DETERMINATION OF PYRANOANTHOCYANINE AND MALVIDIN-3-GLUCOSIDE CONTENT IN RED WINE OF DIFFERENT VINTAGES VIA LC-MS/ESI

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The qualification of pyranoanthocyanins vitisin A, vitisin B and pinotin A regarding indicator for wine age has been discussed controversially for several years. In the present study the amount of malvidin-3-glucoside and pyranoanthocyanin pigments in red wine in general and their suitability as tracer substances of wine age were examined via LC-ESI-MS. The total monomeric anthocyanin content was measured employing the pH differential method. For statistical data evaluation canonical discriminant analysis was applied to the data sets. 124 authentic wines from ten vintage years were analysed. Content of total monomeric anthocyanins and malvidin-3-glucoside in particular decreased over time, whereas neither the absolute nor the relative amount of pyranoanthocyanins related to monomeric anthocyanins showed definite correlation, hence leading to the conclusion that the analysis of pyranoanthocyanins does not allow deductions about the age of authentic wines.
Keywords: malvidin-3-glucosides, pyranoanthocyanins, vitisin A, vitisin B, pinotin A, wine age

Bestimmung der Gehalte an Pyranoanthocyanen und Malvidin-3-glucosid in Rotwein unterschiedlicher Jahrgänge mittels ESI-MS. Die Eignung der Pyranoanthocyane Vitisin A, Vitisin B und Pinotin A als Altersindikator wird schon seit einiger Zeit kontroversiell diskutiert. Aus diesem Grund wurde der Gehalt von Malvidin-3-glucosid und den oben genannten Substanzen in österreichischen Rotweinen unterschiedlicher Jahrgänge mittels LC-ESI-MS bestimmt und die Ergebnisse mit dem Alter der Weine in Beziehung gesetzt. Des Weiteren wurde die Menge an monomeren Anthocyanen im Wein mit der pH-Differential-Methode photometrisch ermittelt. Untersucht wurden insgesamt 124 authentische Weine von zehn unterschiedlichen Jahrgängen. Die Daten wurden mit Hilfe der kanonischen Diskriminanzanalyse statistisch ausgewertet. Die Ergebnisse zeigten generell eine Abnahme der gesamten monomeren Anthocyane und speziell des Malvidin-3-glucosids im Laufe der Jahre, während weder der absolute Gehalt noch der relative Gehalt der einzelnen Pyranoanthocyane im Verhältnis zu den gesamten monomeren Anthocyanen einen Bezug zum Alter der Weine aufwies. Aus diesem Grund ist es nicht möglich diese Substanzen zweifelsfrei als Altersindikatoren heranzuziehen.
Schlagwörter: Malvidin-3-glucoside, Pyranoanthocyane, Vitisin A, Vitisin B, Pinotin A, Weinalter

Much effort is being made to control the colour and taste of red wines. Investigations revealed that coloured phenolic substances, so-called anthocyanins and pyranoanthocyanins, are mainly affecting the organoleptic properties of wine.

Anthocyanins consist of an anthocyanidin (aglycone) and a sugar moiety in 3' or 5' position (Eder, 2000; Vergara et al., 2010).

Anthocyanins can carry different substituents like hydroxyl or methoxyl groups and react readily with other phenolic compounds and small organic molecules in an aqueous environment to e.g. pyranoanthocyanins (Mateus et al., 2003). They are specified by a newly formed ring structure between the C4 and the hydroxyl group at C5. This leads to a broad variety of compounds. Skins of wine grapes contain anthocyanins, which are extracted into the wine during the fermentation process. During wine maturation a progressing chromatic shift from purple-red to orange-red can be observed referring to changing chromophoric properties due to formation of anthocyanin derivatives and condensates (Schwarz et al., 2003a; Mateus et al., 2006). Blanco-Vega et al. (2011) monitored formation of wine pyranoanthocyanins in model wine by HPLC-DAD-ESI-MS/MS using red grape skin extracts and wine fermentation metabolites They found that pyruvic acid reacted quickly with a high product yield, while acetaldehyde induced mainly pigment polymerization. Compared to their anthocyanin precursors, pyranoanthocyanins are characterized by a higher stability over a broad pH range and insensitivity for wine treatment methods like for example bleaching with sulphur dioxide (De Freitas and Mateus, 2010). However, by spiking wines samples with pinotin A it was discovered that this pyranoanthocyanin has no influence on overall perceived colour of red wines (Rentzsch et al.,2007).

