Mitteilungen Klosterneuburg 59 (2009): 121-126

Determination of 2-aminoacetophenone in wines using the Stir Bar Sorptive Extraction method coupled with GC-MS and GC-NPD

Tatjana Košmerl and Emil Zlatić

University of Ljubljana, Biotechnical Faculty
SI-1000 Ljubljana, Jamnikarjeva 101
E-Mail: Tatjana.Kosmerl@bf.uni-lj.si

Untypical ageing off-flavour found in some white wines is due to increased formation of 2-aminoacetophenone (2-AAP). Laborious pre-treatments of the aromatic substance 2-AAP are required prior to analysis as it is present in wine only at trace levels. A stir bar sorptive extraction (SBSE) method was used to detect and quantify the concentration of 2-AAP simultaneously from small samples coupled with gas chromatography (GC) and nitrogen-phosphorous detection (NPD). This simple extraction technique has a low variation coefficient and low detection limits (0.020µg/l). Studies were conducted on widespread Slovenian white wines ('Welschriesling' and 'Chardonnay' from the Podravje winegrowing region, and 'Malvasia' and 'Chardonnay' from the Primorska winegrowing region) of 2005 and 2006 vintages. Under normal storage conditions (constant low cellar temperature and high relative humidity) a maximum concentration of 0.72µg/l 2-AAP was detected which was comparable to the maximum value (0.71µg/l) found after storage under stress storage conditions (higher temperature, lower humidity and UV-light). The average concentration of 2-AAP, however, was about 80 % lower in wines stored under normal conditions than in wines stored under stress conditions. Older wines contained significantly higher concentrations of 2-AAP in comparison to younger ones, but among all wines those from the variety 'Malvasia' exhibited highest values.
Keywords: extraction, GC-MS, 2-aminoacetophenone, 2-AAP, ageing, aroma compounds, white wines

Bestimmung von 2-Aminoacetophenonen in Weinen mittels Stir Bar Sorptive Extraction in Kombination mit GC-MS und GC-NPD. *UTA in Weißweinen entsteht auf Grund einer verstärkten Bildung von 2-Aminoacetophenonen (2-AAP). Da diese Aromasubstanzen in Weinen nur in Spuren vorkommen, sind vor der Analyse aufwändige Vorbehandlungen notwendig. Um das Vorhandensein und den Gehalt an 2-AAP gleichzeitig in geringen Probenmengen zu bestimmen, wurde eine Stir bar sorptive extraction (SBSE)-Methode in Kombination mit GC und NPD angewandt. Diese einfache Extraktionsmethode hat einen niedrigen Variationskoeffizienten und eine niedrige Nachweisgrenze (0,020µg/l). Slowenische Weißweine ('Welschriesling' und 'Chardonnay' aus der Weinbauregion Podravje; 'Malvasia' und 'Chardonnay' aus der Weinbauregion Primorska; Jahrgänge 2005 und 2006) wurden untersucht. Unter normalen Lagerungsbedingungen (konstant niedrige Kellertemperatur und hohe relative Luftfeuchtigkeit) wurde eine maximale Konzentration von 0,72µg/l 2-AAP bestimmt, was in etwa den Maximalwerten (0,71µg/l), die bei nachteiligen Lagerungsbedingungen (höhere Temperatur, niedrige Luftfeuchtigkeit und UV-Licht) festgestellt wurden, entspricht. Die durchschnittliche Konzentration von 2-AAP war jedoch in den Weinen aus normalen Lagerungsbedingungen um ca. 80 % niedriger. Ältere Weine enthielten signifikant höhere Konzentrationen an 2-AAP als jüngere, Weine der Sorte 'Malvasia' zeigten die höchsten Werte.*
Schlagwörter: Extraktion, GC-MS, 2-Aminoacetophenone, 2-AAP, ageing, Aromasubstanzen, Weißwein

