Analysis of underivatized amino acids in wines by solid phase extraction and HILIC-MS

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Keywords: Underivatized amino acids, HILIC, single quadrupole mass detection, solid phase extraction, wine, HyperSep C18, ISQ EM, ISQ EC

Application benefits

- Solid phase extraction cleans up wines reproducibly and reliably, facilitating analysis of underivatized amino acids in wines.
- Mass detection enables the quantification of co-eluting amino acids, in comparison with method involving derivatization and UV or FL detection.
- Single ion monitoring (SIM) mode delivers superior sensitivity and area reproducibility compared to scan mode.

Goal

To demonstrate a straightforward and reliable solid phase extraction method to clean up wine for analysis of underivatized amino acids.

Introduction

Amino acids are the most important nitrogen source during the wine making process, accounting for 30% to 40% of the total wine nitrogen. The amino acid content in wine is influenced by the grape variety, geographical origin, fermentation condition, vintage year, etc.[1] Wine is a complex sample, containing many types of compounds (such as organic acids, polyphenolic compounds, protein, lipid, and pigments). The amino acid analysis in wine can therefore be a challenging task due to possible interferences and/or contaminants present.

Solid phase extraction (SPE) is one of the most popular sample pre-treatment techniques. It is used either to remove sample matrix interferences or to enrich analytes, both of which result in more sensitive detection. Coupling SPE with liquid chromatography/mass spectrometry (LC/MS) analysis can also minimize ion-suppression and make the method more robust. Typically, there are two types of SPE: the analytes of interest are either adsorbed (or retained) on the sorbent of the SPE cartridge or they pass through the cartridge with the sample solvent while

undesired compounds or contaminants are retained on the sorbent.[2] For example, nine catechins and phenolic acids in a red wine were extracted by the former SPE method using Thermo Scientific™ HyperSep™ Retain PEP (polar enhanced polymer) material, prior to high performance liquid chromatography (HPLC) analysis.[3] The latter SPE method has been widely applied to remove interfering matrix compounds (such as pigments, lipids, and proteins) in chromatographic analysis of polar analytes (such as amino acids, organic acids, and polyphenols) in wines. Briefly, the polar analytes are filtered and passed through C18 SPE cartridges while hydrophobic wine components are retained on the SPE cartridges.[2,4]

This application note describes an SPE method to clean up wines for the analysis of underivatized amino acids. The SPE was performed using a Thermo Scientific™ HyperSep™ C18 material, which retained interfering matrix compounds. The amino acids in eluates were separated on a Thermo Fisher™ Accucore™ HILIC column and analyzed with a Thermo Scientific™ ISQ™ EM mass detector.

Experimental details

Chemicals

- Deionized water, 18.2 MΩ·cm at 25 °C, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (P/N 50136149)
- Acetonitrile Optima™ LC/MS grade, Fisher Chemical™ (P/N A955)
- Methanol Optima™ LC/MS grade, Fisher Chemical™ (P/N A456-212)
- Formic Acid, Optima™ LC/MS grade, Fisher Chemical™ (P/N A117)
- Ammonium Formate, Optima™ LC/MS grade, Fisher Chemical™ (P/N A115)
- Pierce™ Amino Acid Standard H, Thermo Scientific™ (P/N PI20088)
- L-Amino acids (purchased from a reputable vendor)
- Hydrochloric acid (HCl), fuming, 37% (purchased from a reputable vendor)

Sample handling

- Fisherbrand™ Mini Centrifuge (P/N 12-006-901)
- Thermo Scientific™ Orion 3 Star™ pH Benchtop Meter (P/N 13-644-928)
- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 100–1000 µL (P/N 4641100N), 10–100 µL (P/N 4641070N), 1–10 µL (P/N 4641030N)
- Vials (amber, 2 mL), Fisher Scientific™ (P/N 15508760)
- Snap Cap with Septum (Silicone/PTFE), Fisher Scientific™ (P/N 10547445)
- Fisherbrand™ Crimp Top Fixed Insert Vial (amber, 0.3 mL) (P/N 03-FIV(A))
- Fisherbrand™ Certified Vial Kit-Clear Glass, PP Screw Cap, Septum (Silicone/PTFE), for preparing samples (P/N 15562320)
- Thermo Scientific™ HyperSep™ C18 (1000 mg/6 mL) (P/N 60108-301)
- Thermo Scientific™ SPE 24-port vacuum manifold (P/N 60104-233)

