RESOLUTION OENO 22/2003

HPLC-DETERMINATION OF NINE MAJOR ANTHOCYANINS IN RED AND ROSÉ WINE

THE GENERAL ASSEMBLY,

CONSIDERING Article 5, paragraph 4 of the International Convention for the Unification of Methods of Analysis and Appraisal of Wine of 13 October 1954,

UPON THE PROPOSAL of the Sub-commission of the Methods of Analysis and Appraisal of Wine,

DECIDES to introduce in Annex A of the Compendium on International Methods of Analysis of Wine and Musts the following method as a Type II method:

1. Field of application

The analytical method concerns the determination of the relative composition of anthocyanins in red and rosé wine. The separation is performed by HPLC with reverse phase column and UV-VIS detection.

Many authors [3, 6-17] have published data on the anthocyanin composition of red wines using similar analytical methods. For instance Wulf et al. [18] have detected and identified 21 anthocyanins and Heier et al. [13] nearly 40 by liquid chromatography combined with mass spectrometry. The anthocyanin composition may be very complex, so it is necessary to have a simple procedure. Consequently this method only determines the major compounds of the whole anthocyanin fraction.

Member states are encouraged to continue research in this area to avoid any non scientific evaluation of the results.

2. Principle

Separation of the five most important non acylated anthocyanins (see Figure 1, peaks 1-5) and four major acylated anthocyanins (see Figure 1, peaks 6-9).

Analysis of red and rosé wine by direct separation by HPLC by using reverse phase column with gradient elution by water/formic acid/acetonitrile with detection at 518 nm [1.2].

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3. Reagents and material

Formic acid (p.a. 98 %) (CAS 64-18-6);

Water, HPLC grade;

Acetonitrile, HPLC grade (CAS 75-08-8);

HPLC solvents:

Solvent A: Water/Formic acid/Acetonitrile 87 : 10 : 3 (v/v/v)

Solvent B: Water/Formic acid/Acetonitrile 40 : 10 : 50 (v/v/v)

Membrane filter for HPLC solvent degassing and for sample preparation to be analysed.

Reference products for peak identification.

The HPLC analysis of anthocyanins in wine is difficult to perform due to the absence of commercially available pure products. Furthermore, anthocyanins are extremely unstable in solution.

The following anthocyanin pigments are commercially available:

Cyanidol-3-glucoside (also couromanin chloride); M = 484.84 g/mol

Peonidol-3-glucoside; M = 498.84 g/mol

Malvidol-3-glucoside (also Oeninchloride); M = 528.84 g/mol

Malvidol-3,5-diglucoside (also Malvinchloride); M = 691.04 g/mol g/mol.

4. Apparatus

HPLC system with:

- binary gradient pump, injection system for sample volumes ranging from 10 to 200 µl,
- diode array detector or a UV detector with a visible range,
- integrator or a computer with data acquisition software,
- furnace for column heating at 40°C,
- solvent degassing system,
- analytical column, for example:

LiChrospher 100 RP 18 (5 µm) in LiChroCart 250-4 guard column: for example RP 18

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(30-40 mm) in a cartridge 2 mm in diameter x 20 mm long

5. Procedure

5.1. Preparation of samples

Clear wines are poured directly without any preparation into the sample vials of the automatic sample changer. Cloudy samples are filtered using a 0.45 µm membrane filter for HPLC sample preparation. The first part of the filtrate should be rejected.

Since the range of the linearity of absorption depending on the concentration of anthocyanins is large, it is possible to modulate the injection volumes between 10 and 200 µl depending on the intensity of the wine colour. No significant difference between the results obtained for different injection volumes was observed.

5.2. Analysis

HPLC conditions

The HPLC analysis is carried out in the following conditions:

Injection Volume:	50 µl (red wine) up to 200 µl (rosé wine)
Flow:	0.8 ml/minute
Temperature:	40°C
Run time:	45 minutes
Post time:	5 minutes
Detection:	518 nm

Gradient elution:	Time (min)	Solvent A % (v/v)	Solvent B % (v/v)
	0	94	6
	15	70	30

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30	50	50
35	40	60
41	94	6

To check the column efficiency, the number of theoretical plates (N) calculated according to malvidol-3-glucoside should not be below 20,000, and the resolution (R) between peonidol-3-coumaryl glucoside and malvidolin-3-coumaryl glucoside should not be lower than 1.5. Below these values, the use of a new column is recommended.