Détection de 2-aminoacétophénones dans les vins à l'aide de la méthode Stir Bar Sorptive Extraction en combinaison avec GC-MS et GC-NPD. *Dans les vins blancs, l'UTA se produit à la suite d'une formation renforcée de 2-aminoacétophénones (2-AAP). Étant donné que ces substances aromatiques sont présentes dans les vins seulement sous forme de traces, des prétraitements sophistiqués sont nécessaires avant l'analyse. Afin de déterminer simultanément*

la présence de et la teneur en 2-AAP dans de petits échantillons, on s'est servi d'une méthode Stir bar sorptive extraction (SBSE) en combinaison avec GC et NPD. Cette méthode d'extraction simple possède un faible coefficient de variationá et une limite de détection basse (0,020µg/l). Les vins slovènes ('Welschriesling' et 'Chardonnay' de la région viticole Podravje; 'Malvasia' et 'Chardonnay' de la région viticole Primorska; millésimes 2005 et 2006) ont été examinés. Dans des conditions de stockage normales (en permanence, une température de cave basse et une humidité relative élevée), une concentration maximale de 0,72µg/l 2-AAP a été constatée, ce qui correspond approximativement aux valeurs maximales (0,71µg/l) constatées dans des conditions de stockage défavorables (température plus élevée, humidité basse et lumière UV). Pourtant, la concentration moyenne des 2-AAP était inférieure de 80 % dans les vins stockés dans des conditions normales. Les vins plus âgés contenaient des concentrations significativement plus élevées en 2-AAP que les vins plus jeunes, les vins du cépage 'Malvasia' présentaient les valeurs les plus élevées.

Mots clés: extraction, GC-MS, 2-aminoacétophénone, 2-AAP, ageing, substances aromatiques, vin blanc

The untypical ageing off-flavour of wine is due to increased levels of 2-aminoacetophenone (2-AAP). Previous research has found, that nitrogen fertilization, availability of water to the vine and climatic conditions significantly increase the concentration of 2-AAP in wine. This increase, however, was not significantly correlated with the concentration of the plant growth regulator (indol-3-acetic acid), which is the principal precursor of 2-AAP. Additionally, no indicator substance for a potential formation of 2-AAP has been identified in either grape must or wine of the variety 'Riesling' in Germany (Sponholz et al., 1997; Linsenmeier et al., 2007). In relations to plant physiology and microbiology it seems that the formation of 2-AAP is linked to plant stress (Huehn et al., 1999).

It is known from practical experience that an insufficient nitrogen fertilization of the vine can affect the sensory properties of wines resulting in bitter, astringent and unpleasant notes caused by increased concentrations of 2-AAP. The formation of these off-flavours can be prevented with an addition of ascorbic acid to musts and wines (Lorenzini, 2002) or by timely usage of „Würzburger UTAFIX-Test" for sensory detection of the untypical ageing note in young wines already after completion of alcoholic fermentation (Gessner et al., 1999). Investigations showed that the concentration of 2-AAP is usually very low immediately after alcoholic fermentation, but increases during subsequent storage especially at higher temperatures. Yeast can be an additional factor for the increased formation of 2-AAP because of its stress during alcoholic fermentation (Ciolfi et al., 1995; Dollmann et al., 1996; Huehn et al., 1999). Sponholz et al. (1997) reported that particularly yeasts of the species *Hanseniaspora (Kloeckera) apiculata* and *Metschnikowia pulcherrima* form higher amounts of 2-AAP in comparison to *Saccharomyces cerevisiae*. In model systems it was proved that 2-AAP was not formed during fermentation by *Saccharomyces cerevisiae* from L-tryptophane (Ciolfi et al., 1995) but from L-kynurenine (Dollmann et al., 1996).

The quantitative and qualitative analysis of volatile aroma compounds present in low concentrations in alcoholic drinks like wine is nowadays often performed using stir bar sorptive extraction (SBSE) or other extraction methods coupled with GC-MS (Atsushi et al., 2005; Kishimoto et al., 2005; Caven-Quantrill and Buglass, 2006 and 2007; Fan et al., 2007; Schmarr et al., 2007). According to literature the SBSE-GC-MS technique has generally a low variation coefficient, high accuracy and low detection limits (Kishimoto et al., 2005).

Materials and methods

Samples

Twenty-one samples of bottled white wines were supplied from three Slovenian wineries (Ptujska klet, Vinakoper and Brič). Studies were conducted with wines from the varieties 'Welschriesling' and 'Chardonnay' from the cooler Podravje-Štajerska Slovenija winegrowing district, and 'Malvasia' and 'Chardonnay' from the warmer Primorska-Slovenska Istra winegrowing district of 2005 and 2006 vintages. Wines were stored at two different conditions: under normal cellar storage conditions (constant low temperature of 8 °C, relative high humidity) and under stress storage condition (high temperature above 20 °C, low humidity, UV-light).

