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Analysis and Chemistry of Migrants from Wine Fining Polymers

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Fining of wine is prerequisite for the long-term stability of wine. Methods used are based not only on natural products, for example, proteins, but also on synthetic polymers such as polyvinylpolypyrrolidone (PVPP). Recently, new materials have been developed to overcome the disadvantageous use of traditional bluefining. These include polyvinylimidazole−polyvinylpyrrolidone copolymers (PVI/PVP) to combine the benefits of PVPP with selective binding of metals such as copper or iron. This work developed a HPLC-MS^2 method to monitor the potential migration of monomers and respective degradation products *N*-vinylimidazole, *N*-vinyl-2-pyrrolidone, imidazole, and 2-pyrrolidone in wine. Use of 0.5 g/L PVPP led to <83 μg/L 2-pyrrolidone in a wine model solution within 30 min, whereas PVI/PVP resulted in nondetectable quantities of 2-pyrrolidone and 18 μg/L imidazole. Unexpectedly, the analysis of 140 wines revealed 2-pyrrolidone as a natural constituent. Independent model incubations verified 4-aminobutyramide and 4-aminobutyric acid as the immediate precursors.

KEYWORDS: Fining; migrants; PVPP; imidazole; 2-pyrrolidone; *N*-vinylimidazole; *N*-vinyl-2-pyrrolidone; wine

INTRODUCTION

The production of wine includes fining as an essential step to improve color, flavor, and stability. Besides clarification proteins, tannins and tannin-like structures such as polymerized anthocyanins, low molecular polyphenols, off-flavors, and metals are targets to be removed. The reagents include not only inorganic materials, for example, bentonite, silica sol, and potassium hexacyanoferrate(II), but also organic materials, for example, activated charcoal, gelatin, isinglass, casein, egg albumin, and algae extracts. Also, synthetic polymers such as polyvinylpolypyrrolidone (PVPP) were developed to improve reproducibility and handling. Fining has to be used with great care, and often only combinations of different materials result in optimized wines.

Increased concentrations of copper or iron cause metallic taste and changes in color or precipitates and have to be reduced to below 0.5 and 5 mg/L, respectively (*1*). Bentonites and proteins, two of the most widely applied fining materials, also have the ability to remove metals. However, the metal-binding capacity strongly depends on the used type, and concentrations of other metals such as calcium and zinc increase during treatment (*2*, *3*). The traditional method to remove metals from wine quantitatively is bluefining with potassium hexacyanoferrate(II) (*4*). Bluefining has major disadvantages. Fining of wines with high copper but low iron concentrations is almost impossible. For quantitative precipitation of copper, the iron content has to be elevated. This can only be achieved by blending with wines of high iron concentration as direct addition is legally prohibited. Disposal of toxic waste and overfining are further problems. Excessive use of potassium hexacyanoferrate(II) causes the formation of free toxic cyanides in wine. As a result, overfined wines are no longer marketable. Thus, only enologists and trained technicians are allowed to apply bluefining (*5*). A promising alternative is polyvinylimidazole−polyvinylpyrrolidone copolymers (PVI/PVP), which are yet not approved within the European Union (EU). These resins consist of *N*-vinylimidazole and *N*-vinyl-2-pyrrolidone in the ratio of 9:1 and hold functional groups, which, in addition to PVPP-like characteristics, selectively bind metals such as copper or iron. Previous studies confirmed that small amounts (50 g/hL) of PVI/PVP are indeed sufficient to reduce concentrations of metals to the levels mentioned above. At the same time, important flavor ingredients such as organic acids or polyphenols were hardly influenced (*1*, *5*−*9*).

All synthetic polymers contain residual monomers, impurities, or degradation products (*10*). Thus, with the use of established and new polymers in the making of wines, and also juices and beer, analytical methods to monitor the potential migration of possible residual monomers need to be developed. With the present work a sensitive LC-MS^2 procedure was established with minimal sample workup. PVPP and PVI/PVP polymers were monitored for migrants in incubations of wine model solutions. 2-Pyrrolidone was verified as a degradation product of *N*-vinyl-2-pyrrolidone in fining polymers and as a natural wine ingredient originating from the biogenic amines of glutamine and glutamic acid.