Instrumentation

- Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system consisting of:
  - Vanquish Flex System Base (P/N VF-S01-A)
  - Vanquish Quaternary Pump F (P/N VF-P20-A)
  - Vanquish Split Sampler FT (P/N VF-A10-A)
  - Vanquish Column Compartment H (P/N VH-C10-A) with active pre-heater (P/N 6732.0110)
  - Thermo Scientific™ ISQ™ EM Single Quadrupole Mass Spectrometer (P/N ISQEM-ESI)

Sample preparation

Standard solutions were prepared with the Pierce Amino Acid Standard H, containing 16 target amino acids of interest (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, and Val), and a stock solution of one additional amino acid Hyp (see Table 2 for full names of amino acids). The 16 amino acids in Pierce Amino Acid Standard H are present in 0.1 N HCl at a concentration of 2.5 mM. The stock solution of Hyp was prepared at a concentration of 10 mM in 0.1 N HCl solution.

Working solutions were prepared by diluting the stock solution with an appropriate volume of 0.1 N HCl solution. For example, 200 µL of the Pierce Amino Acid Standard H (2.5 mM), 50 µL of Hyp (10 mM) and 750 µL of 0.1 N HCl solution were mixed to prepare 500 µM standard mixture for 17 amino acids.

Calibration standard mixtures were prepared at six concentration levels (1, 10, 50, 100, 250, and 500 µM), by performing serial dilutions from the 500 µM working standard solution. Calibration curves for all 17 amino acids were obtained by injections of calibration standards with three injection replicates at each concentration level. The concentration ranges within which reliable quantification of the amino acids was possible were determined (Table 3).

Wine sample preparation by solid phase extraction

A total of six wines were used in this application note and were purchased locally. The wines belong to three white and three red grape varieties, which are all originated from Italy. Table 1 lists grape varieties, geographic origin, and production year of the six wines.

Solid phase extraction using HyperSep C18 cartridges (1000 mg bed/6 mL) was performed to remove interfering matrix compounds (such as pigment, lipid, and protein) in wine. The SPE procedure described by Sanders et al.[4] was used with some modifications. Prior to SPE, 1 mL wine was diluted with 2 mL of 0.1 N HCl in 70/30 (v/v) water/methanol. The HyperSep C18 cartridge was conditioned and equilibrated by consecutively washing it with 20 mL of methanol, 20 mL of 0.1 N HCl in water and 10 mL of 0.1 N HCl in 80/20 (v/v) water/methanol. During the sample loading step, 3 mL of the diluted wine sample was then passed through the SPE cartridge. The abovementioned interfering matrix compounds were retained whereas most amino acids (that are polar) eluted under the acidic condition. In an additional wash step, 1 mL of 0.1 N HCl in 70/30 (v/v) water/methanol was further passed through the cartridge to obtain a better recovery of the amino acids. The final volume of the total eluate (from the sample loading and the column wash step) was recorded for more accurate recovery calculations. The collected sample (i.e., the eluate) was mixed and then injected directly into the chromatography system. The remaining sample was stored in the refrigerator.

Mobile phase preparation

Mobile phase A consisted of acetonitrile/ammonium formate buffer at pH 2.8 (90/10 v/v) and mobile phase B water/ammonium formate buffer at pH 2.8 (90/10 v/v). Stock buffer was prepared at a concentration of 200 mM ammonium formate in water at pH 2.8. The pH was adjusted with formic acid. The stock buffer of 100 mL was then added to acetonitrile of 900 mL for mobile phase A and water of 900 mL for mobile phase B. The final buffer concentration in both mobile phase solvents was 20 mM ammonium formate.