Chemicals

2-aminoacetophenone (Aldrich, Steinheim, Germany), 2,4-dichloroaniline (Merck, Darmstadt, Germany), ethanol (Lichrosolv, Merck, Darmstadt, Germany), so-

diumhydroxid (Lichrosolv, Merck, Darmstadt, Germany), dichloromethane (Merck, Darmstadt, Germany), methanol (Chromasolv, Sigma-Aldrich, Steinheim, Germany).
The stir bars (Twister) coated with PDMS phase (20 mm length, 1 mm thickness), were obtained from Gerstel (Mülheim an der Ruhr, Germany).

Instrumentation

All analyses were performed on a gas chromatograph (Agilent Technology GC 7890, Palo Alto, CA) equipped with a mass selective detector (5975 MSD, Agilent Technology, Palo Alto, CA) and nitrogen-phosphor selective detector (NPD detector, Agilent Technology, Palo Alto, CA). The instrument was equipped with a thermal desorption unit (TDU, Gerstel, Germany) and PTV inlet (CIS4, Gerstel, Germany). Stir bar introduction into the TDU unit was done by a MPS 2 autosampler (Gerstel, Germany).

Sample preparation

All wine samples were adjusted to pH 8.0 with 1 M NaOH before analysis. For each SBSE analysis an aliquot of 10ml of wine sample, spiked with 2,4-dichloroaniline solution at 2 µg/l level was placed in a headspace vial (27 ml volume). The stir bar was added and the sample was stirred at 700 rpm and a temperature of 1 °C for 120 min. After extraction, the stir bar was removed from the sampling vial, rinsed with Mili-Q purified water, dried with a lint free cloth and then introduced in a thermo desorption tube for GC/NPD or GC/MS analysis. Reconditioning of the stir bar was done by soaking in a mixture of dichloromethane and methanol (1:1) and thermal desorption at 300 °C (for one hour) in a flow of helium.

TDU-GC/NPD or MS analysis

The volatile compounds were desorbed from the stir bar at the following conditions: During TDU desorption step the PTV inlet was in solvent vent mode (split ratio 1:50, vent pressure 9.0 psi) and TDU was in splitless mode. The TDU initial temperature was set at 50 °C for 0.5 min, then increased to 280 °C at a rate of 60 °C/min and held for 5 min. The desorbed compounds were trapped in the PTV inlet at -40 °C. After the cryofocusing step the inlet was heated with 12 °C/min to 280 °C (held for 5 min) in splitless mode (splitless time 1.2 min). The GC separation was achieved on a fused silica capillary column (HP-5ms 30m with 0.25 mm internal diameter and 0.25 µm film thickness, Agilent Technology). The GC oven temperature was initially set at 40 °C (2 min isothermal), then ramped at 7 °C/min to 170 °C, followed by a second ramp which had a rate of 120 °C/min to a final temperature of 250 °C (5 min isothermal). Helium was used as a carrier gas at a flow rate of 1.2 ml/min. The NPD was operated at 280 °C with gas flows at 2.5, 7 and 100 ml/min for hydrogen (H_2), 7 ml/min for the make-up gas (nitrogen, N_2) and 100 ml/min for air. The mass selective detector was operated at 70 eV with electron impact ionization. The transfer line was set at a temperature of 280 °C. Electron impact mass spectra were acquired in full-scan (30-300 m/z) and single ion monitoring (SIM) mode (m/z 120, 135 for 2-AAP and m/z 161, 163 for 2,4-dichloroaniline).

Calibration

Calibration was done with synthetic wine solutions (12 % v/v of ethanol, 3.5 g/l of L-tartaric acid, pH 3.5) containing known concentrations of 2-AAP. The calibration covered the range from 0.045 µg/l to 2,280 µg/l (Fig.1) and gave a very good correlation (R^2 = 0.99975). The detection limit was 0.020 µg/l (LOD) and the limit of quantification 0.060 µg/l (LOQ).

