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Method development for the determination of anthocyanins in red wines by high-performance liquid chromatography and classification of German red wines by means of multivariate statistical methods

Bálint Berente*, Demetrio De la Calle García, Manfred Reichenbächer, Klaus Danzer

Friedrich-Schiller-Universität Jena, Institut für Anorganische und Analytische Chemie, Lessingstrasse 8, D-07743 Jena, Germany

Abstract

A simple and fast HPLC method without sample pretreatment is described for the separation of anthocyanins in red wines using a new pH-stable stationary phase. The linearity between peak area and concentration and ruggedness of the method were checked. Investigations were made about the safekeeping of red wine samples concerning anthocyanins. Classification of 52 different wine samples was performed by multivariate statistical methods.

Keywords: Wine; Food analysis; Chemometrics; Method development; Multivariate statistical methods; Anthocyanins

1. Introduction

The quality of red and rosé wines is strongly dependent on their colour. The colour of wines is caused by anthocyanins and their polymers. The free anthocyanins are mono- or diglucosides of five anthocyanidins, namely delphinidin, cyanidin, petunidin, paeonidin and malvidin. The acylated anthocyanins are esters of the glucose part of the free anthocyanins with acetic, *p*-coumaric or caffeic acid [1–3]. Fig. 1 shows the structure of anthocyanins of the most important species *Vitis vinifera* which has only monoglucosides. Investigations of the anthocyanin colour pigments from the grape skin showed that the anthocyanin pattern is characteristic of the cultivar. Classifications of red grape varieties of the species *Vitits vinifera* were performed by means of multivariate statistical methods [4–6]. The typical anthocyanin fingerprint changes during the vinification, maturation and ageing, however, the differences in anthocyanin pattern of wines from different varieties are still noticeable [7].

Illegal improvements of the wine colour can be accomplished in different ways. Potential adulterations with different berries can be proved by high-performance liquid chromatography (HPLC) relatively simply [8]. Another way to enhance the colour density is the adding of a deep coloured other wine, but the part by volume of the added wine must not exceed 15% in quality wines in Germany. The proof of such adulterations may be carried out by investigation of the anthocyanin pattern. The use of analytical methods instead of organoleptic ones for the quality control of wines is of the greatest importance due to their performance and objectivity.

*Corresponding author. Tel.: +49-3641-948-175; fax: +49-3641-948-172.

E-mail addresses: c7beba@uni-jena.de (B. Berente), c7rema@uni-jena.de (M. Reichenbächer)

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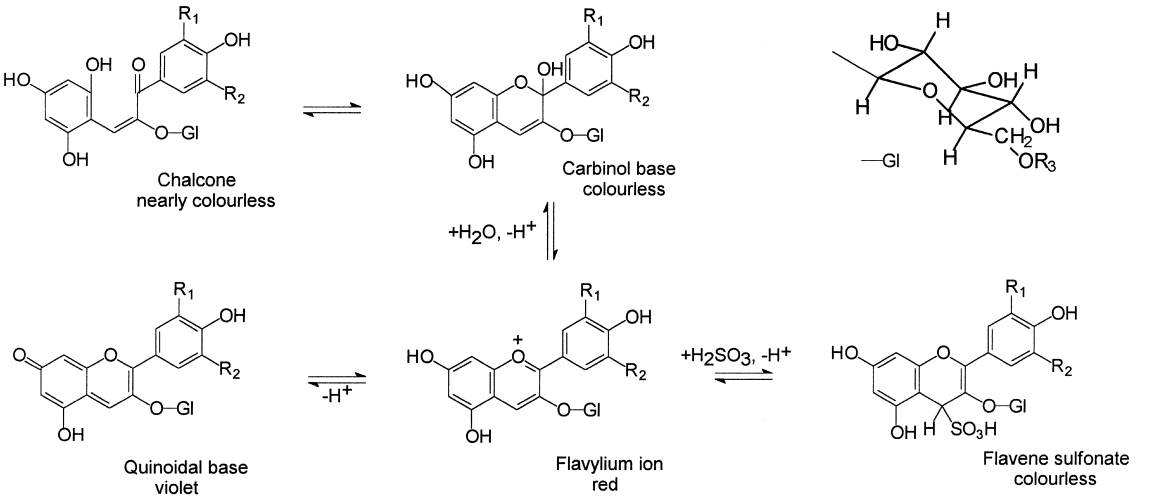

Fig. 1. Structure and equilibria of the anthocyanins present in species *Vitis vinifera* at wine pH. The groups R_1, R_2, R_3 are listed in Table 1.