Table 1. Distribution of the red and white wine samples according to grape variety and production area and year

	Grape variety	Growing region	Production year
White Wine 1 (WW1)	Pinot Grigio	Friuli Grave	2019
White Wine 2 (WW2)	Garganega, Trebbiano di Soave	Veneto	2019
White Wine 3 (WW3)	Vermentino	Apulia	2019
Red Wine 1 (RW1)	Primitivo	Apulia	2016
Red Wine 2 (RW2)	Sangiovese	Umbria	2018
Red Wine 3 (RW3)	Sangiovese, Cannonau	Tuscany	2016

Chromatographic conditions

Parameter	Value
Column	Thermo Scientific™ Accucore™ 150 Amide HILIC (2.1 × 150 mm, 2.6 µm) P/N 16726-152130
Mobile phase A	90/10 (v/v) ACN/200 mM aqueous ammonium formate at pH 2.8
Mobile phase B	90/10 (v/v) H_2O/200 mM aqueous ammonium formate at pH 2.8
Gradient	Time (min) %B 0.0 0.0 5.0 0.0 15.0 15.6 20.0 33.3 30.0 33.3 30.2 0.0 40.0 0.0
Flow rate	0.4 mL/min
Column temperature	30 °C (forced air with active pre-heater at 30 °C)
Sampler temperature	4 °C
Injection volume	0.5 µL
Needle wash solvent	Acetonitrile/water 50:50 (v/v)
Needle wash mode	Before draw

MS detector settings

Parameter	Value
Ionization mode	ESI
Polarity (Spray voltage)	Positive (+2500 V) and Negative (-2000 V)
Full scan	*m/z* 60–350
SIM scan	SIM masses and RT (retention time) windows are listed in Table 2
SIM width	0.1 amu
Lowest dwell time	0.15 s
CID voltage	20 V except for Asp (15 V)
Vaporizer temperature	477 °C
Ion transfer tube temperature	300 °C
Gas flow pressures	Sheath gas: 80.0 psig Auxilliary gas: 7.3 psig Sweep gas: 2.0 psig

Chromatography Data System

Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS) was used for data acquisition and analysis.

Table 2. Molecular weight, SIM mass, SIM RT window, and SIM acquisition polarity for 17 amino acids. SIM mass is $[M+H]^+$ for positive mode and $[M-H]^-$ for negative mode acquisitions.

Name	Acronym	Monoisotopic mass [M]	SIM – RT windows (min)	SIM *m/z*	SIM acquisition polarity
Alanine	Ala	89.05	15.3–17.3	90.0	positive
Arginine	Arg	174.11	20–22	175.1	positive
Aspartic acid	Asp	133.04	18.8–20.8	132.0	negative
Glutamic acid	Glu	147.05	17–19	146.1	negative
Glycine	Gly	75.03	16.5–18.5	76.0	positive
Histidine	His	155.07	20–22	156.1	positive
Hydroxyproline	Hyp	131.06	15.2–17.2	132.1	positive
Isoleucine	Ile	131.09	8.5–11	132.1	positive
Leucine	Leu	131.09	6.7–8.8	132.1	positive
Lysine	Lys	146.11	20.5–22.5	147.1	positive
Methionine	Met	149.05	10–12	150.1	positive
Phenylalanine	Phe	165.08	6–8	166.1	positive
Proline	Pro	115.06	13.2–15.2	116.1	positive
Serine	Ser	105.04	17–19	106.0	positive
Threonine	Thr	119.06	16–18	120.1	positive
Tyrosine	Tyr	181.19	11.5–13.5	182.1	positive
Valine	Val	117.15	12–14	118.1	positive