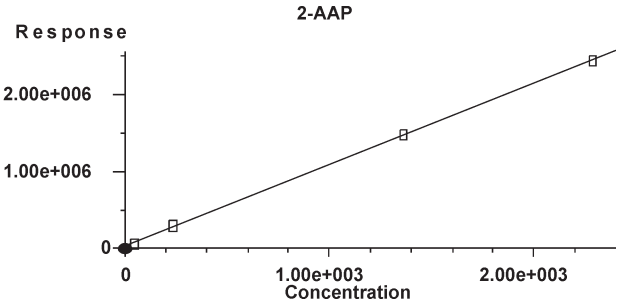

Fig. 1: Calibration curve for the determination of 2-AAP with TDU-GC/MS analysis

Results and discussion

The influence of sample volume, salt addition, extraction temperature and pH-value on the determination of 2-AAP with TDU-GC/MS analysis is shown in Table 1. The reference value, normalized to the value 1.0, was defined as a sample volume of 10 ml and an extraction temperature of 1 °C. The addition of 10 % NaCl had a strong negative influence on the result (0.16), but also increased sample volume (20 ml) at higher and low temperature (0.48 and 0.67, respectively) and redu-

ced the area response of 2-AAP in the GC analysis. Only in the case of a bigger sample volume (20 ml) and pH adjustment to 8.0 a significantly higher response (4.28) was observed. In general a Twister with the PDMS phase is used for the extraction of more non-polar analytes. Partitioning of polar compounds ($K_{OW} < 2$; octanol water partition coefficient) into the PDMS phase can be enhanced by adjusting the sample pH-value before extraction and salt addition.

Table 1: The influences of sample volume, salt addition, extraction temperature and pH value on normalized relative peak area of 2-AAP

Influence			Area response
Sample volume (10 ml) at	1 °C		1.00
Sample volume (10 ml) at	1 °C + 10 % NaCl		0.16
Sample volume (20 ml) at	1 °C		0.67
Sample volume (20 ml) at	25 °C		0.48
Sample volume (20 ml) at	1 °C	pH 8.0	4.28

The influence of the pH-value on the total ion chromatogram (TIC) of the GC-MS analysis is presented in Figure 2 where the upper blue curve represents the chromatogram achieved at the native pH-value of wine and the lower black one that with the wine adjusted to pH 8.0. The biggest effects of the pH adjustment were found for the extraction of octanoic acid (black arrow) and decanoic acid (red arrow). This is important because wine samples contain matrix compounds like octanoic, nonanoic and decanoic acid that partition into Twister PDMS phase and may interfere with the target substance during analysis. Partitioning of ionizable organic compounds into the PDMS phase of a Twister stir bar can be controlled by adjusting the pH-value of the sample before extraction (Fig. 2). Ionized organic species such as carboxylic acids and amines will not readily partition into the non-polar PDMS Twister phase at increased pH-values. Contrary, at the native pH-value of wine the extraction of organic acid into the PDMS phase will be enhanced. By raising the pH-value of the wine samples the amines are deprotonated and there extraction into the PDMS phase of the Twister will be enhanced while the extraction of organic acids will be reduced.

In Figure 3 the GC results achieved with the NPD equipment are shown, the upper curve represents the response for the wine sample at pH 8.00 and the lower chromatrogram that at native pH-values. The peak of 2-AAP is marked with the red arrow.

Reconditioning of the stir bar is a very important step to avoid carryover problems between different samples. A combined process consisting of a cleaning with a solvent mixture (dichloromethane/methanol) and a thermal desorption was best suited to avoid carryover by stir bar; just one thermal desorption during several analyses is not sufficient. The thermal desorption characteristics of the stir bar were assessed at two different tem-