MATERIALS AND METHODS

Materials. The following chemicals of analytical grade were commercially available: heptafluorobutyric acid (Acros Organics, Beerse, Belgium); acetic acid, ammonium solution, formic acid, and methanol (Aldrich/Sigma-Aldrich, Steinheim, Germany); 4-aminobutyric acid and tartaric acid (Merck, Darmstadt, Germany); ethanol, glutamic acid, L-glutamine, and sodium hydroxide (Roth, Karlsruhe, Germany); and 4-aminobutyramide (Enamine, Kiev, Ukraine).

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Divergan HM (both PVI/PVP samples) and Divergan F (PVPP 1) as well as imidazole, 2-pyrrolidone, *N*-vinylimidazole, and *N*-vinyl-2-pyrrolidone were kindly supplied by BASF SE (Ludwigshafen, Germany). PVPP 2−6 were obtained from Jarchem Industries Inc. (Newark, NJ).

Analytical HPLC-MS^2. A Jasco PU-2080 Plus quaternary gradient pump unit, with degasser, and a Jasco AS-2057 Plus autosampler (Jasco, Gross-Umstadt, Germany) were used. Chromatographic separation was carried out on a stainless steel column (Eurospher 100-5 C18, 250 × 4.6 mm) by Knauer (Berlin, Germany) using a flow rate of 1.0 mL/min at 25 °C. The mobile phase used was water (solvent A) and water/methanol 3:7 (v/v, solvent B). To both solvents heptafluorobutyric acid (0.6 mL/L) was added. Samples were injected at 15% B (held for 10 min); the gradient then changed linearly to 100% B in 5 min (held for 10 min) and then changed to 15% B in 5 min (held for 15 min). The mass analyses were performed using an Applied Biosystems API 4000 quadrupole instrument (Applied Biosystems, Foster City, CA) equipped with an API source using an electrospray ionization (ESI) interface. The LC system was connected directly to the probe of the mass spectrometer. Nitrogen was used as sheath and auxiliary gas. The optimized parameters for mass spectrometry (positive ion mode) to achieve maximum sensivitiy were as follows: curtain gas, 40 psi; collision gas, medium; ion spray voltage, 2500 V; temperature, 550 °C; ion source gas 1, 60 psi; ion source gas 2, 60 psi; entrance potential, 10 V; collar 2, 0 V. The remaining parameters are given in **Table 1**. Samples were diluted to proper concentrations with water and subjected directly into the HPLC-MS^2 without any workup. For quantification a matrix calibration in white and red wine according to the standard addition method was used. Data obtained by HPLC-MS^2 showed coefficients of variation of < 5%.

Wine Model Solution. Four grams of tartaric acid, 0.1 g of acetic acid, and 120 mL of ethanol were dissolved in 800 mL of water. After adjustment of the pH value to 3.2 with 2 N sodium hydroxide, the solution was made up to 1000 mL.

Migration Tests. Amounts of 0.5 and 10 g of PVI/PVP or PVPP were stirred in 1 L of wine model solution for 5, 15, and 30 min at 5 °C. Prior to analysis, solutions were filtered.

Incubations of 2-Pyrrolidone, 4-Aminobutyric Acid, and 4-Aminobutyramide. Five hundred milligrams of 2-pyrrolidone, 500 mg of 4-aminobutyric acid, and 2 mg of 4-aminobutyramide were dissolved in 1 L of wine model solution, respectively, and incubated for 190 days at 50 °C.

