 Pyramid, 250 x 4,0mm (*Macherey-Nagel*), oven temperature 35°C, flow rate 0,5ml/min, detection on 210nm, eluents: 1 – 0,2% H_3PO_4, 2 – 5mM H_2SO_4, 3 – 12,5mM H_2SO_4) it can be noticed that there was no significant difference in the signals, neither by retention times, nor by area of the chromatographic peaks.

Nevertheless there were some differences in chromatograms, when real sample of wine with complex matrix was analyzed. Thus, the signal of tartaric acid could not be considered correctly in the case of using 12.5mM H_2SO_4 as eluent, due to the merging of vicinal peaks of substances, which were present in wine matrix and absent in standard solution.

The pair of acids – lactic and acetic – was not separated satisfactory for quantification in any described chromatographic conditions. While the value of resolution factor (Rs) for this pair was the biggest in the first chromatographic system with 0.2% H_3PO_4 solution as eluent, intensity of signals were the least. When the signal of acetic acid was maximum (using 12.5mM H_2SO_4 as eluent), %RSD$_{peak's\ area}$ of ten measures was more than 10%. %RSD of retention times in all three cases of chromatographic systems for all analytical signals were small and meet the requirements for HPLC analysis.

Taking into account results of described study it can be noticed that the "clearest" signals of organic acids, which are present in wine with complex matrix, can be obtained in the case of using 0.2% H_3PO_4 solution as eluent.

References

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