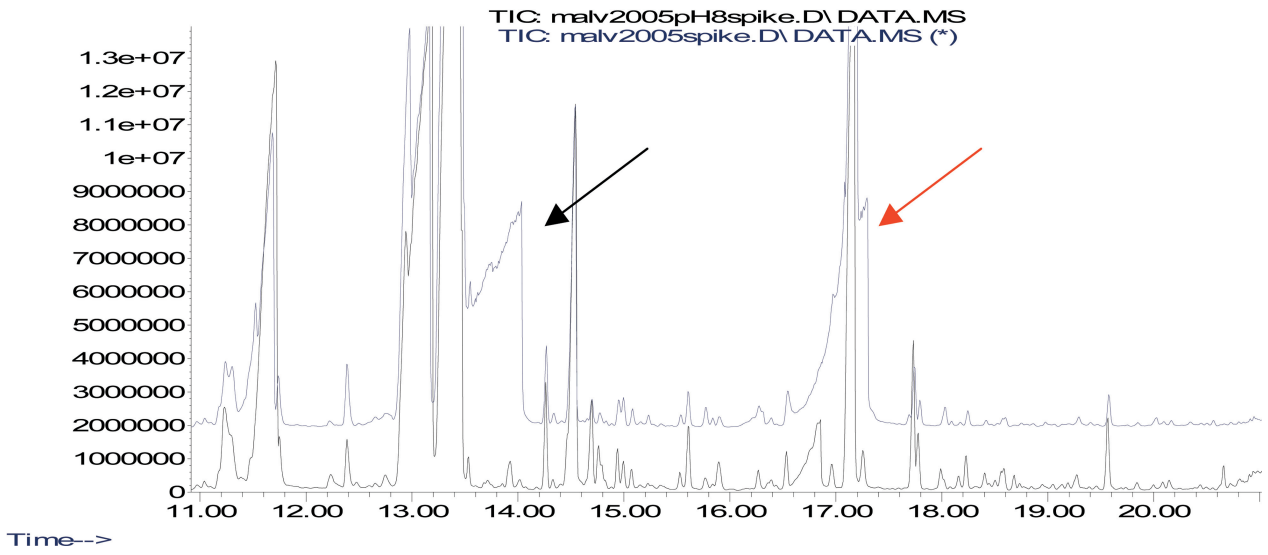

Fig. 2: The influence of pH-value on the total ion chromatogram (TIC) of a wine sample

