Native amino acid analysis in wine by HILIC separation and detection with single quadrupole mass spectrometry

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ABSTRACT

This work describes a sensitive and reproducible LC method with single quadrupole mass detection for the analysis of 22 underivatized amino acids. Since amino acids are quite polar, we used a hydrophilic interaction liquid chromatography (HILIC) column to retain and separate them. The use of single quadrupole mass detector allowed straightforward analysis of the amino acids without further sample preparation such as pre- or post column derivatization. Under the optimized gradient conditions, all the amino acids except five peak pairs (*i.e.*, Tyr/Val, Ala/Hyp, Gln/Ser, Asn/Glu, His/Arg) were baseline-separated within 22 minutes, including the separation of isomers of Leu and Ile (R_s >5). Generally, good linearity (R^2 >0.991) was obtained for a range of 0.1 to 500 µM standard amino acids. The developed method was successfully applied to detect 17 amino acis in wine, found in Ref. [1]. Finally, proline, which can be used as a discriminator of wine from different grape varieties and areas [1], was quantified using an internal standard (proline-2,2,5-d_3).

INTRODUCTION

Amino acid levels in wine are of increased interest due to significant roles they play: they are a source of nitrogen for yeast during fermentation and have a direct effect on the aromatic composition of wines [1]. The amino acid content of wine is influenced by yeast strain, treatments used during fermentation, the grape variety, and production area [2]. Proline is the most abundant amino acid present in wines, containing around 30% to 85% of total amino acid content in wine. Since (the amino acid) proline is not metabolized during yeast fermentation, it can be used as a diagnositc marker for different wine varieties and areas, and also be useful for QA/QC purposes [2].

For the analysis of amino acids in food and beverage, liquid chromatography (LC) methods with pre-column derivatization have been most widely used due to a relative simplicity of the apparatus and the increased sensitivity. However, the derivatization technique requires handling of toxic chemicals and analyst's additional labor, which can negatively affect method robustness and reproducibility due to reagent interference, systematic and random errors during sample handling step [3]. LC combined with mass detector (MD) is an attractive approach where native (or underivatized) amino acids can be determined without further sample preparation. In addition, MS can provide accurate, sensitive and robust detection of analytes. Furthermore, single quadrupole (SQ) MD is easy to operate and control.

In this work, we developed a sensitive and reproducible HILIC method with SQMD to determine 22 native amino acids, applicable to a variety of samples such as food, beverages, and fermentation media. The developed method was then applied to analyze 17 amino acid components in a white wine. Finally, proline, a possible diagnostic marker for wine, was quantified using an isotopically labelled internal standard.

MATERIALS AND METHODS

Sample Preparation

Standard solutions were prepared with the Pierce amino acid standard H, containing 17 amino acids (Ala, Arg, Asp, Cystine, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr and Val) and 5 additional amino acids (Asn, Gln, Nva and Hyp) from Sigma-Aldrich. The Pierce amino acid standard H contains 2.5 mM of each of all the amino acids except cystine (1.25 mM, oxidized cysteine dimer) in 0.1N HCl. Stock solutions were prepared at a concentration of 2.5 mM, by mixing the Pierce amino acid H with the 5 amino acids. Working solutions and calibration standards were prepared by diluting the stock solution with the appropriate volume of 0.1 N HCl solution. Internal standard solutions for proline quantification were prepared by adding the same concentration of proline-2,2,5-d_3 to each concentration level for the calibration. A white wine was purchased from a local supermarket. An appropriate amount of wine was filtered using a 0.20 µm syringe filter and was then diluted 5-fold and 8-fold in 0.1N HCl, prior to injection.

Instrumentation

Chromatographic separation was performed on a Thermo Scientifc™ Vanquish™ Flex UHPLC system and detection with Thermo Scientifc™ Vanquish™ ISQ Single Quadrupole Mass Detector.