A typical chromatogram is given in Figure 1, where the following anthocyanins are separated:

		Peak-N°
Group 1: "Nonacylated anthocyanidin-3-glucosides":	delphinidol-3-glucoside	1
	cyanidol-3-glucoside	2
	petunidol-3-glucoside	3
	peonidol-3-glucoside	4
	malvidol-3-glucoside	5
Group 2: "Acetylated anthocyanidin-3-glucosides":	peonidol-3-acetylglucoside	6
	malvidol-3-acetylglucoside	7
Group 3: "Coumarylated anthocyanidin-3-glucosides":	peonidol-3-coumarylglucoside	8
	malvidol-3-coumarylglucoside	9

6. Expression of results

Note that the values are expressed as relative amounts of the sum of the nine anthocyanins defined in this method.

7. Fidelity parameters

The repeatability (r) and the reproducibility (R) values for the nine anthocyanins are given in Table 2 and depend on the amount of the peak area. The uncertainty measurement of a particular peak area is determined by the value of r and R which

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corresponds to the nearest value given in Table 2.

The values made up of validation data can be calculated by following the appropriate statistical rules. To calculate the total error (s_r) for example of the sum of acetylated anthocyanins, the variances (s_r^2) of specific the total error of ratios, for example, that of acetylated to coumarylated anthocyanins the square of relative errors ($=s_r/a_i$) are to be added. By using these rules, all the fidelity values can be calculated by using the data in Table 2.

Annex A

Bibliography

1. Marx, R., B. Holbach, H. Otteneder; Determination of nine characteristic Anthocyanins in Wine by HPLC; OIV, F.V.N° 1104 2713/100200
2. Holbach, B., R. Marx, M. Ackermann; Bestimmung der Anthocyanzusammensetzung von Rotwein mittels Hochdruckflüssigkeitschromatographie (HPLC). Lebensmittelchemie (**1997**) 51: 78 - 80
3. Eder, R., S. Wendelin, J. Barna; Auftrennung der monomeren Rotweinanthocyane mittels Hochdruckflüssigkeitschromatographie (HPLC).Methodenvergleich und Vorstellung einer neuen Methode. Mitt. Klosterneuburg (**1990**) 40: 68-75
4. ISO-5725-2: **1994** "Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility"
5. Otteneder, H., Marx, R., Olschimke, D.; Method-performance study on the determination of nine characteristic anthocyanins in wine by HPLC. O.I.V. F.V.N° 1130 (**2001**)
6. Mattivi F.; Scienza, A.; Failla, O.; Vika, P.; Anzani, R.; Redesco, G.; Gianazza, E.; Righetti; P. Vitis vinifera - a chemotaxonomic approach: Anthocyanins in the skin. *Vitis (special issue)* **1990**, 119-133
7. Roggero, I.P.; Larice, I.L.; Rocheville-Divorne, C.; Archier, P.; Coen, V. Composition Antocyanique des cepages. *Revue Francaise d'Oenologie* **1998**, *112*, 41-48
8. Eder, R.; Wendelin, S; Barna, J. Classification of red wine cultivars by means of anthocyanin analysis. *Mitt. Klosterneuburg* **1994**, *44*, 201-212

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9. Arozarena, I.; Casp, A.; Marin, R.; Navarro, M. Differentiation of some Spanish wines according to variety and region based on their anthocyanin composition. *Eur. Food Res. Technol.* **2000**, *212*, 108-112

10. Garcia-Beneytez, E.; Revilla, E.; Cabello, F. Anthocyanin pattern of several red grape cultivars and wines made from them. *Eur. Food Res. Technol.* **2002**, *215*, 32-37

11. Arozarena, I.; Ayestarán, B.; Cantalejo, M.J.; Navarro, M.; Vera, M.; Abril, K.; Casp, A. *Eur. Food Res. Technol.* **2002**, *214*, 313-309

12. Revilla, E.; Garcia-Beneytez, E.; Cabello, F.; Martin-Ortega, G.; Ryan, J-M. Value of high-performance liquid chromatographic analysis of anthocyanins in the differentiation of red grape cultivars and red wines made from them. *J. Chromatogr A* **2001**, *915*, 53-60

13. Heier, A.; Blaas, W.; Droß, A.; Wittkowski, R.; Anthocyanin Analysis by HPLC/ESI-MS, Am.J.Enol.Vitic, **2002**, 53, 78-86