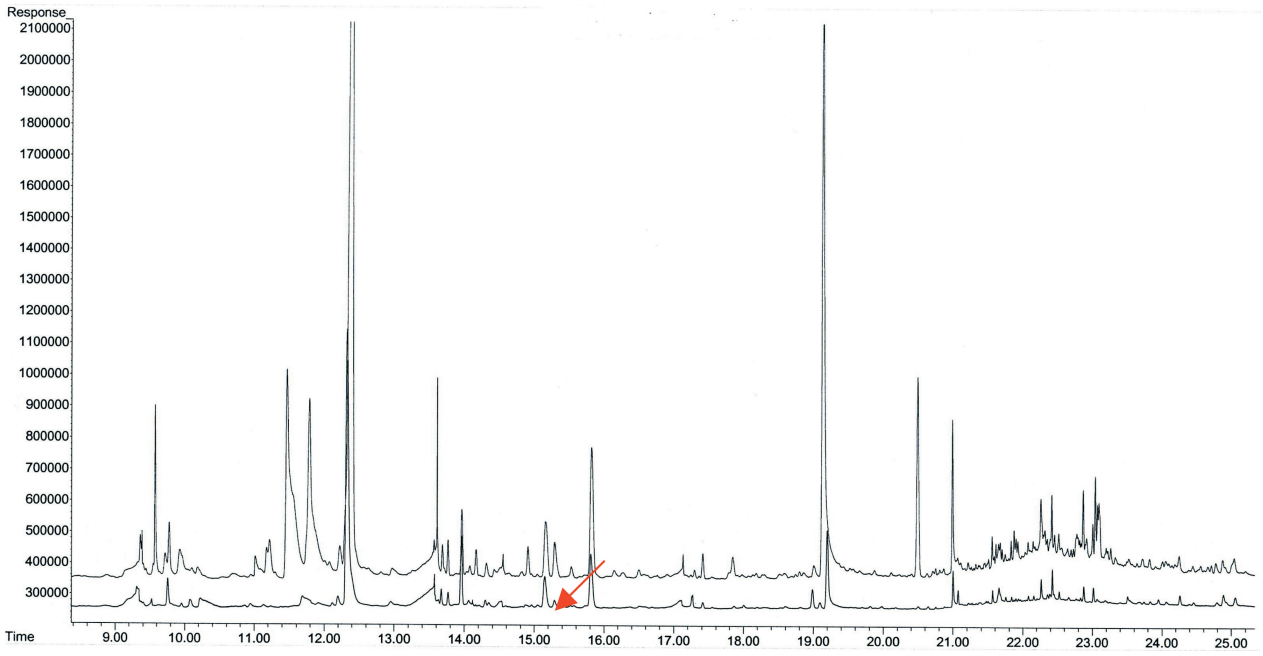

Fig. 3: The influence of pH-value on the NPD chromatogram of a wine sample

Table 2: The influence of temperature and desorption numbers on desorption percentages of 2-AAP and Internal Standard (IS)

Number of desorption	230 °C/5 min		280 °C/5 min	
	Desorption of			
	2-AAP (%)	IS (%)	2-AAP (%)	IS (%)
1	78.9	79.2	96.6	97.3
2	19.3	19.2	3.4	2.6
3	1.8	1.7	0.0	0.1

Table 3: Recovery study of 2-AAP in concentration range from 45 to 2,280 ng/l

Added 2-AAP (ng/l)	Determined 2-AAP (ng/l)	Recovery (%)
45	32.7	72.7
228	213.5	93.6
1,360	1,156	85.0
2,280	1,882	82.5

peratures and the results expressed as percentage desorption of 2-AAP and internal standard (2,4-dichloroaniline) are shown in Table 2. The results reveal that the higher temperature (280 °C) and at least two desorption steps are necessary for a satisfying recovery of 2-AAP. In a recovery study an average percentage of 2-AAP recovery of 83.5 % ± 8.6 ng/l (Tab.3) was measured.

The concentration of 2-AAP varied in the investigated wines from not detectable to 0.84 µg/l (Tab.4). Its average concentration for wines stored under normal (marked with A) and stress (marked with B) conditions was 0.23 µg/l and 0.42 µg/l, respectively. So one can conclude that storage of wine at ambient (stress) conditions causes an increase of the 2-AAP values by 0.19 µg/l (in average), that represents an increase of 82.6 %. The last three samples in Table 4 (number 10 to 12), however, have until now only been stored under normal conditions and among them sample 10 contained the highest 2-AAP content (0.84 µg/l).

Conclusions

The work has shown that the proposed TDU-GC/MS method is simple and sensitive and can eliminate laborious solvent extraction or other sample preparation steps.

In eighteen wine samples, stored under normal storage conditions (constant low cellar temperature and high relative humidity) a maximum concentration of 2-AAP of 0.72 µg/l was determined, which is comparable to the maximum value (0.71 µg/l) found under stress stor-

Table 4: Content of 2-AAP (µg/l) in wines investigated (n = 3)

Sample sign	Variety	Vintage	2-AAP (µg/l)
1A	Malvasia	2005	0.04
1B	Malvasia	2005	0.53
2A	Welsh Riesling	2005	0.33
2B	Welsh Riesling	2005	0.42
3A	Welsh Riesling	2006	0.08
3B	Welsh Riesling	2006	0.27
4A	Chardonnay	2005	0.35
4B	Chardonnay	2005	0.57
5A	Chardonnay	2006	0.07
5B	Chardonnay	2006	0.33
6A	Malvasia	2005	0.72
6B	Malvasia	2005	0.71
7A	Malvasia	2006	0.07
7B	Malvasia	2006	0.25
8A	Chardonnay	2005	0.30
8B	Chardonnay	2005	0.45
9A	Chardonnay	2006	0.15
9B	Chardonnay	2006	0.25
10	Malvasia	2005	0.84
11	Malvasia	2006	n.d.
12	Chardonnay	2006	n.d.

n.d. = not detectable

age conditions (exposed to higher ambient temperature, lower humidity and UV-light). The average concentration of 2-AAP, however, was more than 80 % (0.23 and 0.42 µg/l, respectively) lower in wines stored under normal storage conditions than in wines stored under stress conditions. Older wines of the 2005 vintage contained significantly higher 2-AAP contents than younger ones of the 2006 vintage (0.48 and 0.15 µg/l, respectively). Among the varieties, 'Malvasia' wines contained in average the highest concentration under both storage conditions, 0.40 µg/l in comparison to all the others varieties investigated with average content 0.27 µg/l.