Table 1. Instrument module and part number

Module	Part number (P/N)
Vanquish Flex System Base	VF-S01-A-01
Vanquish Quaternary Pump F	VF-P20-A
Vanquish Split Sampler FT	VF-A10-A
Vanquish Column Compartment H	VH-C10-A
ISQ EM Single Quadrupole Mass Detector	ISQEM-ESI

Final Method

Table 2. LC conditions

Parameter	Value	
Column	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 ammonium formate pH 2.8	
Mobile phase B	90/10 (v/v) H_2O/200 mM ammonium formate pH 2.8	
Flow rate	0.4 mL/min	
Column temp.	30 °C (forced air with active pre-heater at 30 °C)	
Sampler temp.	4 °C	
Injection vol.	0.5 µL	
Gradient	Time (min)	%B
	0	0.0
	5	0.0
	15	15.6
	20	33.3
	30	33.3
	30.2	0.0
	40	0.0

Table 3. MS conditions

Parameter	Value	
Ionization mode	ESI positive except for Asp & Glu (ESI negative)	
Source voltage	+ 2500 V; - 2000 V	
Full scan	*m/z* 60-350	
SIM widths	0.2 amu	
CID	20 V except for Asp (15 V)	
Vaporizer temp.	477 °C	
Ion transfer tube temp.	300 °C	
Gas flow pressure	Sheath gas	80.0 psig
	Auxiliary gas	7.3 psig
	Sweep	2.0 psig