Based on these observations anthocyanin derivatives became a target of intense research as putative chemical marker substances in the context of wine age. The anthocyanin malvidin-3-glucoside and some of its related pyranoanthocyanins such as vitisin A (a reaction product with pyruvic acid), vitisin B (a reaction product with acetaldehyde) and pinotin A (malvdin-3-glucoside-4-vinylcatechol - a reaction product with caffeic acid), have been investigated in several studies (Schwarz et al., 2003b; Aquirre et al., 2011). Pinotin A was isolated in "Pinotage" red wine by Rentzsch et al. (2007) and Asenstorfer et al. (2003). Schwarz et al. (2003b) found a maximum of vitisin A during fermentation which was stable up to 12 months and continued to decrease over the following years. Rentzsch et al. (2010) confirmed that the content of vitisin A is also decreasing during the maturation of 'Tempranillo' wines, whereas the content of hydroxyphenyl pyranoanthocyanins increases. In contrary, Aguirre et al. (2011) found an increase of vitisin A in 6, 7 and 8 year old 'Cabernet Sauvignon' red wine, concomitant with a decrease of free malvidin-3-glucoside. Vitisin B was investigated less frequently. It is usually present in concentrations of 0 to 2 mg/l shortly after fermentation depending on the applied yeast strains (Morata et al., 2003) or winemaking technology (Chinnici et al., 2009). Marx et al. (2003) noticed a relative increase of pinotin A compared to total anthocyanin over four years of vintages of 'Pinotage' wines. This data is in agreement with the results of Rentzsch et al. (2010), who found an overall increase over 10 years of vintages of 'Tempranillo' wines. Additionally investigations regarding monomeric anthocyanins showed that the substances decrease by 50 % after seven years, while the polymeric fraction doubled (Aguirre et al., 2011).

Assessment of these contradicting findings made it plain that closer investigation has to be conducted. This work aims to clarify the relationship between wine age and selected polyphenols. An increase of pyranoanthocyanins with a concomitant decrease of monomeric anthocyanins was expected.

MATERIAL AND METHODS

CHEMICALS AND STANDARDS

Methanol was purchased from J.T. Baker (Deventer, The Netherlands), acetonitrile from Promochem (Wesel, Germany) and malvidin-3-glucoside from Sigma Aldrich (Vienna, Austria). All chemicals were p.a. grade and all solvents were of high-performance liquid chromatography (HPLC) quality.

SAMPLE PREPARATION

124 authentic, unfined and microvinificated varietal red wines of ten different vintage years were vinified by the Federal College and Research Institute of Viticulture and Pomology in Klosterneuburg.

According to a standard protocol approximately 50 kg of grapes were destemmed, crushed and 30 mg/l SO_2 were added. A quick alcoholic fermentation was achieved by adding 20 g/hl of a selected dry yeast preparation (Oenoferm Klosterneuburg; Erbsloeh, Geisenheim, Germany). The end of alcoholic fermentation was detected by analysis of residual sugar with FT-IR with a Winescan 120 (Foss, Rellingen, Germany). Malolactic fermentation was induced by addition of a culture of

Oenococcus oeni. After a natural settling period of two weeks at 4 °C the wines were filtrated through K150 filter sheets (Seitz, Bad Kreuznach, Germany). The wines were stored in darkness at 4 °C.
Before analysis, the samples were diluted 1:2 in methanol and filtration by syringe filters (PTFE, 13 mm x 0.2 µm; Whatman, Piscataway, New Jersey, USA) was performed. The filtrate was diluted 1:10 by 10 mM ammonium formate buffer pH 3.75.

INSTRUMENTATION

Analysis was performed with a single quad HPLC-ESI-MS system (Shimadzu LCMS 2010 A; Shimadzu, Kyoto, Japan). Ionisation of the analytes was obtained via electrospray ionisation (ESI). Nitrogen served as desolvation and drying gas. Nebulizing gas flow was 2.0 l/min and drying gas flow 14.5 psi. Interface voltage was set to 500 V. The heat block and CDL temperature were 300 °C. The mass spectrometer was connected to a liquid chromatography system of Shimadzu (Kyoto, Japan), which consisted of a degasser DGU-14A, a binary gradient pump LC-10AD$_{VP}$ in low pressure mixing mode by a FCV-10AL$_{VP}$ valve, a SCL-10 A$_{VP}$ controller, an SIL-10 AP autosampler and a CTO-10 A$_{VP}$ column oven. The injector was set to full loop injection with 10 times overfill. Separation of the analytes was achieved with a C-18 reversed phase column (Rapid Resolution HT 2.1 x 50 mm, 1.8 µm particle diameter, Agilent). Solvent A was 98 % water, 1 % acetonitrile and 1 % formic acid. Solvent B was 98 % acetonitrile, 1 % water and 1 % formic acid. The flow rate was set to 0.5 ml/min, the column temperature was set to 60 °C and the injection volume was 5 µl. The gradient profile was 0.01 to 1.01 min 5 % B, from 1.01 to 7.10 to 32 % B, 7.11 to 8.60 95 % B and 8.61 to 5 % B. The total run time was 13 min.
LCMSolution software, version 3.40.299 was used for data processing and system control. Naringenin was added as internal standard. The mass to charge ratios (M+) were malvidin-3-glucoside (493), vitisin A (561), vitisin B (517), pinotin A (625) and naringenin (273). All substances were identified by the mass spectra of molecular and fragment ions and quantified by the ratio of area to the area of the internal standard. Samples were measured in triplicate and every fifth measurement a pooled control sample was analysed. Long-term stability was approx. 4 % RSD (24 h sequence).