Wine Samples. One hundred and forty wine samples (72 white, 62 red, 6 rosé) produced in several regions (Australia (3), Austria (1), Bulgaria (1), Chile (5), France (14), Germany (70), Hungary (7), Italy (20), Macedonia (1), Mexico (1), Portugal (1), Romania (1), Spain (7), South Africa (4), United States (4)) from various grape varieties were analyzed: 92 of them were dry, 22 were semisweet, and 26 were sweet wines (classification based on labeling information). Samples included 1 wine of 1991 vintage, 1 wine of 1995, 1 wine of 1997, 1 wine of 2000, 2 wines of 2001, 2 wines of 2002, 2 wines of 2003, 4 wines of 2004, 10 wines of 2005, 33 wines of 2006, 66 wines of 2007, and 11 wines of 2008; the remaining 4 were of unknown year of production. Finally, 3 wines originated from organic vineyards (EcoVin). (See the Supporting Information for more details regarding the studied wines.)

Table 1. Mass Spectrometer Parameters[a]

compound	Q1 mass (amu)	Q3 mass (amu)	dwell time (ms)	DP	CE	CXP
imidazole	69.08	42.20	75.00	81.00	31.00	2.00
2-pyrrolidone	86.10	44.10	75.00	66.00	31.00	6.00
	86.10	69.00	75.00	66.00	23.00	4.00
N-vinylimidazole	95.09	41.10	75.00	71.00	33.00	0.00
	95.09	69.20	75.00	71.00	29.00	12.00
N-vinyl-2-pyrrolidone	112.08	69.20	75.00	51.00	21.00	4.00
	112.08	84.00	75.00	51.00	17.00	14.00

[a] DP, declustering potential; CE, collision energy; CXP, cell exit potential.

Incubation of *N*-Vinyl-2-pyrrolidone. A solution of 2 mg/L *N*-vinyl-2-pyrrolidone in wine model solution (pH 3.2) was incubated for 86 h at ambient temperature.

Statistics. Data are given as means of measurement of three independent incubations. In-house validation was based on OENO-7-2000 (OIV).

By means of multivariate data analysis correlations between wine parameters (color, vintage, producing area, alcoholic strength, sugar content, vintage wine) and levels of target compounds (2-pyrrolidone, 4-aminobutyric acid, 4-aminobutyramide, glutamine, glutamic acid) were established. β coefficients qualify the impact of one variable on another by adjusting all other variables and are given in parentheses. The maxima are −1.000 for a negative correlation and +1.000 for a positive correlation. All reported correlations are significant ($p < 0.05$). Chemometric analyses of the data were performed using SPSS 17.0.

RESULTS AND DISCUSSION

Method Validation. To study the migration of imidazole, 2-pyrrolidone, and *N*-vinylimidazole as well as *N*-vinyl-2-pyrrolidone, measurements in MRM-mode of HPLC-MS^2 were done. The retention times of imidazole, 2-pyrrolidone, *N*-vinylimidazole, and *N*-vinyl-2-pyrrolidone are 7.3, 8.4, 14.3, and 17.6 min, respectively. For every compound, except imidazole, two mass transfers, a quantifier and a qualifier, were selected. Due to the small molecular mass of imidazole, there was only one useable mass transfer. In addition, peak retention times were used for verification.

The method was validated in-house. Validation data can be gathered from **Table 2**. Reproducibility, repeatability, and recovery were ascertained with three different fortification limits (20, 50, and 80 μg/L for imidazole; 90, 150, and 250 μg/L for 2-pyrrolidone; 8, 12, and 16 μg/L for *N*-vinylimidazole and *N*-vinyl-2-pyrrolidone) in white, red, dry, and sweet wines on four different days. For determination of the limits of detection and quantification resolution OENO-7-2000 (The International Organisation of Vine and Wine (OIV); http://news.reseau-concept.net/images/oiv/client/Resolution_Oeno_FR_2000_07.pdf) was used. Interestingly, *N*-vinyl-2-pyrrolidone resulted in unsatisfactory values for reproducibility, repeatability, and recovery.