14. Arozarena, I.; Casp, A.; Marin, R.; Navarro, M. Multivariate differentiation of Spanish red wines according to region and variety. *J. Sci. Food Agric*, **2000**, *80*, 1909-1917

15. Anonymous. Bekanntmachung des Bundesinstituts für gesundheitlichen Verbraucherschutz und Veterinärmedizin. *Bundesgesundheitsbl. Gesundheitsforsch. Gesundheitsschutz,* **2001**, *44*, 748

16. Burns, I.; Mullen, W.; Landrault, N.; Teissedre, P.-L.; Lean, M.E.I.; Crozier, A. Variations in the Profile and Content of Anthocyanins in Wines made from Cabernet Sauvignon and hybrid grapes. *J. Agric. Food Chem.* **2002**, *50*, 4096-4102

17. Otteneder, H.; Holbach, B.; Marx, R.; Zimmer, M. Rebsortenbestimmung in Rotwein mittels Anthocyanspektrum. *Mitt. Klosterneuburg*, **2002**, *52*, 187-194

18. L.W. Wulf and C.W. Nagel; High-Pressure liquid chromatographic separation of Anthocyanins of Vitis vinifera. Am.J.Enol.Vitic **1978**, 29, 42-49

Annex B

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Statistical results

Method performance study and evaluation

17 laboratories from 5 European Nations participated in the validation study of the method under the coordination of the German Official State Laboratory for Food Chemistry in Trier. The participants are listed in Table 3. An example of a chromatogram is presented in Figure 1 and the detailed results are given in Table 2.

The statistical evaluation followed the Resolution 6/99 and the Standard ISO 5725-1944 **[4.5]**.

The chromatograms sent back with the results sheets fulfilled all requirements concerning the performance of the analytical column. No laboratory had to be completely eliminated, for example, because of a wrong peak identification.

The outlier values were searched using Dixon and Grubbs outlier testing according to the procedure for "Harmonised Protocol – IUPAC 1994" and the OIV Resolution OENO 19/2002. The values of s_r, s_R, r and R were calculated for 9 major anthocyanins at 5 content levels. For analytical results, the values of the closest levels should be used.

In order to have a global vision of the method performance, all the values RSD_r- et RSD_R- gathered are grouped by range of areas in the following table:

Table 1: Summary of the results of the method performance study

Range of relative peak areas*[%]	Range of RSD_r [%]	Range of RSD_R [%]
>0.4 – 1.0	6.8 - 22.4	20.6 - 50.9
>1.1 – 1.5	4.2 - 18.1	11.8 - 28.1
>1.5 – 3.5	2.1 – 7.7	10.6 - 15.6
>3.5 – 5.5	2.7 – 5.7	18.7 – 7.5
>5.5 – 7.5	2.4 – 3.9	6.5 - 10.0
>10 – 14	1.1 – 2.9	3.7 - 9.2

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>14 – 17	1.0 - 3.9	3.2 - 5.4
>50 – 76	0.3 - 1.0	2.1 - 3.1
* independent of anthocyanin		

This leads to the conclusion that repeatabilities and reproducibilities depend on the total sum of the relative peak areas. The higher they are, the better are RSD_r and RSD_R. For anthocyanin contents close to the detection limit (e.g. Cyanidin-3-glucoside) with small relatives areas (less than 1%) the RSD_r et RSD_R values can rise significantly. For anthocyanin whose relative areas are more than 1%, the RSD_r and RSD_R values are reasonable.