PH-DIFFERENTIAL METHOD

Total monomeric anthocyanin content was measured with an Agilent 8453 UV-VIS Spectroscopy System (Agilent technologies, Santa Clara, California, USA) controlled by UV-VIS ChemStation Rev. B.01.01 [21] software. According to Lee et al. (2005), the absorbance was measured at 510 nm and 700 nm at pH 1.0, pH 3.5 and pH 4.5 each. Total anthocyanin content was quantified as malvidin-3-glucoside (extinction coefficient ε = 28,000 l/mol/cm).

STATISTICAL DATA EVALUATION

Statistical analysis was carried out by using SPSS 12.0 for windows (IBM, Armonk, New York, United States). Data were subjected to analysis of variance, and means were compared by t-test. Concerning discriminant analysis malvidin-3-glucoside, vitisin A, vitisin B and pinotin A were defined as variables. Wines of vintage years were arranged in groups (2000 to 2003, 2004 to 2007, 2008 to 2010).

RESULTS AND DISCUSSION

Equally treated and unfined wines from one single vineyard were investigated during long-term storage in a vertical row in this research. The concentrations of malvidin-3-glucoside, vitisin A, vitisin B and pinotin A were measured. In the ideal case, these concentrations are determined from one single bottle over years of storage, but this is not feasible due to the long study time and the possible impact on the wine sample by the sampling procedure. Accordingly, some compromises had to be made respecting climatic condition, harvesting time and the fermentation process. Nevertheless the influences of winemaking technique, soil, microclimatic conditions and growing site were kept to a minimum as wines are derived from the same vineyard each. The highest content of pyranoanthocyanins was 3.59 mg/l and most of the values are in the range from 0.1 mg/l to 0.6 mg/l (Table 1), which is lower than the results reported by Rentzsch et al. (2007).

ANALYSIS OF MALVIDIN-3-GLUCOSIDE AND PYRANOANTHOCYANINS VIA LC-MS

Thanks to the sub 2 µm-particle-diameter-stationary-phase, a fast separation could be obtained providing satisfying chromatographic separation of all investigated analytes as shown in Figure 1. Especially malvidin-3-glucoside (5.6 min), vitisin A (6.0 min) and vitisin B (6.2 min) exhibit a very similar retention behaviour due to their structural homology. Pinotin A (7.9 min) is far

Fig. 1: LC-MS-chromatogram of an authentic wine sample (for better visualisation magnification was applied, factors are shown corresponding to the M+)