The first application of the HPLC for determination of anthocyanins from grape skin was described by Wulf and Nagel using mobile phases at pH 1.6 with 10% formic acid [9]. The low pH of the mobile phase is required to keep the anthocyanins homogeneous in the flavylium form according to the pH-dependent equilibria; otherwise peak broadening is obtained due to the presence of other forms of anthocyanins (Fig. 1). The other benefit is the increased sensitivity of the selective detection of the flavylium form at near 520 nm [1–4,7,8,10]. However, information of the lifetime of the columns for long periods of time at pH 1.6 is not given by the manufacturers.

The aim of our work was to improve the HPLC separation of anthocyanins in red wines to get a method which is more suitable for the routine work than the methods described earlier. A pH-stable stationary phase was used to avoid rapid loss of column performance. Phosphate buffer was used to obtain the best stabilisation of the pH and consequently a best repeatability and ruggedness. Sample pretreatment was not applied and the analysis time was reduced to 45 min while keeping good resolutions. The characterisation of the method includes the test of linearity between peak area and concentration and the test of ruggedness by two-level fractional factorial design. Furthermore, a classification of 52 different German wine samples was performed by cluster analysis and principal components analysis.

2. Experimental

2.1. Chemicals and standards

Solvents used for chromatography were acetonitrile of HPLC ultra gradient grade supplied by Mallinckrodt Baker (Deventer, The Netherlands) and deionised water. Chemicals used for adjusting the pH of the mobile phase were 85% H_3PO_4 of analytical-reagent grade and KH_2PO_4 for preparation of pH standard buffer solutions supplied by Merck (Darmstadt, Germany). Standard substances of cyanidin-3-glucoside, paeonidin-3-glucoside and malvidin-3-glucoside were delivered by Roth (Karlsruhe, Germany). Ethanol of Uvasol grade from Merck was used for preparing standard solutions. 0.45-μm pore size membrane filters from Schleicher&Schuell (Dassel, Germany) were used for filtration of the mobile phase and the samples.

2.2. Apparatus

The equipment used in this work was a Shimadzu liquid chromatograph consisting of an online degasser (ERC 3520), two LC-8A pumps, an eluent

mixing chamber, a manual injector with 20-μl loop (Rheodyne 7125), a CTO-6A column oven, a SPD-10AV Dual UV–Vis detector, a CBM-10AV communication bus module and Class-LC10 Vers.1 chromatographic data processing software. Statistica 5.0 statistics software from Statsoft (Tulsa, OK, USA) was used for the multivariate data analysis.

2.3. Chromatographic conditions

The separation was performed using an UltraSep ES RP18 MLD II, 5 μm, 250×4 mm column preceded by an UltraSep ES RP18 5 μm, 10×4 mm precolumn, both supplied by Sepserv (Berlin, Germany). The following mobile phases were used: buffer: 10 m*M* $KH_2PO_4+H_3PO_4$ to pH 1.6, solvent A: acetonitrile–buffer (5:95), solvent B acetonitrile–buffer (50:50). The oven temperature was 50°C. Gradient elution was applied at 1 ml/min flow-rate according to the following program: linear gradient from 10% B to 45% B in 30 min, from 45% B to 100% B in 1 min, 100% B for 3 min, from 100% B to 10% B in 1 min, 10% B for 10 min (dwell volume: 1.4 ml). Samples (20 μl) were injected, UV–Vis detection was applied at 518 nm. The wine samples, standard solutions and mobile phases were filtered by a 0.45-μm pore size membrane filter.