Figure 1: Separation of 9 anthocyanins in red wine

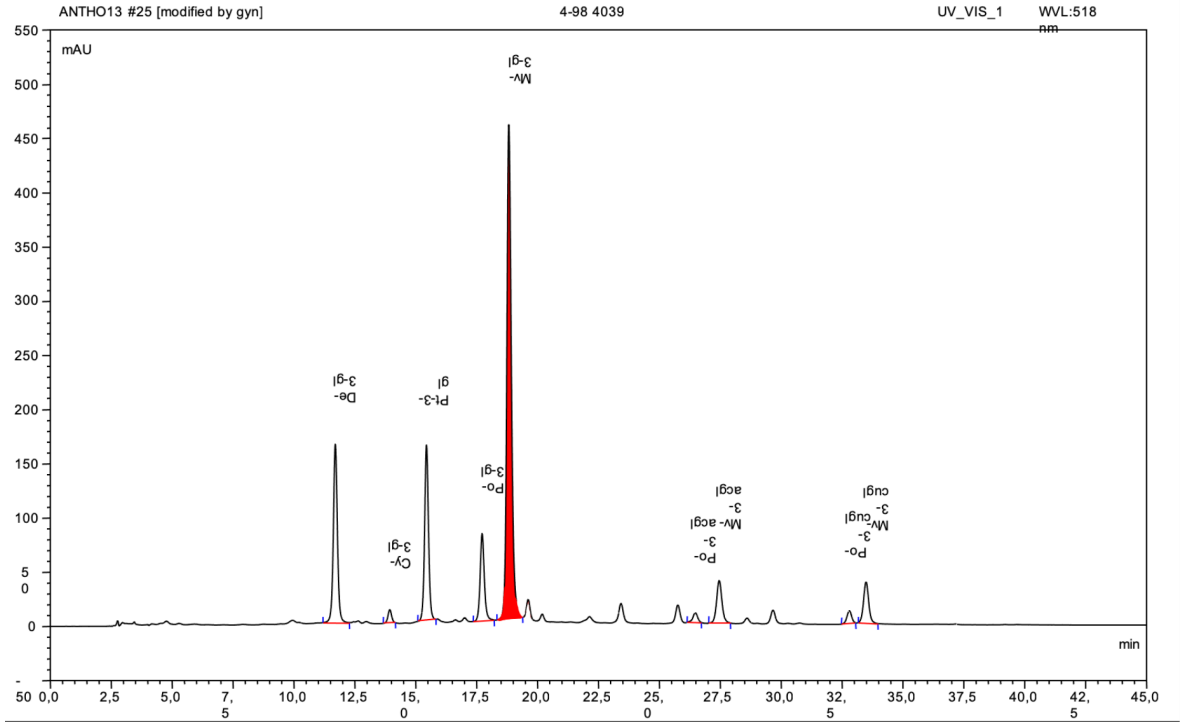

Table 2: Results of the method performance study Page 1 of 2

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Anthocyanin	sample 1	sample 2	sample 3	sample 4	sample 5
Delphinidol-3-glucoside					
n	14	14	16	15	16
mean	6.75	14.14	3.45	16.68	3.54
s_r	0.163	0.145	0.142	0.142	0.108
RSD_r(%)	2.4	1.0	4.1	0.8	3.1
r	0.46	0.41	0.40	0.40	0.30
s_R	0.544	0.462	0.526	0.704	0.490
RSD_R(%)	8.1	3.3	15.2	4.2	13.8
R	1.52	1.29	1.47	1.97	1.37
Cyanidol-3-glucoside					
n	16	17	16	15	14
mean	2.18	1.23	0.61	1.46	0.34
s_r	0.086	0.053	0.043	0.110	0.031
RSD_r(%)	4.0	4.3	7.1	7.5	9.2
r	0.24	0.15	0.12	0.31	0.09
s_R	0.460	0.211	0.213	0.180	0.158
RSD_R(%)	21.2	17.2	34.9	12.3	46.7
R	1.29	0.59	0.60	0.50	0.44
Petunidol-3-glucoside					

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n	15	17	16	14	15
mean	10.24	14.29	5.75	12.21	6.19
s_r	0.233	0.596	0.157	0.097	0.196
RSD_r(%)	2.3	4.2	2.7	0.8	3.2
r	0.65	1.67	0.44	0.27	0.55
s_R	0.431	0.996	0.495	0.469	0.404
RSD_R(%)	4.2	7.0	8.6	3.8	6.5
R	1.21	2.79	1.39	1.31	1.13
Peonidol-3-glucoside					
n	16	15	17	17	16
mean	11.88	6.23	13.75	7.44	4.12
s_r	0.241	0.166	0.144	0.232	0.174
RSD_r(%)	2.0	2.7	1.0	3.1	4.2
r	0.68	0.47	0.40	0.65	0.49
s_R	0.981	0.560	1.227	0.602	0.532
RSD_R(%)	8.3	9.0	8.9	8.1	12.9
R	2.75	1.57	3.44	1.69	1.49
Malvidol-3-glucoside					
n	16	15	17	16	16
mean	55.90	55.04	76.11	52.60	61.04