Results and discussion

Method linearity and repeatability

The method in this application was determined by modifying a previous method.[5] Briefly, MS acquisition was in component mode, targeting 20 scans per peak with an assumed peak width (at the base) of 24 s. The method repeatability was evaluated by injecting successively seven times a 100 µM standard mixture of 17 amino acids. Very good method repeatability was observed with low values of relative standard deviation (RSD) for retention time and peak area (Table 3). The RSD of retention time were less than 0.1% for all amino acids except for phenylalanine (0.14%), leucine (0.12%) and isoleucine (0.1%). The area RSD were less than 2.6% except for hydroxyproline (3.5%), glutamic acid (7.9%) and aspartic acid (9.2%). The greater area RSD for the two acids (Glu and Asp) most likely results from their relatively lower signal to noise ratio (SNR of 41 and 113). These acidic amino acids (Glu and Asp) showed the first and third lowest SNR values among all the 17 amino acids.

Calibration curves were obtained using averaged peak areas from three replicate injections of standard mixtures, without both any weighting and forcing through the origin. Calibration results (such as calibration range, curve fit type, and R^2) for all 17 amino acids were summarized in Table 3. All 17 amino acids showed excellent method linearity and curve fits, with the coefficient of determination R^2 >0.997. Twelve amino acids out of 17 showed R^2 greater than or equal to 0.999.

Solid phase extraction method characterization

Figure 1a shows a typical SIM chromatogram for all 17 standard amino acids in 250 µM standard solution, and Figure 1b and Figure 1c display the chromatograms of a white and a red wine sample where the SPE was performed. While almost the same results (such as the analysis time of <22 min and the baseline separation of the isomer peak pairs Leu/Ile) as the one reported in reference 5 were observed with the standard mixture (Figure 1a), all 17 amino acids in wines were well detected without any spiking after the sample pretreatment with SPE. (Figure 1b and Figure 1c)

Table 3. Retention time (RT), precision of RT and peak area (n =7), and calibration parameters

				Calibration		
Name	Average RT (min)	RT RSD (%)	Area RSD (%)	Concentration range (µM)	Curve fit type	R^2
Phe	7.10	0.137	2.33	1–250	Linear	0.9990
Leu	8.46	0.115	1.73	1–250	Linear	0.9998
Ile	9.50	0.103	0.65	1–250	Linear	0.9988
Met	10.82	0.035	1.31	1–250	Linear	0.9992
Tyr	12.47	0.000	1.47	1–250	Linear	0.9999
Val	12.67	0.030	1.46	1–250	Linear	0.9992
Pro	13.86	0.000	2.35	1–500	Linear	0.9994
Ala	15.94	0.024	1.07	10–250	Linear	0.9994
Hyp	16.02	0.000	3.46	1–250	Linear	0.9990
Thr	16.51	0.032	0.87	1–250	Linear	0.9987
Gly	16.94	0.022	2.28	10–250	Linear	0.9987
Ser	17.77	0.000	2.50	10–250	Linear	0.9994
Glu	18.15	0.000	7.92	10–250	Quadratic	0.9999
Asp	19.54	0.027	9.24	10–250	Quadratic	0.9980
His	20.35	0.053	1.77	10–250	Linear	0.9996
Arg	20.32	0.000	2.52	10–500	Linear	0.9976
Lys	20.91	0.000	2.40	10–250	Quadratic	0.9999