3. Results and discussion

3.1. Chromatographic separation

For the method development a Cabernet Sauvignon wine was used (1996, Nicolas Napoleon, Pay d'Oc, France), because this variety has large amounts of anthocyanins [7,9]. The optimisation started with solvents containing phosphate buffer at pH 2, the other conditions were taken from Holbach et al. [7]. At first the peak broadening was decreased by setting the pH as low as the column allowed (pH≥1.5). Further improvement of the efficiency was obtained by increasing the temperature to 50°C. In the end, flow-rate and gradient program were optimised systematically. Identification of nine peaks was performed by measuring the UV–visible spectra on the peak maxima and comparing with literature data; the compounds are given in Table 1. cy-3-gl, pa-3-gl and ma-3-gl were additionally identified by standards. Fig. 2 shows the chromatogram of the test wine containing the identified compounds. The three peaks situated between peaks 5 and 6 were expected, respectively, as de-3-acgl, cy-3-acgl and pe-3-acgl which were not taken into consideration because of not resolved interfering substances. The reproducibility of the method was estimated by $n=5$ measurements: although gradient elution was applied the relative standard deviation (RSD) of the retention times was $<0.5\%$ and the RSD of the peak areas was $<1\%$.

3.2. Test of linearity

The anthocyanin content of red wines depends on variety, vintage and technology and decreases with time. The determination of absolute concentrations gives little information regarding variety because the

Table 1
Anthocyanins investigated in this study[a]

Peak	t_R (min)	Anthocyanin	Abbreviation	R_1	R_2	R_3
1	5.3	Delphinidin-3-glucoside	de-3-gl	–OH	–OH	
2	7.7	Cyanidin-3-glucoside	cy-3-gl	–OH	–H	
3	9.3	Petunidin-3-glucoside	pe-3-gl	–OH	$-OCH_3$	–H
4	11.9	Paeonidin-3-glucoside	pa-3-gl	–H	$-OCH_3$	
5	13.2	Malvidin-3-glucoside	ma-3-gl	$-OCH_3$	$-OCH_3$	
6	22.1	Paeonidin-3-acetylglucoside	pa-3-acgl	–H	$-OCH_3$	–Acetyl
7	23.0	Malvidin-3-acetylglucoside	ma-3-acgl	$-OCH_3$	$-OCH_3$	
8	29.5	Paeonidin-3-coumarylglucoside	pa-3-cugl	–H	$-OCH_3$	–Coumaryl
9	30.1	Malvidin-3-coumarylglucoside	ma-3-cugl	$-OCH_3$	$-OCH_3$	

[a] Peaks are shown in Fig. 2. The positions of groups R_1, R_2, R_3 are shown in Fig. 1

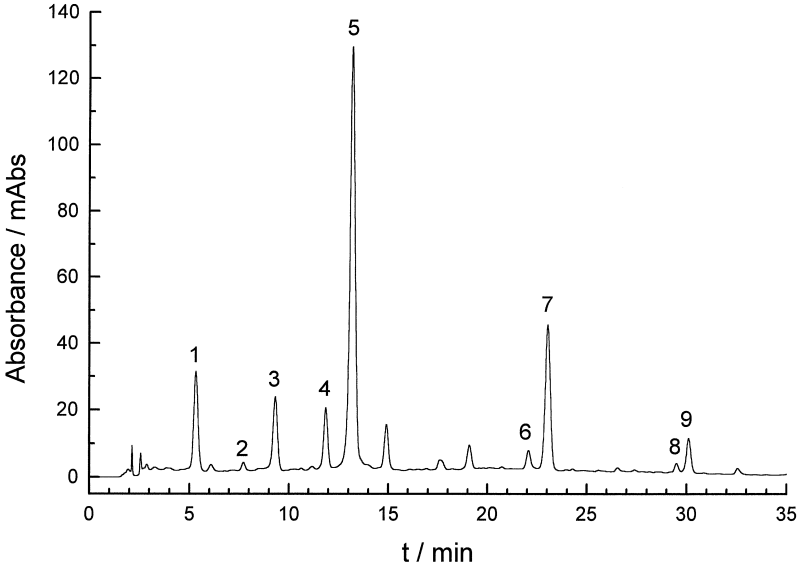

Fig. 2. Chromatogram of the Cabernet Sauvignon wine used for the method optimisation.