Degradation of *N*-Vinyl-2-pyrrolidone in Wine. Detailed experiments revealed that *N*-vinyl-2-pyrrolidone is not stable in acidic aqueous−ethanolic solutions such as wine in contrast to the other structures measured in the method mentioned above. This resulted in poor recoveries for *N*-vinyl-2-pyrrolidone during validation. Three different compounds with molecular masses of 85, 129, and 157 could be identified. Denisov (*11*) verified the reaction between *N*-vinyl-2-pyrrolidone and methanol as well as water in acidic solutions via nuclear magnetic resonance spectrometry. According to this, m/z 130 $[M + H]^+$ was assigned to the water adduct and m/z 158 $[M + H]^+$ to the ethanol adduct by HPLC-MS^2 (**Figure 1**). In addition, m/z 86 $[M + H]^+$ was verified as 2-pyrrolidone by authentic standard. The formation of 2-pyrrolidone from *N*-vinyl-2-pyrrolidone was explained by Denisov (*11*) by loss of acetaldehyde

Table 2. Validation Data

compound	limit of detection (μg/L)	limit of quantification (μg/L)	reproducibility (%)	repeatability (%)	recovery (%)	linearity
imidazole	5	12	5	3	101	0.9996
2-pyrrolidone	25	83	16	9	102	0.9988
N-vinylimidazole	2	6	4	3	102	0.9993
N-vinyl-2-pyrrolidone	2	6	29	24	65	0.9993

from the water adduct. The kinetics of degradation of *N*-vinyl-2-pyrrolidone and of formation of the follow-up products are shown in **Figure 2** and confirm the published data. *N*-Vinyl-2-pyrrolidone was degraded almost completely within 85 h to 2.5% of the initial concentration. At the same time, water and ethanol acetal adducts as well as small amounts of 2-pyrrolidone (2 mol % based on *N*-vinyl-2-pyrrolidone) emerged. Exact determination of the acetals was impossible because reference standards of the adducts were not available. In addition, no *N*-vinyl-2-pyrrolidone but the acetal degradation products were found in wine migration tests with fining polymers.

Migration Tests of PVPP and PVI/PVP. In EU directive 97/48/EC conditions for migration tests of materials in contact with foods, for example, polymers or films, are given. Following the directive an acidic−ethanolic solution at 5 °C for 30 min had to be used as conditions relevant to the present study. The maximum amount of application of PVI/PVP is 0.5 g/L. To exceed the limit of quantification, 10.0 g/L was therefore used for the migration tests. For comparison, PVPP, a frequently used fining polymer, was tested under the same conditions. **Table 3** gives the complete results of migration tests for 8 different samples of both polymers (2 × PVI/PVP, 6 × PVPP). In PVPP samples concentrations of 2-pyrrolidone between 69 and 108 mg/kg were detected. In contrast, during contact with PVI/PVP, 2-pyrrolidone was not quantifiable. This must be due to the low contents of *N*-vinyl-2-pyrrolidone in this polymer (ratio 9:1). However, 34−36 mg/kg imidazole migrated into the wine model solution. The concentrations stayed almost the same after 5 and 30 min. These results clearly demonstrate that major parts evolve already within the first 5 min, after which levels increase only very slowly. This might be relevant to the actual wine production, when contact times are shorter than 30 min. Comparative migration tests with ethanol-free solutions led to similar concentrations of the migrants.

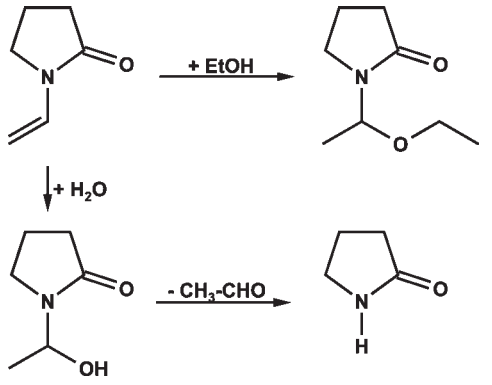

Figure 1. Reactions of *N*-vinyl-2-pyrrolidone with water and ethanol.