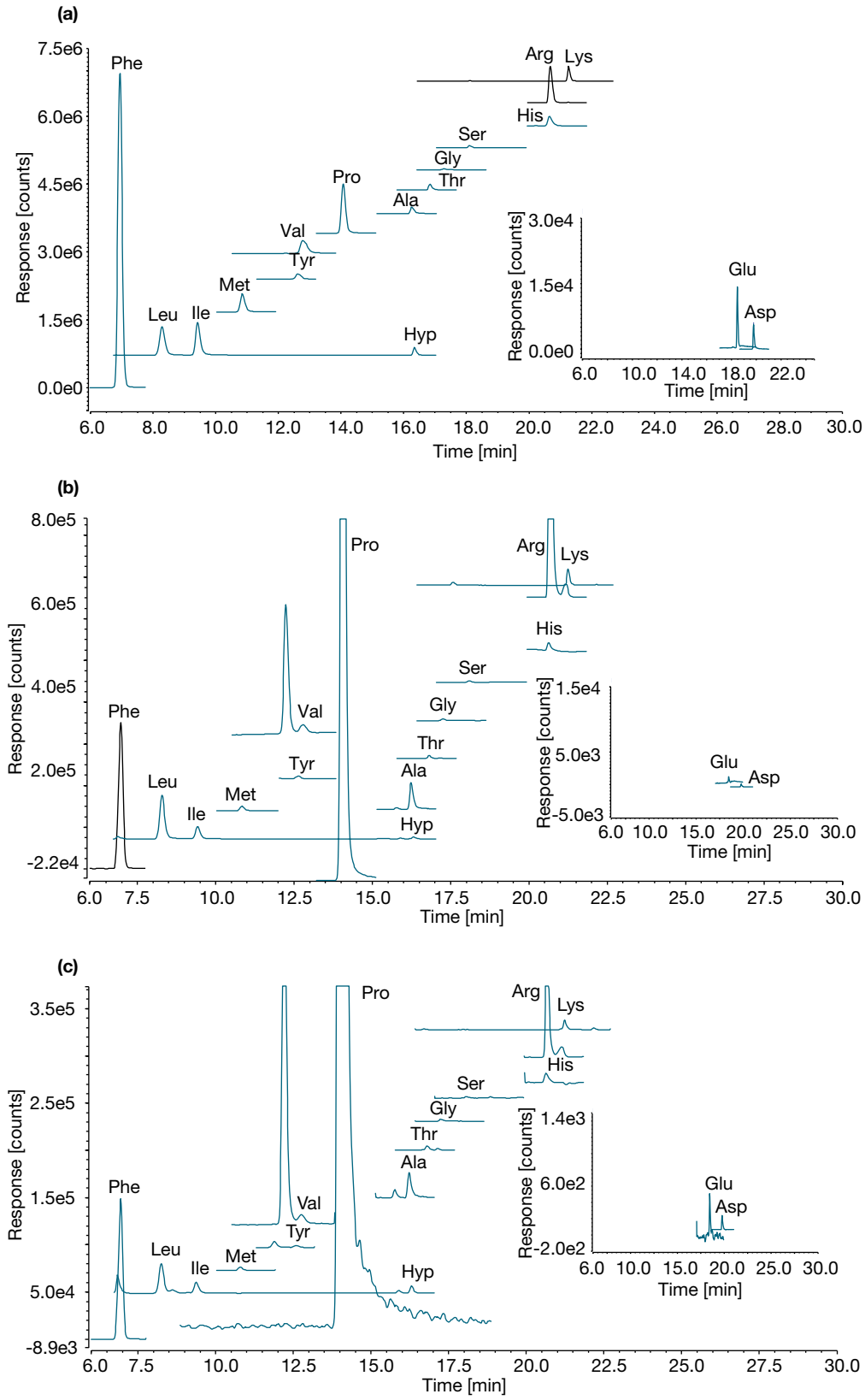

Figure 1. SIM chromatograms for 17 standard amino acids in (a) 250 µM standard solution, (b) white wine 1, and (c) red wine 1. The inserted figures show glutamic acid and aspartic acid.

SPE repeatability was checked for three test wines (WW3, RW1, RW2) by injecting three different SPE samples for each wine and evaluating peak area RSD for all 17 amino acids. Table 4 summarizes RSD values for peak area of the 17 amino acids in each test wine. The SPE method shows good repeatability, with the area RSD <6% except for several amino acids (such as Asp in red wines, Glu and His in red wine 1, and Val in red wine 2). Aspartic acid in red wine 1 and 2, glutamic acid in red wine 1, and histidine in red wine 1 were detected at near the lowest concentrations of the corresponding calibration curves.