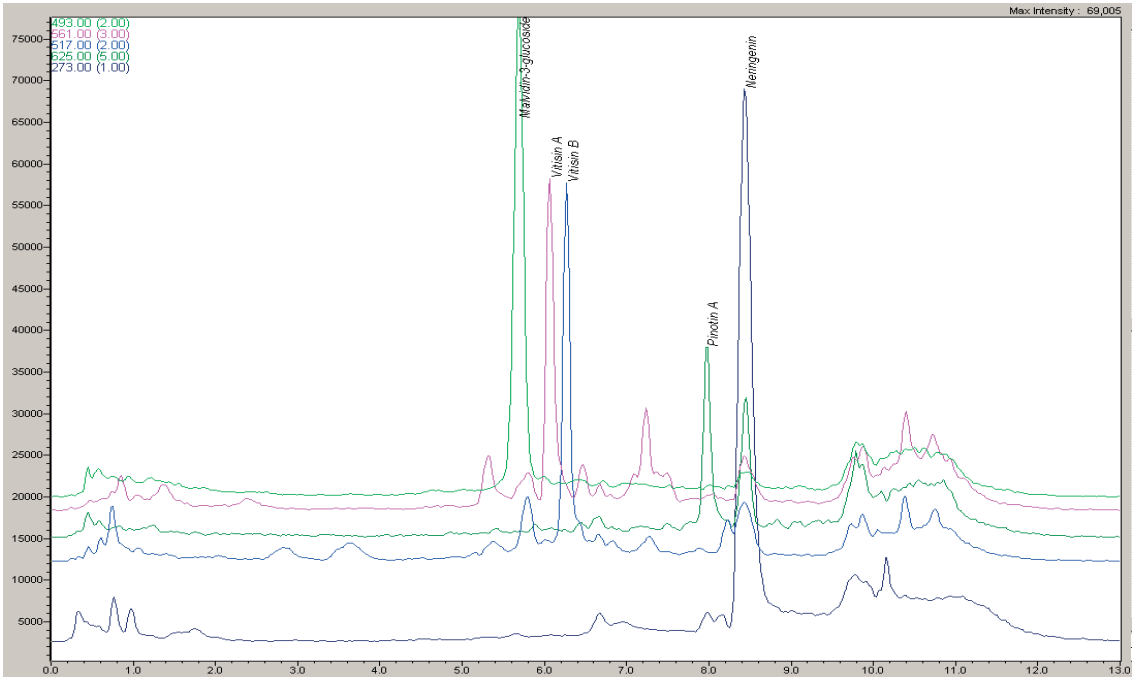
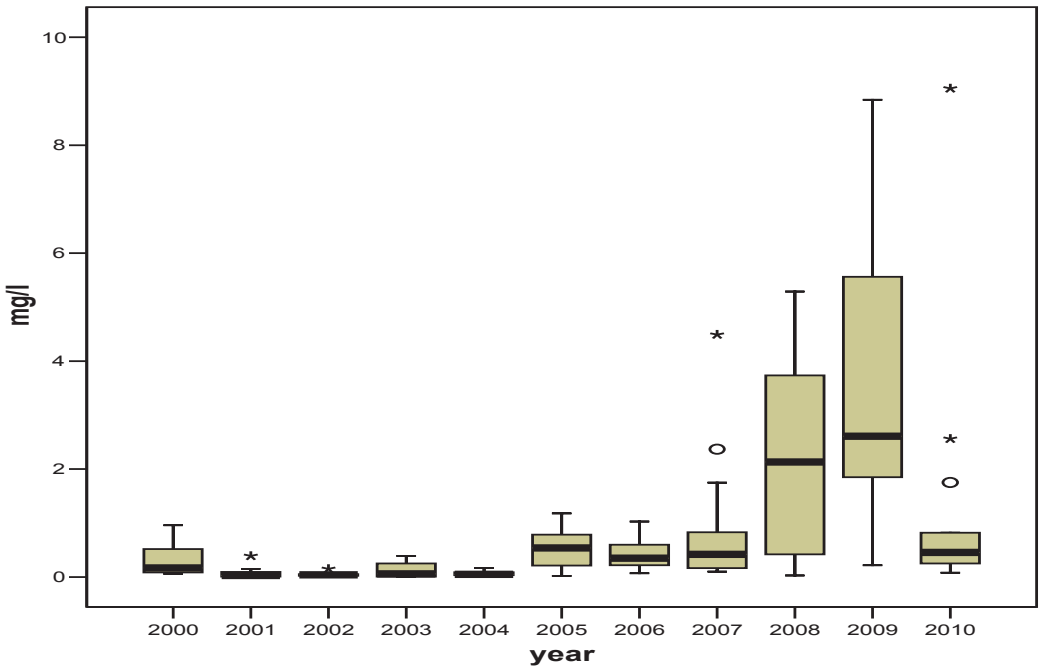


Fig. 2: Malvidin-3-glucoside content by different vintage years



more hydrophobic due to its dihydroxyphenyl moiety and thus represents the last eluting compound. Although naringenin (8.4 min) takes even longer to elute, it was the most appropriate internal standard because of its similar chemical nature and the fact that it is not present in wine samples.

Vitisin A, vitisin B, and pinotin A were identified according to their molecular ions (M+) and the main fragments in their mass spectra. The spectrum for vitisin A showed an M+ of 561 mass units and a fragment ion of 399 (aglycon fragment). This agrees with previous mass spectral analyses of vitisin A (Heier et al., 2002; Morata et al., 2007). The vitisin B spectrum showed an M+ of 517 mass units and a fragment ion of 355 (aglycon) (Heier et al., 2002; Morata et al., 2007). The molecular mass of pinotin A (M+) was found to be 625, what is in accordance with the identification of pinotin A made by Schwarz et al. (2003c).