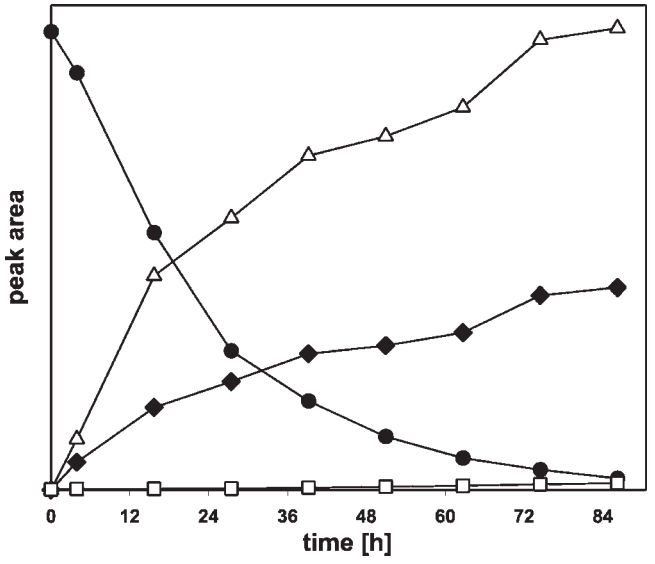

Figure 2. Formation of reaction products of *N*-vinyl-2-pyrrolidone (●): (△) water adduct; (◆) ethanol adduct; (□) 2-pyrrolidone.

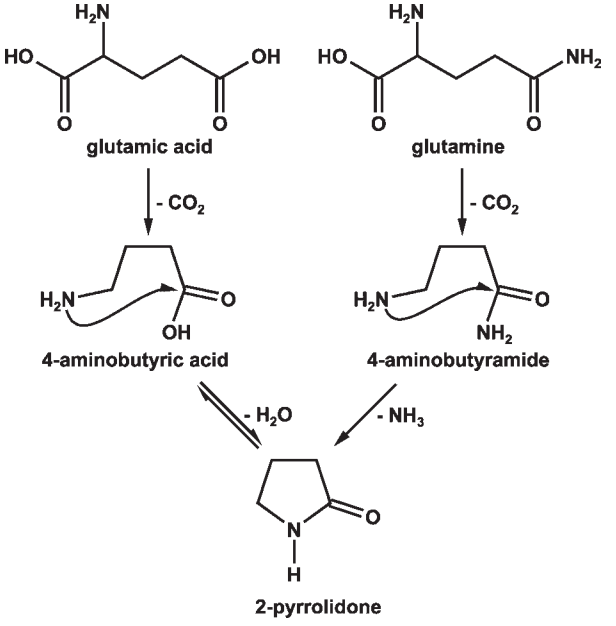

Figure 3. Formation of 2-pyrrolidone from glutamic acid and glutamine.

Table 3. Results of Migration Tests of PVPP and PVI/PVP (Milligrams per Kilogram), As Determined by Migration Experiments with Model Wine

	PVI/PVP 1		PVI/PVP 2		PVPP 1		PVPP 2	
contact time (min)	imidazole	2-pyrrolidone	imidazole	2-pyrrolidone	imidazole	2-pyrrolidone	imidazole	2-pyrrolidone
5	31	<8	34	<8	nd	64	nd	112
15	33	<8	35	<8	nd	68	nd	109
30	34	<8	36	<8	nd	69	nd	108
	PVPP 3		PVPP 4		PVPP 5		PVPP 6	
contact time (min)	imidazole	2-pyrrolidone	imidazole	2-pyrrolidone	imidazole	2-pyrrolidone	imidazole	2-pyrrolidone
5	nd	104	nd	98	nd	78	nd	61
15	nd	105	nd	101	nd	78	nd	82
30	nd	103	nd	103	nd	77	nd	79