Table 4. Area precision of SPE method (n = 3)

Name	WW4	RW1	RW2
Ala	0.44	3.13	3.34
Arg	5.36	2.49	4.84
Asp	2.47	7.16	8.57
Glu	3.05	9.58	4.39
Gly	2.35	2.92	5.93
His	1.20	6.60	ND
Leu	1.57	3.23	3.08
Ile	2.53	2.75	4.32
Hyp	0.68	2.24	4.27
Lys	3.74	4.63	2.22
Met	2.12	3.15	4.21
Phe	2.05	1.33	5.58
Pro	0.39	1.02	4.44
Ser	5.09	4.58	5.15
Thr	1.72	1.19	1.19
Tyr	2.17	3.73	5.10
Val	3.54	5.39	6.10

ND denotes not detected

In addition, the recovery of spiked matrix (RSM) test was performed to evaluate the reliability of the SPE method in the presence of sample matrix. The RSM was calculated by the difference in the measured amount of amino acids in spiked and unspiked wine sample, compared to the spiked known amount, as shown in the following equation:

$$RSM\ (\%) = \frac{amount\ (mg/L)\ of\ spiked\ sample - amount\ (mg/L)\ of\ unspiked\ sample}{spiked\ amount\ (mg/L)} \times 100$$

For this purpose, a 50 µM standard mixture (as a final concentration) was spiked into each wine. The two types of samples (i.e., the spiked- and unspiked wine samples) were analyzed after the SPE. The amino acid concentrations were determined using the resulting area, molecular weight and the calibration equation parameters of Table 3. Table 5 lists the RSM values of 13 amino acids in WW1 and RW3 samples. The four amino acids (Pro, Arg, His, Lys) were excluded, in case either the quantification was not possible (proline, see more details in the section of quantification) or the RSM values were higher than the upper limit (120%) of a generally acceptable RSM range (Arg, His, Lys). This may have been caused by matrix effect. The unspiked Ser and Val were not detected in red wine 3. The RSM values ranged from 71% to 103% for WW1 and from 73% to 113% for RW3, respectively, showing the SPE method is generally reliable to determine amino acids in wine.

Table 5. Recovery of spiked matrix (RSM)

Name	WW1 - RSM (%)	RW3 - RSM (%)
Ala	100	80
Asp	82	93
Glu	71	78
Gly	94	97
Leu	98	73
Ile	97	85
Hyp	96	93
Met	103	84
Phe	98	75
Ser	95	106
Thr	99	86
Tyr	98	86
Val	93	113

Quantification of amino acids in wines

Figure 2 shows the amount of 16 amino acids in wines, which include Ala, Arg, Asp, Glu, Gly, His, Hyp, Ile, Leu, Lys, Met, Phe, Ser, Thr, Tyr, Val. All 16 amino acids in six different wines (WW1, WW2, WW3, RW1, RW2, RW3) were quantified using calibration curves. Proline was excluded since the concentrations of proline were far beyond the upper llimit (i.e., 500 µM) of the calibration curve. The result implies that proline was observed to be the most abundant amino acid in all the six wines. This is in line with previously reported results.[1] It is worth noting that it is necessary to appropriately dilute wine samples, prior to the SPE, to quantify proline. In addition, regarding the abovementioned three amino acids (Arg, His, Lys) with high RSM values, more careful calibrations (e.g., using isotopically labeled internal standards) are recommended for more accurate quantification. The calibration standards were injected together with the SPE-treated wine samples to provide the calibration curves with less variations of signal responses. The amounts of amino acids in the three white wines were within the range of values reported in Reference 6. The amounts of hydroxyproline in all six wines ranged from 6.1 mg/L to 11 mg/L, within the range reported in the reference.[7,8] All 17 amino acids were detected in all six wines, with the exception of three amino acids (i.e., histidine in red wine 2, serine and valine in red wine 3). In addition, it was clearly shown that higher amounts of leucine and tyrosine are observed in white wines (Figure 2, inset). Considering the relative area values (data not shown), it can be assumed that higher amounts of proline were found in red wines.