For malvidin-3-glucoside the limit of detection was 3.57 µg/l and the limit of quantification was 12 µg/l. The absolute amounts of vitisin A and B and pinotin A were determined semi-quantitive by using the calibration of malvidin-3-glucoside. The limits of quantification were estimated as 50 µg/l. The method repeatability of the analysis was within 15 %.

RESULTS OBTAINED VIA LC-MS

The concentrations of the pigments in the wines were grouped according to vintage year and drawn as boxblots. Figure 2 clearly indicates the decrease of the malvidin-3-glucoside content during long-term storage. Only the value of the young wines from 2010 is untypical but can be explained by low wine quality due to unripe grapes. The average amount decreases within ten years to nearly 5 % of the original value. A similar behaviour was found for the content of vitisin A, which also decreased significantly with the age of the wines (Fig. 3). Since there is a big variability in the content of vitisin B and pinotin A in the younger wines, the correlation between age of wine and content was not so clear (Fig. 4 and Fig. 5).

These observations lead to the assumption that pyranoanthocyanines, especially vitisin A, are formed during and shortly after fermentation and are stable within the first years of storage, whereas the compounds are transformed or degraded after this period. These findings are coherent with Aguirre et al. (2011) only to some extent where concomitant to the decline of malvidin-3-glucoside no increase of vitisin A occurred. The reason for this may be found in the instance that only the variety 'Cabernet Sauvignon' of one particular winemaker was analysed thus providing a poor basis for comparison with authentic wines of different grape varieties. The work of Schwarz et al. (2003) confirms that the maximum content of vitisin A is reached within the first year of storage. In this report however vitisin A was still found in wine at 50 % of the initial concentration after 15 years of storage.

Considering the absolute content of anthocyanins in the wines, it can be asserted that in general the content of pinotin A is slightly lower than that of vitisin A and B (Table 1). Alcalde-Eon et al. (2006) do not substantiate this result because they found extraordinary high pinotin A levels in their samples. On the contrary Chinnici et al. (2009) determined pinotin A as the pyranoanthocyanine with the lowest concentration among the malvidin-3-glucoside derivatives. Since the formation of different pyranoanthocyanins is dependent on concentration of different wine substances as reaction partners (e.g. caffeic acid, pyruvate), the reasons for these differences could be explained by different varieties and vinification techniques (Eder et al., 2004).

Table 1: Mean concentrations of analytes according to different vintage years

Year	Mean [mg/l]			
	Mv3gl	Vit A	Vit B	Pin A
2010	31,82	0,65	1,28	0,32
2009	36,55	1,58	3,59	0,54
2008	28,90	0,61	2,22	0,19
2007	18,83	0,85	0,77	0,23
2006	20,00	1,33	0,44	0,18
2005	10,12	0,60	0,53	0,18
2004	11,06	0,50	0,07	0,35
2003	0,89	0,14	0,13	0,07
2002	0,75	0,08	0,05	0,29
2001	0,91	0,18	0,08	0,13
2000	1,62	0,42	0,32	0,13

DETERMINATION OF TOTAL MONOMERIC ANTHOCYANINS USING PH DIFFERENTIAL METHOD

There is a distinct decrease of the monomeric anthocyanin fraction by time as shown in Figure 6. The concentration of monomeric anthocyanins in young wines is as high as 150 mg/l and is reduced by more than 50 % after two years. This finding is in accordance with Aguirre et