effects of technology and age are greater. However, the anthocyanin composition, that is, the ratio of anthocyanins to each other, is determined by the variety and shows more stability in time. The proportional peak areas obtained after normalisation by the total peak area of anthocyanins are able to characterise the anthocyanin pattern if the linearity between concentration and peak area is ensured. This must be checked because the anthocyanins have equilibrium reactions and association phenomena which can cause a deviation from Lambert–Beer's Law [3]. The total anthocyanin content in red wines varies between 0 and 250 μg/ml except for very young wines which can contain several times more anthocyanins [1–3]. The linearity was investigated for ma-3-gl in the range of 5–125 μg/ml at seven concentration levels. The standard solutions were prepared by weighing the standards with a Sartorius MC1 microbalance and dissolving in 12% ethanolic solution at pH 3. All standard solutions were measured twice. The linearity between concentration and peak area was estimated by the Mandel test [11], outliers were not found with probability $P=0.99$. Slope=124 707 counts·ml/μg, intercept=−55 183 counts which is not significantly different from zero proofed by the *t*-test [12]. The RSD of the fitting was 1.59%.

3.3. Test of ruggedness

Information about the effects caused by changes in chromatographic conditions are important for the decision of which factors must be set more carefully. The measurement parameters were chosen according to the development of the method. Table 2 shows the lower and upper levels of the factors, which were set relatively narrow to check only the realistic deviations from the standard conditions. Fractional factorial design with eight runs was used [12,13], all runs were measured three times. Changes in the elution order of anthocyanins were not obtained. The proportional peak areas in percentages and the resolutions between critical peak pairs were taken as dependent variables for the statistical analysis. The computed effects are given in Table 3. The significance of an effect was estimated by a *t*-test, comparing the effect with the experimental error [13]. The in-run standard deviation computed from the triplicate measurements was used as experimental error. The test showed, that the factors pH and temperature

Table 2
The investigated factors with lower and upper levels and the factorial design for the runs

Factors and levels	Lower level (−1)	Upper level (+1)
pH	1.6	1.7
Acetonitrile (ACN) in B (%)	48	52
Temperature (°C)	45	55
Flow-rate (ml/min)	0.9	1.1

Runs	pH	ACN (%)	Temperature	Flow
1	−1	−1	−1	−1
2	−1	−1	1	1
3	−1	1	−1	1
4	−1	1	1	−1
5	1	−1	−1	1
6	1	−1	1	−1
7	1	1	−1	−1
8	1	1	1	1

are more important than acetonitrile content of mobile phase B and flow-rate. The equilibrium of each anthocyanin depends differently on these first two factors [10], it means that not only is the separation affected by acetonitrile content and flow-rate but the sensitivity is also.

3.4. Investigations on sample storage

Since the anthocyanin content of red wines decreases continuously, the common ways of wine storage were compared to determine which conditions are required to store the wine samples without loss of anthocyanins over long periods of time. For the examinations a commercial Beaujolais Primeur wine (1997, Clochemerle, France) was chosen, because these wines come early onto the market and the initially high anthocyanin content decreases relatively rapidly. From the first bottle opened the following samples were prepared: 150 ml of wine in 250-ml Erlenmeyer flasks for storing at room temperature and at 4°C in refrigerator, 15 ml of wine in closed 20-ml GC headspace vials for storing at −18°C in a freezer. One of the frozen samples was melted, analysed and then frozen again on each day when analyses were performed to check if repeated melting and freezing of a sample affects the anthocyanin content by possible irreversible precipitation reactions. The other frozen samples were measured just once. Furthermore, new bottles were

Table 3
Results of the test of ruggedness[a]