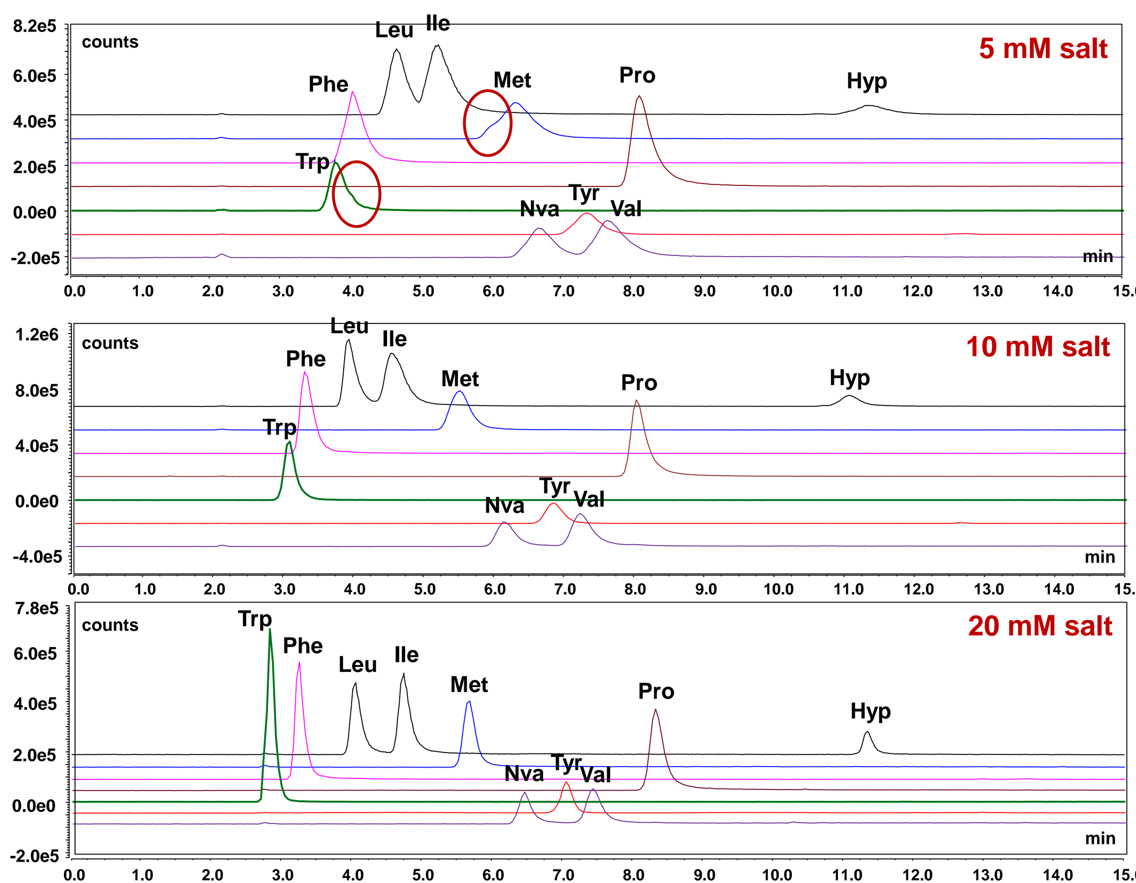
Data Analysis

Thermo Scientific™ Chromeleon™ software (version 7.2.8) was used for instrument control and data acquisition and processing.

Results

Optimization of buffer salt content

Figure 1. Optimization of buffer salt content on HILIC, showing the enhancement of peak shape and retention for ten selected amino acids at the optimal concentration of 20 mM.