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s_r	0.545	0.272	0.251	0.298	0.377
RSD_r(%)	1.0	0.5	0.3	0.6	0.6
r	1.53	0.76	0.70	0.83	1.06
s_R	2.026	2.649	2.291	1.606	1.986
RSD_R(%)	3.6	4.8	3.0	3.1	3.3
R	5.67	7.42	6.41	4.50	5.56

n	= N° of laboratories retained after eliminating outliers
s_r	= standard deviation of repeatability
RSD_r(%)	= relative standard deviation of repeatability
r	= repeatability
s_R	= standard deviation of reproducibility
RSD_R(%)	= relative standard deviation of reproducibility
R	= reproducibility

Table 2: Results of the method performance study Page 2 of 2

Anthocyanin	sample 1	sample 2	sample 3	sample 4	sample 5
Peonidol-3-acetylglucoside					
n	14	16		14	16
mean	1.16	1.44		0.59	3.74
s_r	0.064	0.062		0.059	0.215

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RSD_r(%)	5.5	4.3		10.1	5.8
	0.18	0.17		0.17	0.60
s_R	0.511	0.392		0.272	0.374
RSD_R(%)	43.9	27.2		46.4	10.0
R	1.43	1.10		0.76	1.05
Malvidol-3-acetylglucoside					
n	16	17		17	16
mean	5.51	4.84		3.11	15.07
s_r	0.176	0.167		0.088	0.213
RSD_r(%)	3.2	3.4		2.8	1.4
r	0.49	0.47		0.25	0.60
s_R	0.395	0.366		0.496	0.617
RSD_R(%)	7.2	7.6		16.0	4.1
R	1.11	1.02		1.39	1.73
Peonidol-3-coumarylglucoside					
n	16	14		17	16
mean	1.26	0.90		0.89	1.32
s_r	0.130	0.046		0.060	0.058
RSD_r(%)	10.3	5.1		6.8	4.4
r	0.36	0.13		0.17	0.16
s_R	0.309	0.109		0.204	0.156

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RSD_R(%)	24.5	12.2		23.0	11.8
R	0.86	0.31		0.57	0.44
Malvidol-3-coumarylglucoside					
n	17	17		17	16
mean	4.62	2.66		4.54	4.45
s_r	0.159	0.055		0.124	0.048
RSD_r(%)	3.4	2.1		2.7	1.1
r	0.45	0.15		0.35	0.13
s_R	0.865	0.392		0.574	0.364
RSD_R(%)	18.7	14.7		12.6	8.2
R	2.42	1.10		1.61	1.02

n	= N° of laboratories retained after eliminating outliers
s_r	= standard deviation of repeatability
RSD_r(%)	= relative standard deviation of repeatability
r	= repeatability
s_R	= standard deviation of reproducibility
RSD_R(%)	= relative standard deviation of reproducibility
R	= reproducibility

Table 3: List of participants

ABC Labor Dahmen, Mülheim/Mosel	**D**

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Chemisches Landes- und Staatliches Veterinäruntersuchungsamt Münster	**D**
Institut für Lebensmittelchemie Koblenz	**D**
Institut für Lebensmittelchemie Speyer	**D**
Institut für Lebensmittelchemie Trier	**D**
Institut für Lebensmittelchemie und Arzneimittel Mainz	**D**
Labor Dr. Haase-Aschoff, Bad Kreuznach	**D**
Labor Dr. Klaus Millies, Hofheim-Wildsachsen	**D**
Labor Heidger, Kesten	**D**
Landesveterinär- und Lebensmitteluntersuchungsamt Halle	**D**
Staatliche Lehr- und Forschungsanstalt für Landwirtschaft, Weinbau und Gartenbau, Neustadt/Weinstraße	**D**
Staatliches Institut für Gesundheit und Umwelt, Saarbrücken	**D**
Staatliches Medizinal-, Lebensmittel- und Veterinäruntersuchungsamt, Wiesbaden	**D**
Laboratoire Interrégional de la D.G.C.C.R.F de Bordeaux, Talence/France	**F**
Unidad de Nutricion y Bromotologia, Facultad de Farmacia, Universidad de Salamanca, Salamanca/Espana	**E**
University of Glasgow, Div. of Biochem. and Molek. Biology	**UK**
Höhere Bundeslehranstalt und Bundesamt für Wein- und Obstbau, Klosterneuburg	**A**

17 Laboratories D (13); A (1); F (1); E (1); UK (1)

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