Acknowledgement

The authors would like to express their sincere gratitude to the main enologists of Slovenian wine cellars (Mr. BOJAN KOBAL from the winery Ptujska klet vinarstvo d.o.o., Mr. IZTOK KLENAR from Vinakoper and Mr. BOŠTJAN ZIDAR from Wine cellar Brič), who provided the wine samples for this study. For financial support of application research project No. V4-0317 entitled "Prevention of untypical ageing off-flavour in Slovenian white wines" thanks to the Ministry of Agriculture, Forestry and Food and to the Slovenian Research Agency.

References

ATSUSHI, M., ATSUKO, I., HITOSHI, U. and HIROSHI, I., 2005: Identification of 2,4,6-trichloroanisole (TCA) causing a musty/muddy off-flavor in sake and its production in rice koji and *moromi* mash. *J. Biosci. Bioeng.* 100(2): 178-183

DOLLMANN, B., SCHMITT, A., KOECHLER, H. und SCHREIER, P. 1996: Zur Entstehung des „untypischen Alterstones" in Wein: Bildung von 2-Aminoacetophenon in Modellstudien mit Saccharomyces cerevisiae. *Wein-Wiss.* 51(2): 122-125

CAVEN-QUANTRILL, D.J. and BUGLASS, A.J. 2006. Comparison of micro-scale simultaneous distillation-extraction and stir bar sorptive extraction for the determination of volatile organic constituents of grape juice. J. Chromatogr. A 1117: 121-131

CAVEN-QUANTRILL, D.J. and BUGLASS, A.J., 2007: Determination of volatile organic compounds in English vineyard grape juices by immersion stir bar sorptive extraction-gas chromatography/mass spectrometry. Flavour and Fragrance J. 22(3): 206-213

CIOLFI, G., GAROFOLO, A. and DI STEFANO, R. 1995: Identification of some o-aminophenones as secondary metabolites of *Saccharomyces cerevisiae*. Vitis 34(3): 195-196

FAN, W., TSAI, I.M. and QIAN, M.C. 2007: Analysis of 2-aminoacetophenone by direct-immersion solid-phase microextraction and gas chromatography-mass spectrometry and its sensory impact in Chardonnay and Pinot gris wines. Food Chemistry 105(3): 1144-1150

GESSNER, M., KOEHLER, H.J., NAGEL-DERR, A., CHRISTOPH, N., SIMAT, T. and KRELL, K. 1999: The atypical ageing note. Dt. Weinmagazin (19): 12-14, 16-17

HUEHN, T., SPONHOLZ, W.R. und GROßMANN, M. 1999: Freisetzung unerwünschter Aromastoffe aus Pflanzenhormonen bei der alkoholischen Gärung. Wein-Wiss. 54(4): 105-113

KISHIMOTO, T., WANIKAWA, A., KAGAMI, N. and KAWATSURA, K. 2005: Analysis of hop-derived terpenoids in beer and evaluation of their behavior using the stir bar-sorptive extraction method with GC-MS. J. Agric. Food Chem. 53(12): 4701-4707

LINSENMEIER, A., RAUHUT, D., KUERBEL, H., SCHUBERT, S. and LOHNERTZ, O. 2007: Ambivalence of the influence of nitrogen supply on o-aminoacetophenone in 'Riesling' wine. Vitis 46(2): 91-97

LORENZINI, F. 2002: Effect of ascorbic acid on the 'stress characteristic' of wines. Tests on Chasselas. Rev. Suisse Vitic. Arboric. Hortic. 34(5): 299-302

SCHMARR, H.G., GANß, S., SANG, W. and POTOURIDIS, T. 2007: Analysis of 2-aminoacetophenone in wine using a stable isotope dilution assay and multidimensional gas chromatography-mass spectrometry. J. Chromatogr. A 1150(1/2):78-84

SPONHOLZ, W.R., HUEHN, T., ENGELMANN, A. und SIBEN, A. 1997: Mögliche Einflüsse weinbaulicher Parameter auf die Ausbildung des „Untypischen Alterungstons" bei Rieslingweinen. Wein-Wiss. 52(1): 41-50

Received September 09, 2009