Conclusion

- A simple and reproducible solid phase extraction method was developed and applied to six wines for analysis of underivatized amino acids.
- The method using HyperSep C18 material generally shows good area precision and recovery of spiked matrix.
- Amino acids can be analyzed with high area and retention time precision with the presented HILIC-MS method.

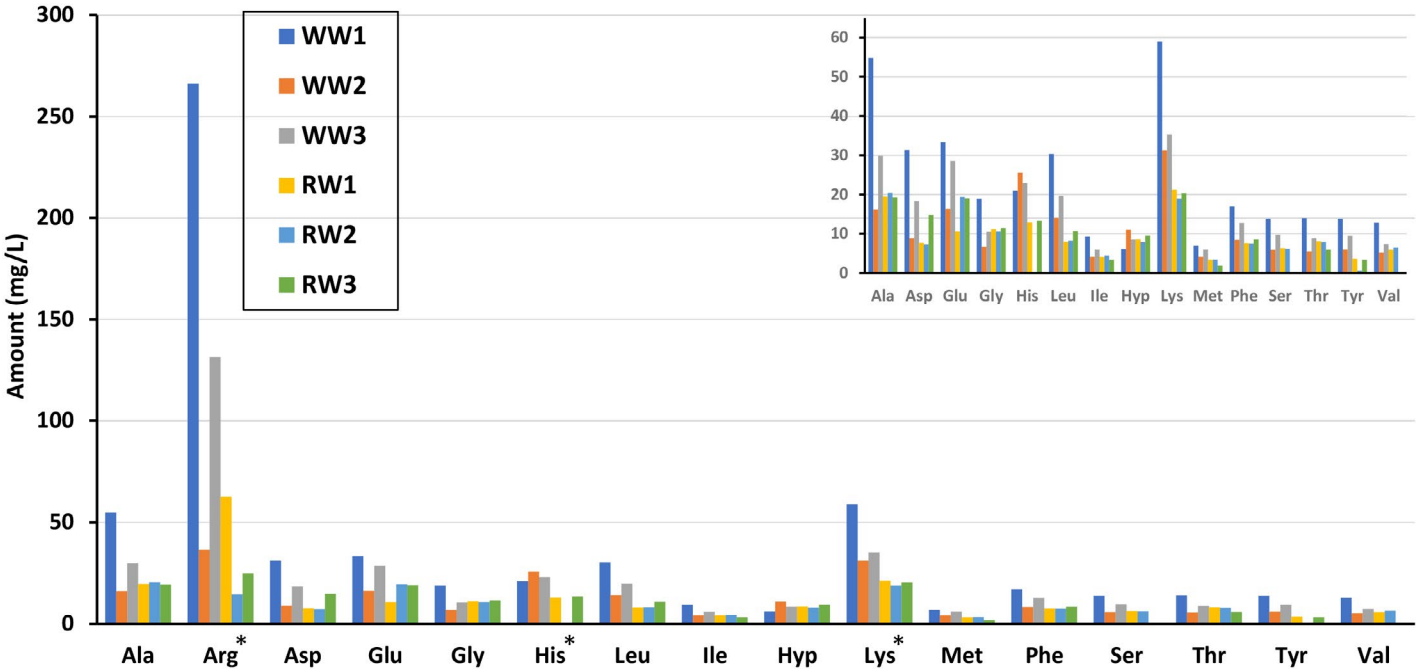

Figure 2. Amount of amino acids in six wines. Proline, with concentrations far beyond the upper limit of the calibration range, is excluded. Three amino acids (Arg, His, Lys), with RSM values larger than the upper limit of a generally acceptable RSM range, are indicted with asterisk. The amino acids contents (mg/L) were quantified using calibration curves. Six wines, treated with SPE, were injected three times each and average concentrations of the amino acids are presented. The inserted figure shows amounts of 15 amino acids except arginine.

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