Variables	Mean values on standard conditions	pH	Effects on the variables factor		Flow	In-run SD
			ACN (%) in B	Temperature		
Proportional peak areas in %						
de-3-gl	9.62	**0.176**	**−0.155**	**−0.290**	0.097	0.070
cy-3-gl	0.85	**0.028**	**−0.049**	**−0.152**	**−0.010**	0.004
pe-3-gl	7.99	**0.256**	−0.073	**−0.336**	−0.059	0.038
pa-3-gl	6.22	**0.059**	**0.075**	**0.081**	**−0.038**	0.017
ma-3-gl	52.23	**−0.669**	**0.443**	**1.319**	**−0.359**	0.036
pa-3-acgl	2.05	**−0.077**	**0.040**	**−0.040**	**−0.071**	0.011
ma-3-acgl	16.62	0.006	−0.099	**−0.565**	**0.370**	0.076
pa-3-cugl	0.90	**0.042**	**−0.054**	**0.041**	0.019	0.014
ma-3-cugl	3.52	**0.179**	**−0.127**	**−0.058**	**0.053**	0.024
R_S between peaks						
6/7	2.2	**−0.418**	0.103	**0.406**	0.038	0.102
8/9	1.6	**−0.413**	**−0.191**	**0.464**	**0.120**	0.027

[a] Significant effects ($P=0.95$) are written in bold type.

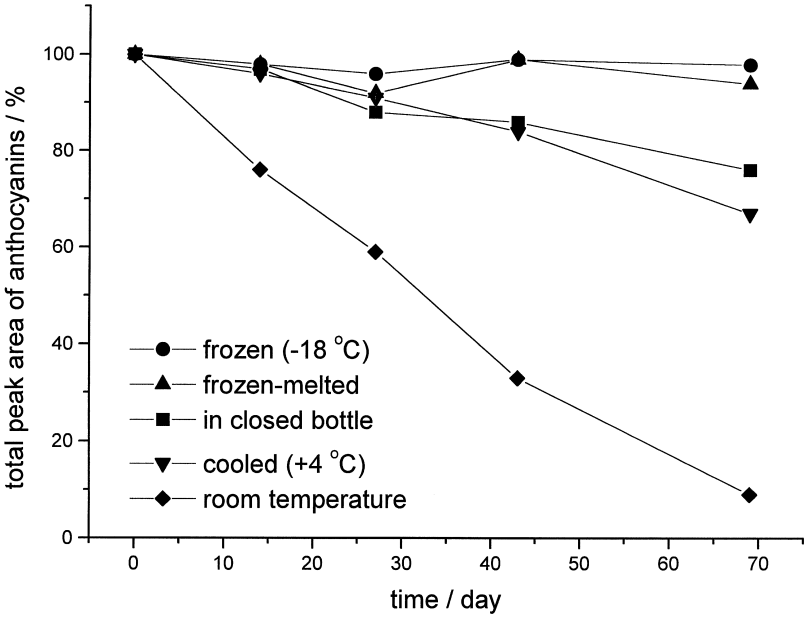

Fig. 3. Breakdown of the anthocyanins under different storage conditions. 100%=Initial content.

opened on each day when analyses were performed to investigate the anthocyanin degradation in the original bottles. The storage conditions were tested over 69 days. All samples were measured in duplicate and the sum of the peak areas of the nine anthocyanins was used to characterise the degradation. Fig. 3 shows that both higher temperature and contact with air accelerate the breakdown of anthocyanins: the loss of anthocyanins in 69 days was 24% in the original bottles which were stored at room temperature but had no contact with air, 33% in the samples which were cooled to 4°C but had contact with air in the refrigerator and 91% in samples stored at room temperature where both effects occurred. Storage at −18°C in a freezer is appropriate for keeping the samples for long periods of time, significant losses of anthocyanins were not obtained even if the sample was melted and frozen several times.