Fig. 3: Vitisin A content by different vintage years

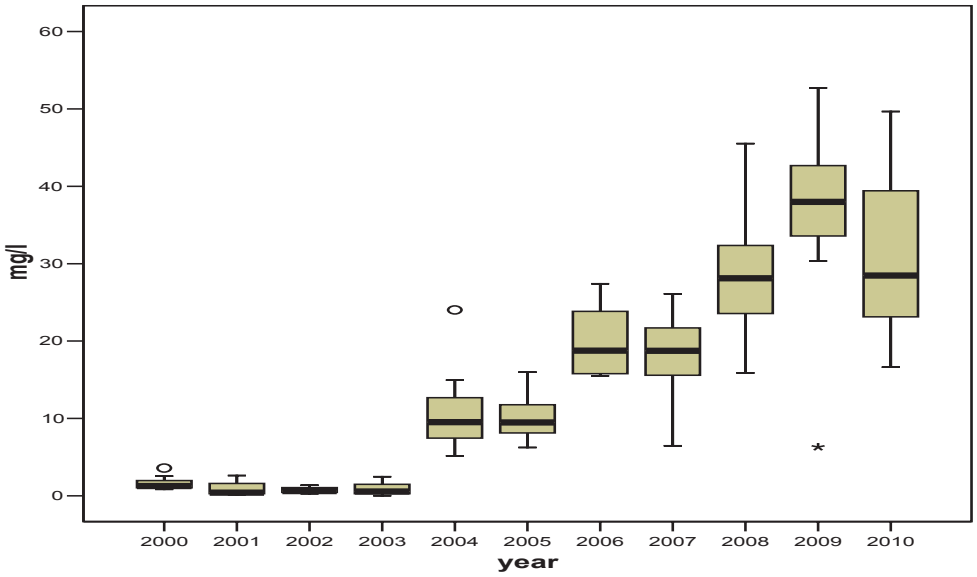


Fig. 4: Vitisin B content by different vintage years

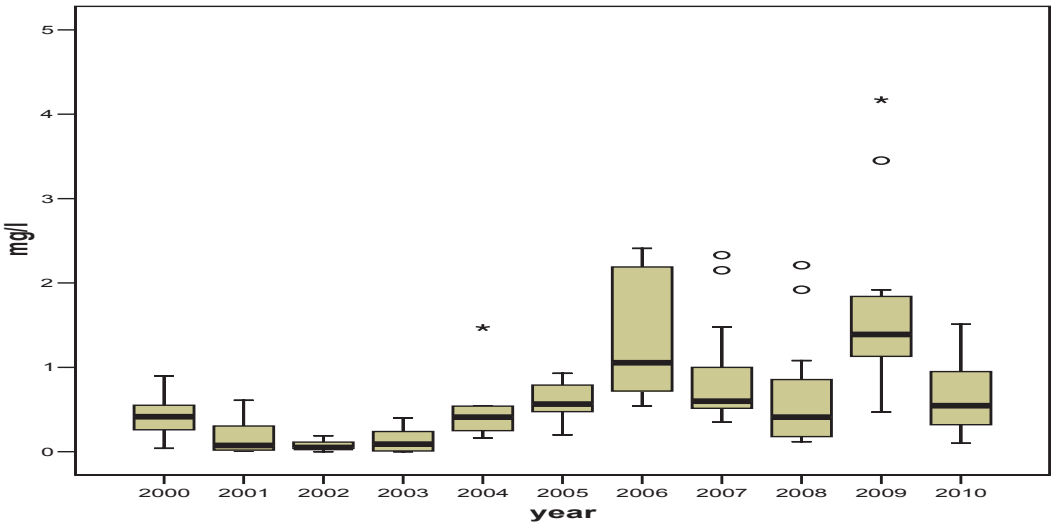


Fig. 5: Pinotin A content by different vintage years

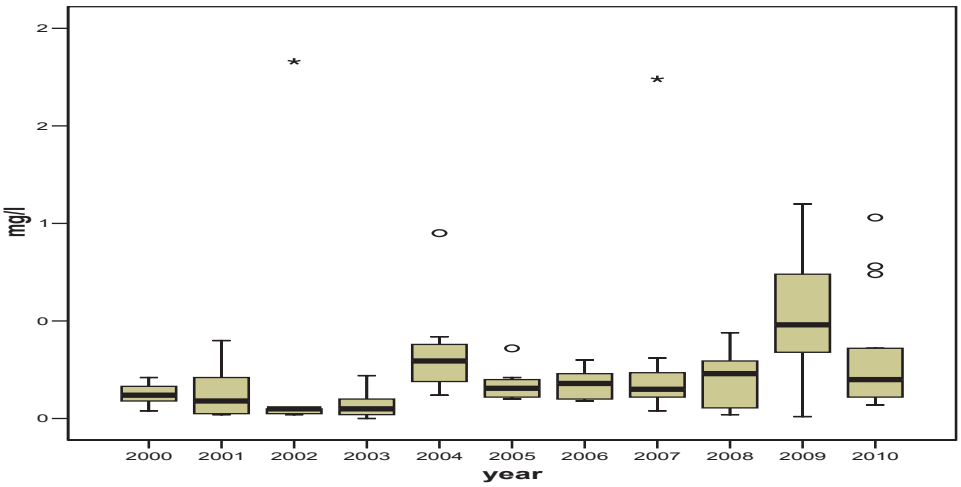


Fig. 6: Total monomeric anthocyanin content by different vintage years

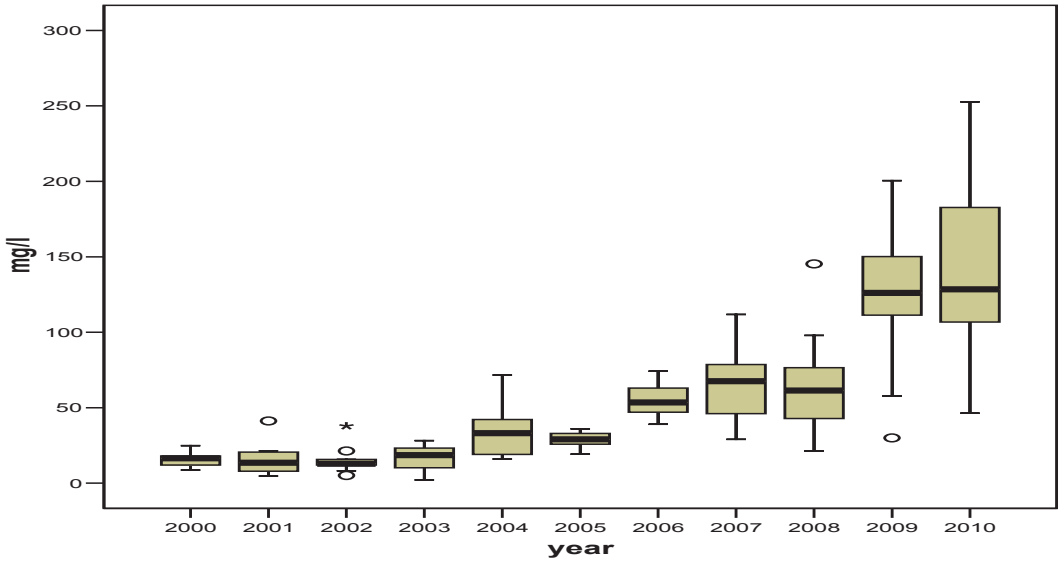
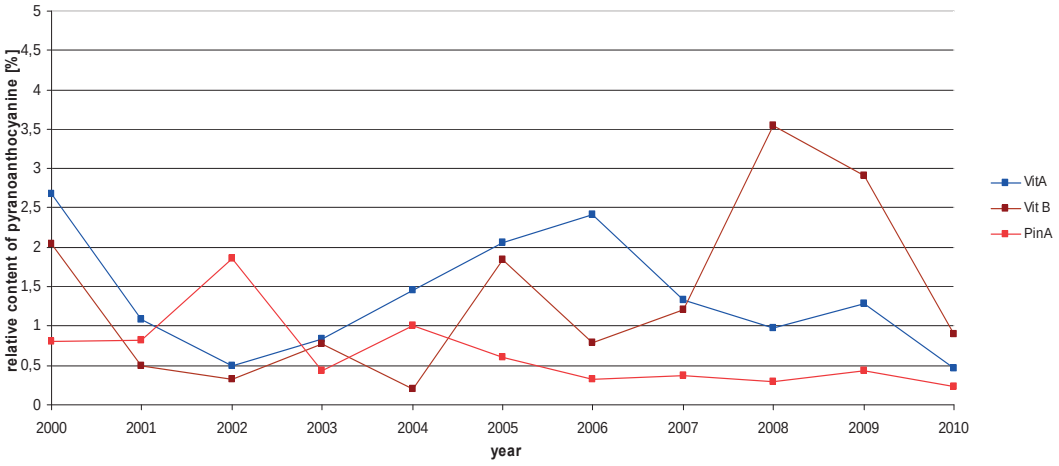


Fig. 7: Relative content of pyranoanthocyanins related to total monomeric anthocyanins



ASSESSMENT OF CHANGES REGARDING WINE AGE

al. (2011), who have also included the amount of polymeric anthocyanins in their work. They found that the monomeric fraction declines by 50 % during an eight years storage period while the polymeric fraction increases constantly.

The monomeric anthocyanins consist amongst others of malvidin-3-glucoside which accounts for 5 to 50 % of the monomeric anthocyanins depending on variety and vintage year (Eder et al., 1994). The percentage of the pyranoanthocyanins vitisin A, B and pinotin A is at maximum 3.5 % and seems to be independent from wine age (Fig. 7).