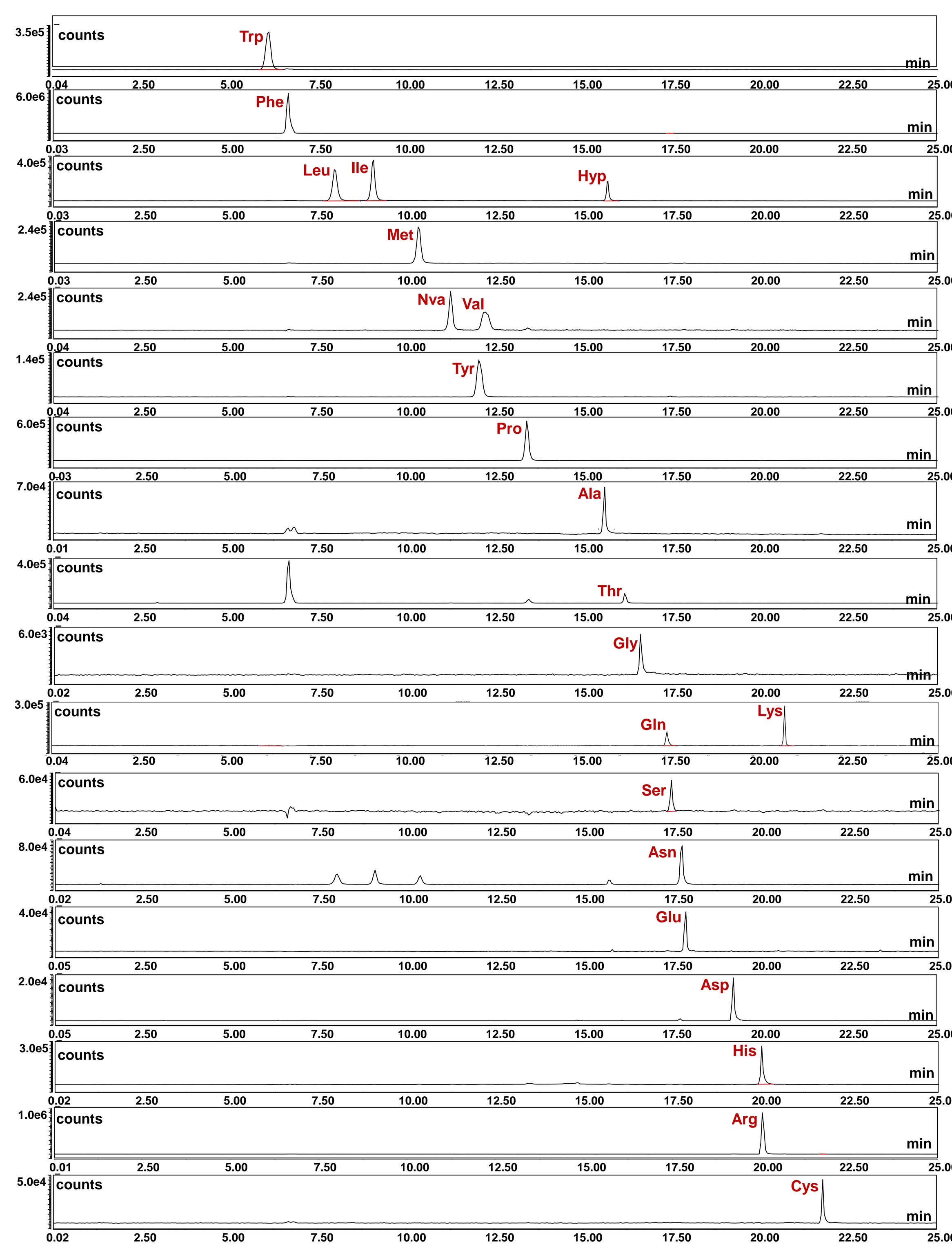


Ammonium formate buffer (of 5, 10, and 20 mM) at pH 3 was used in the mobile phase. The standard mixture of 250 µM was injected with the injection volume of 1 µL for 5 and 10 mM, and of 0.5 µL for 20 mM at mobile phase flow rate of 0.9 mL/min.

Chromatograms of SIM scan for 22 standard amino acids under final method

All the amino acids except five peak pairs (*i.e.*, Tyr/Val, Ala/Hyp, Gln/Ser, Asn/Glu, His/Arg), including two isomer pairs (*i.e.*, Leu/Ile, Nva/Val) were baseline-separated within 22 minute, on the Accucore™-150-Amide-HILIC column, within 22 minute.

Figure 2. Chromatograms of SIM scan of 22 standard amino acids at a concentration of 100 µM. Amino acids with same m/z values (Leu/Ile/Hyp, Nva/Val, and Gln/Lys) are detected under the same SIM channels (m/z 132.1, 118.1, and 147.1).

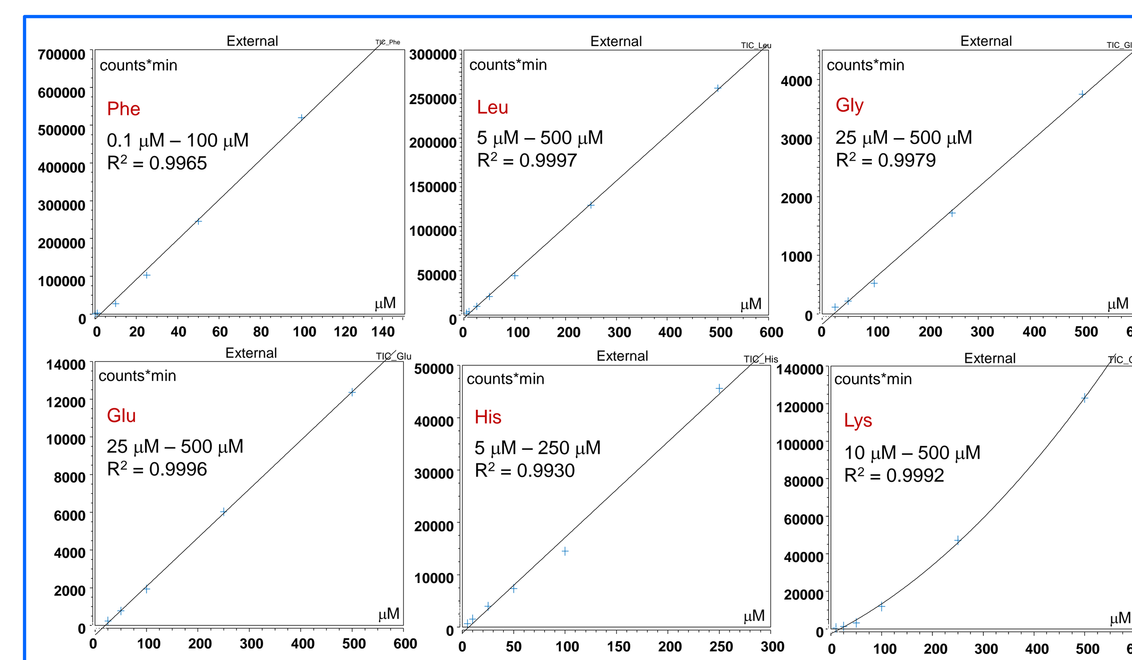


Method linearity and reproducibility

Table 4. Calibration parameters, retention time (RT), relative standard deviation (RSD) of RT and peak area for 22 standard amino acids, along with their *m/z* values (n = 10). For the repeatability tests, a 100 µM standard mixture except for Phe (25 µM) was injected consecutively ten times.