3.5. Classification of wine samples by multivarietal data analyses

Fifty-two German wine samples were analysed, the distribution of the samples is given in Table 4. Fifteen samples had guaranteed authenticity: four

Table 4
List of the 52 samples with regard to variety, vintage, origin and age

Number of the wine samples concerning							
Variety		Vintage		Origin		Age (year)	
Blauer Portugieser	7	1989	1	Alzey	4	0.5	16
Blauer Spätburgunder	31	1992	2	Blankenhorsberg	6	1.5	23
Domina	7	1993	5	Durbach	4	2.5	5
Frühburgunder	1	1994	6	Nordheim	7	3.5	5
Schwarzriesling	3	1995	7	Thüringsheim	4	4.5	2
Zweigeltrebe	2	1996	20	Erlabrunn	4	6.5	1
Dornfelder	1	1997	11	Other small vineries	23		

Blauer Portugiesers from Alzey, six Blauer Spätburgunders from Blankenhorsberg, four Blauer Spätburgunders from Durbach and one Blauer Spätburgunder from Würzburg.

The proportional peak areas of the nine anthocyanins were computed at each sample, and these variables were standardised by autoscaling [13]. At first the variables were investigated to obtain which variables correlate the most, that means which variables have similar contributions to the classification of the samples. For this purpose cluster analysis of the variables was performed over all samples by Ward's method with squared euclidean distances [13]. Fig. 4 shows that the proportional peak areas of de-3-gl and pe-3-gl, pa-3-acgl and ma-3-acgl, pa-3-cugl and ma-3-cugl correlate most of all. Reduction of the number of variables was performed by computing the sum of them pairwise. These new variables were standardised.

The classification of the wine samples was performed by principal components analysis, which is appropriate to extract the relevant information from the original variables into fewer new variables, called principal components. The investigation of the objects, i.e., the wines, can be performed visually using the scoreplot where the objects are represented in function of the first principal components (PCs) [13]. Fig. 5 shows that the PC 1 correlates positively with acylated anthocyanins in contrast to ma-3-gl, the PC 2 is affected more by changes between free anthocyanins. The first two principal components extract 70% of the total variance. Fig. 6 shows the scoreplot. The varieties Spätburgunder, Frühburgunder and Schwarzriesling are biologically related to each other [7]; they could not be separated. The lack of acylated anthocyanins in these wines indicates the negative factor scores of PC 1. It is noteworthy that the authentic wines of the different varieties Spätburgunder and Portugieser can be separated with the first two principal components. The two separate groups of genuine Spätburgunders are determined by the origin Blankenhorsberg and Alzey, respectively. The Domina wines have intensive colour as well as the Dornfelder, they have higher factor scores of both principal components. The few samples of Zweigeltrebe and the non-authentic Portugieser wines do not form a group on the scoreplot.

4. Conclusions

The HPLC separation of anthocyanins described in this study is suitable for routine analysis of red wines. No loss of column performance was obtained using the pH-stable stationary phase during the

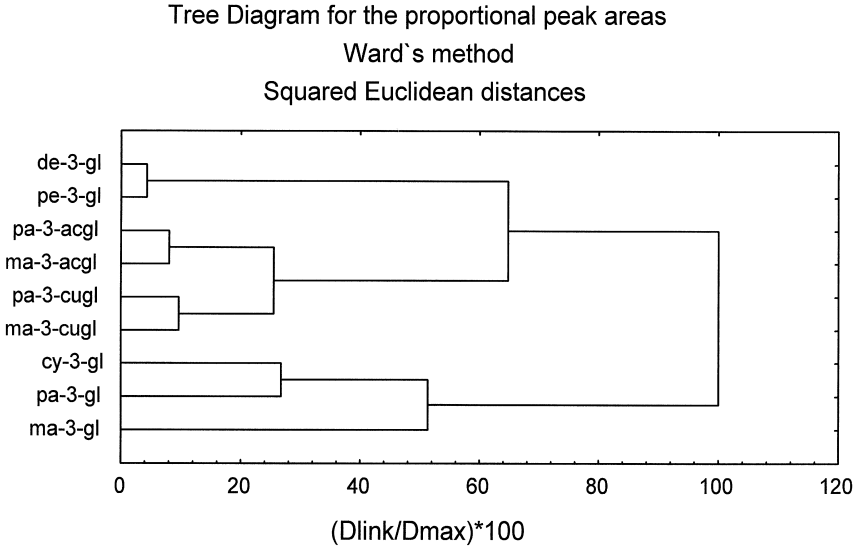

Fig. 4. Correlation between the proportional peak areas of the wine samples.