Finally it can be concluded that there is no connection between neither the absolute nor the relative amount of pyranoanthocyanins to vintage year (Fig. 3 to 5 and Fig. 7). Schwarz et al. (2003) reported a steady decline of vitisin A during 15 years of storage but remarked that the levels are slightly rising in the beginning. The fact that pinotin A concentrations are going up in dependence of wine age (Marx et al., 2003) could not be confirmed, though Marx et al. (2003) concede that total anthocyanins degrade at long term storage. In Figure 8 the result of the classification of the wines according to their age based on the content of pyranoanthocyanin is

shown. Even by expanding the time range to 2 to 3 years by grouping those wines in one class, no sufficient assignment is achieved. 65.6 % correctly classified cases without including the malvidin-3-glucoside proved to be not sufficient in a practical approach to determine the age of an unknown wine. The instability compared to their polymeric forms (De Freitas and Mateus, 2010) may be one reason against the suitability of monomeric pyranoanthocyanins as indicators of wine age. Since malvidin-3-glucoside correlates well with the age of the wine it contributes positively to the discriminant analysis shifting towards a better separation efficiency of the grouping functions (Fig. 9). Using the content of pyranoanthocyanins as well as that of malvidin-3-glucoside it was possible to correctly classify 88.8 % of the original cases according to their age.

Fig. 8: Canonical discriminant analysis of vitisin A, vitisin B and pinotin A

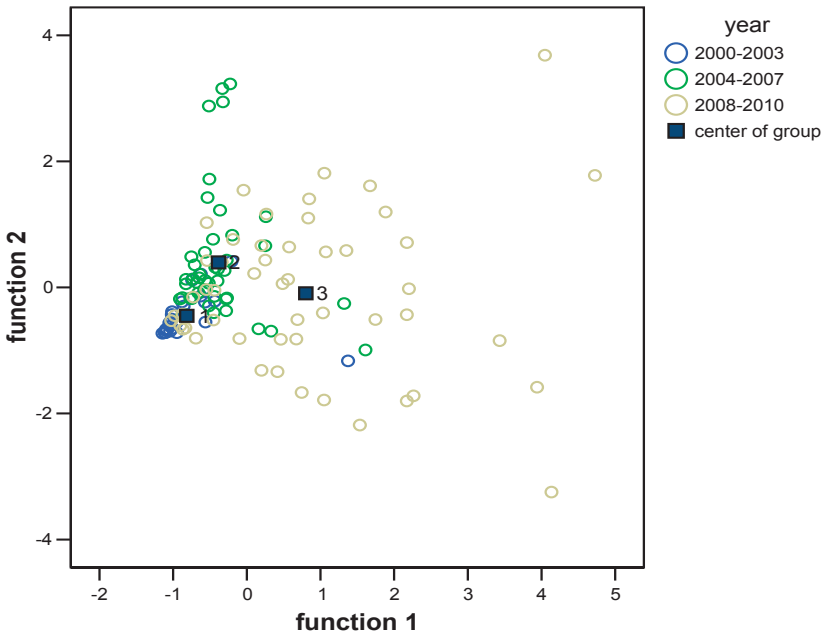
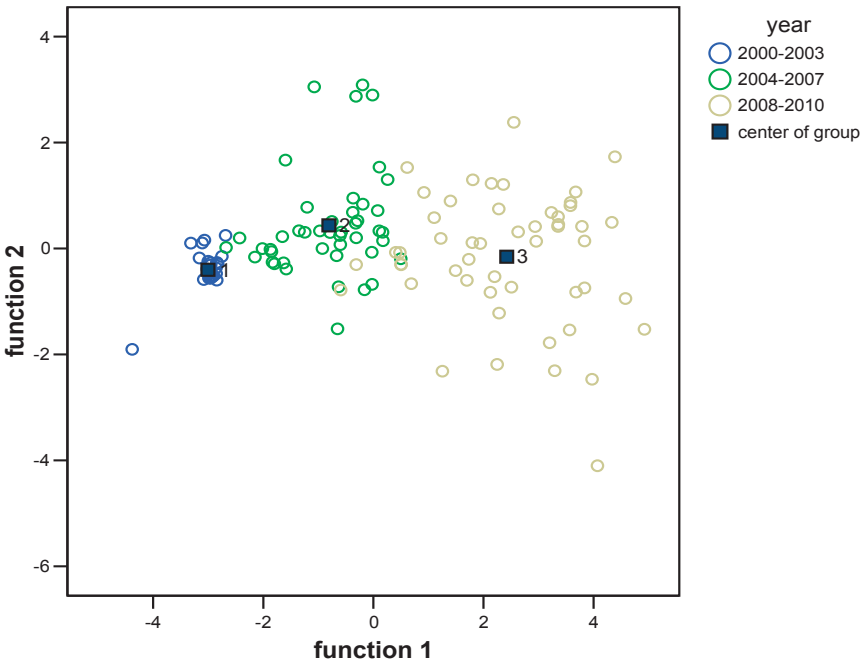


Fig. 9: Canonical discriminant analysis of vitisin A, vitisin B, pinotin A and malvidin-3-glucoside



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