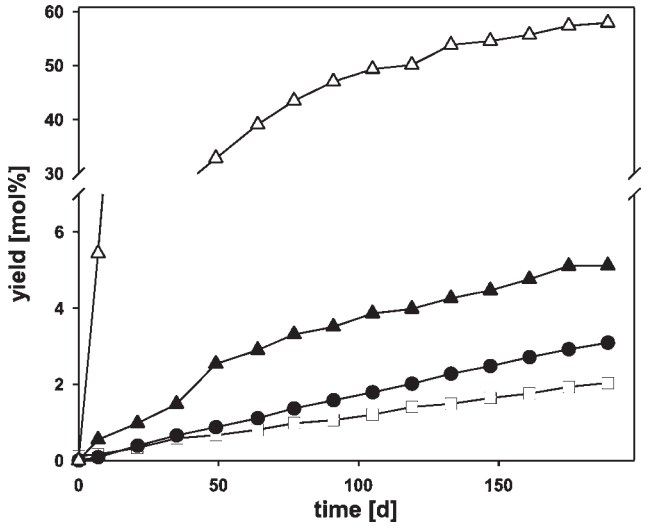

Figure 4. Formation of 2-pyrrolidone (□) in 4-aminobutyric acid incubation, 4-aminobutyric acid (●) in 2-pyrrolidone incubation, and 2-pyrrolidone (△) and 4-aminobutyric acid (▲) in 4-aminobutyramide incubation (wine model solution, pH 3.2, 50 °C).

Table 4. Contents of Glutamic Acid, Glutamine, 4-Aminobutyric Acid, 4-Aminobutyramide, and 2-Pyrrolidone in Wine (Micrograms per Liter) (n = 140)

	minimum	maximum	mean	median	standard deviation
glutamic acid	6636	163603	50295	47645	24500 (49%)
glutamine	6	13998	837	222	1620 (194%)
4-aminobutyric acid	5711	214831	59287	45003	45453 (77%)
4-aminobutyramide	4	2616	402	219	510 (127%)
2-pyrrolidone	43	1910	274	213	239 (87%)

Formation of 2-Pyrrolidone in Wine and Must. During method validation it became obvious that 2-pyrrolidone naturally occurs in wine; that is, wines not treated with PVPP (EcoVin label) showed similar contents of 2-pyrrolidone. This prompted an investigation on the molecular basis of 2-pyrrolidone formation. We hypothesized two formation pathways that are shown in **Figure 3**. 4-Aminobutyric acid is formed from glutamic acid via enzymatic decarboxylation (*12*) and cyclizes to 2-pyrrolidone under water elimination. An alternative pathway is the enzymatic decarboxylation of glutamine to 4-aminobutyramide, cyclizing to 2-pyrrolidone under elimination of ammonia. To verify the proposed formation pathways, 4-aminobutyric acid, 4-aminobutyramide, and 2-pyrrolidone were incubated separately.

Incubations led to the results depicted in **Figure 4**. 2-Pyrrolidone could be found in 4-aminobutyric acid incubations. On the other hand, 4-aminobutyric acid evolved in the 2-pyrrolidone incubations. This clearly supports the notion that both compounds are present in a chemical equilibrium in wine and can be converted into each other. However, after 190 days at 50 °C, only 2 mol % of 4-aminobutyric acid and 3 mol % of 2-pyrrolidone were converted into the respective counterpart. Thus, the rate of reaction is extremely slow. The reaction of 4-aminobutyramide to 2-pyrrolidone proceeded significantly more quickly. After 190 days, 58 mol % of 4-aminobutyramide was converted. Furthermore, in this incubation, small amounts of 5 mol % of 4-aminobutyric acid were found. Comparison of reaction rates therefore established 4-aminobutyramide as the major source of 2-pyrrolidone formation. Finally, the similar reaction rates of 4-aminobutyramide to 4-aminobutyric acid and of 2-pyrrolidone to 4-aminobutyric acid in separate incubations strongly suggested the formation of 4-aminobutyric acid from 4-aminobutyramide via 2-pyrrolidone as an intermediate.