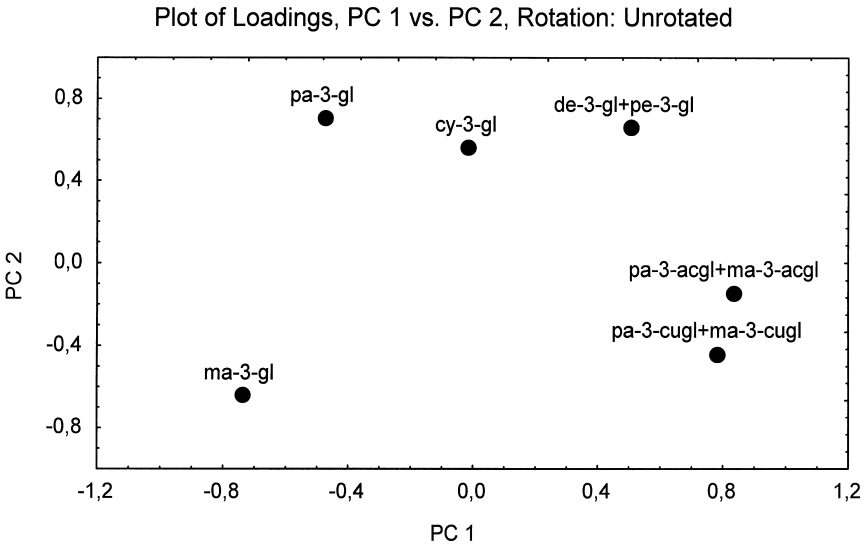

Fig. 5. Correlation between the first two principal components and the variables.

experiments, ca. 400 h. The test of ruggedness showed that pH of the mobile phase and temperature must be adjusted properly for the correct determination of anthocyanins. The anthocyanin pattern helped to establish the genuineness regarding variety. Further investigations are required to determine the effects of geographical origin, ageing and wine technology using authentic wine samples.

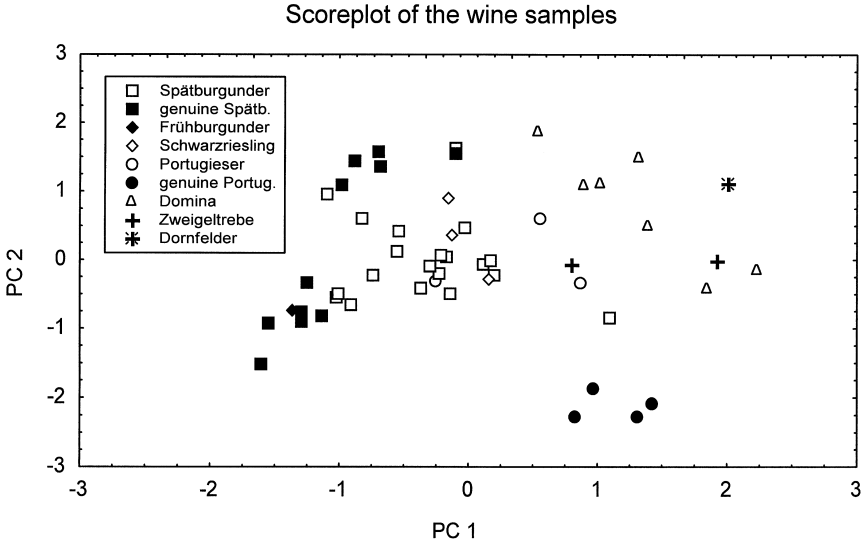

Fig. 6. Classification of the wine samples by principal components analysis using the variables given in Fig. 5.

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