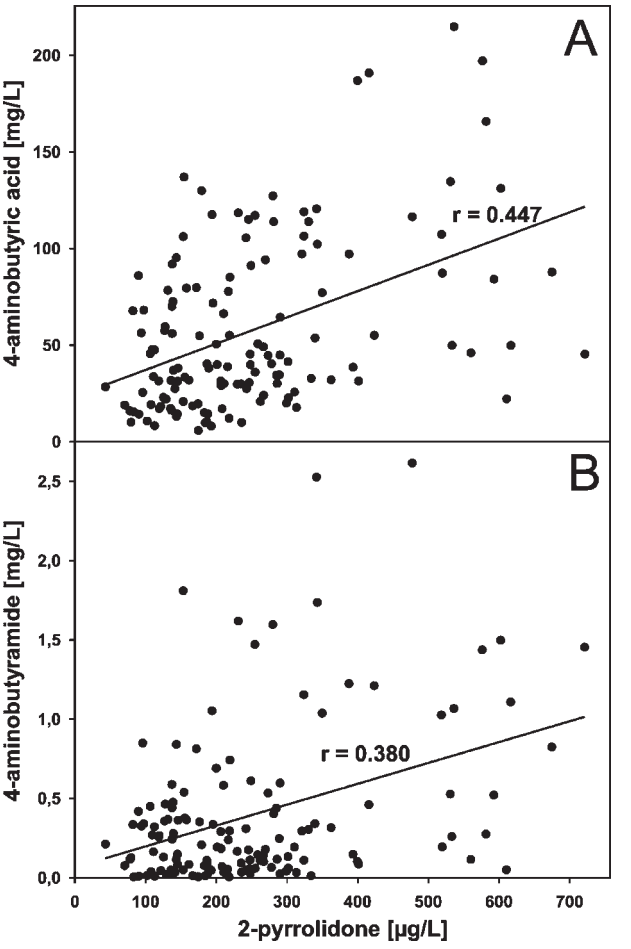

Figure 5. Correlation of 2-pyrrolidone and 4-aminobutyric acid (**A**) and of 2-pyrrolidone and 4-aminobutyramide (**B**).

In addition, 140 different wines were tested for their contents in glutamic acid, 4-aminobutyric acid, glutamine, 4-aminobutyramide, and 2-pyrrolidone (**Table 4**). In **Figure 5A** direct correlation between 2-pyrrolidone and 4-aminobutyric acid (r = 0.447, siginificant) can be seen. Also, in multivariate data analyses contents of 2-pyrrolidone positively correlated to contents of 4-aminobutyric acid (β = 0.291).

Correlation between 2-pyrrolidone and 4-aminobutyramide (r = 0.380, siginificant) is shown in **Figure 5B**. By trend, wines with higher levels of 4-aminobutyric acid and 4-aminobutyramide possessed higher levels of 2-pyrrolidone (β = 0.191). This fact in combination with the strong correlation of 4-aminobutyric acid with 4-aminobutyramide (β = 0.444) was in-line with results of the above model incubations. Older wines contained more 2-pyrrolidone than younger wines (β = 0.325). Again, this confirmed the proposed reaction mechanism. Correlation between age of wines and content of 4-aminobutyric acid or of 4-aminobutyramide was unverifiable. As expected, there was a positive correlation between glutamic acid and the corresponding biogenic amine 4-aminobutyric acid (β = 0.338).

In summary, with the established LC-MS^2 method migrants from PVPP and PVI/PVP could be determined in wine conveniently at trace level concentrations. In addition, the formation of naturally occurring 2-pyrrolidone in wine was linked mechanistically to 4-aminobutyric acid and 4-aminobutyramide.

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Supporting Information Available: A table providing characteristics of various wines used in this study.This material is available free of charge via the Internet at http://pubs.acs.org.

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