Name	*m/z*	RT	Calibration range (µM) & type	Regression	RT RSD (%)	Area RSD (%)
Trp	205.1	6.11	1 – 50 (Linear)	1.0000	0.008	2.15
Phe	166.1	6.64	0.1 – 100 (Linear)	0.9965	0.005	9.50
Leu	132.1	7.96	5 – 500 (Linear)	0.9997	0.012	2.80
Ile	132.1	9.07	5 – 500 (Linear)	0.9993	0.008	2.49
Met	150.1	10.31	5 – 250 (Linear)	0.9994	0.000	2.10
Nva	118.1	11.26	5 – 250 (Linear)	0.9983	0.007	2.06
Tyr	182.1	12.05	5 – 500 (Linear)	0.9991	0.000	1.85
Val	118.1	12.20	10 – 250 (Linear)	0.9997	0.177	2.43
Pro	116.1	13.42	1 – 500 (Linear)	0.9974	0.000	1.86
Ala	90.0	15.62	10 – 250 (Linear)	0.9984	0.000	1.89
Hyp	132.1	15.68	5 – 250 (Linear)	0.9968	0.041	2.00
Thr	120.1	16.17	5 – 250 (Linear)	0.9952	0.003	3.22
Gly	76.0	16.60	25 – 500 (Linear)	0.9979	0.000	6.44
Gln	147.1	17.36	10 – 250 (Quadratic)	0.9991	0.025	2.47
Ser	106.0	17.46	25 – 500 (Linear)	0.9971	0.000	3.98
Asn	133.1	17.73	5 – 250 (Linear)	0.9919	0.120	2.99
Glu	146.1	17.87	25 – 500 (Linear)	0.9996	0.078	4.05
Asp	132.0	19.24	10 – 500 (Linear)	0.9981	0.003	9.46
His	156.1	20.01	5 – 500 (Quadratic)	0.9998	0.000	1.85
Arg	175.1	20.05	5 – 500 (Linear)	1.0000	0.002	2.96
Lys	147.1	20.68	10 – 500 (Quadratic)	0.9992	0.000	3.81
Cystine	241.1	21.78	5 – 250 (Quadratic)	0.9982	0.002	8.24

Figure 3. Calibration curves of selected amino acids



Quantification of amino acids in wine

All 17 amino acids (Arg, Hyp, Ser, Asp, Glu, Thr, Gly, Ala, Pro, Met, Val, Phe, Leu, Ile, His, Lys, Tyr) were successfully detected [1].

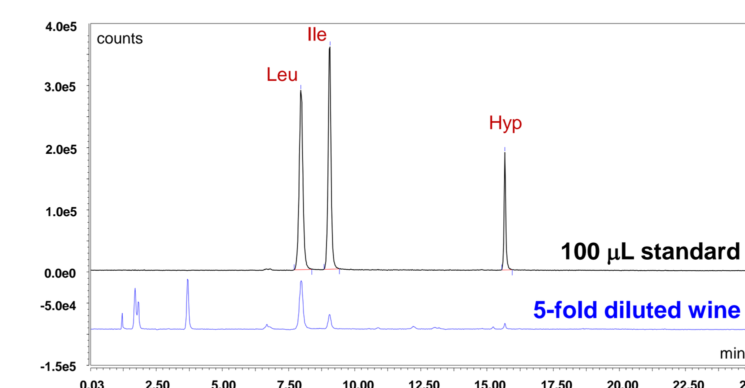
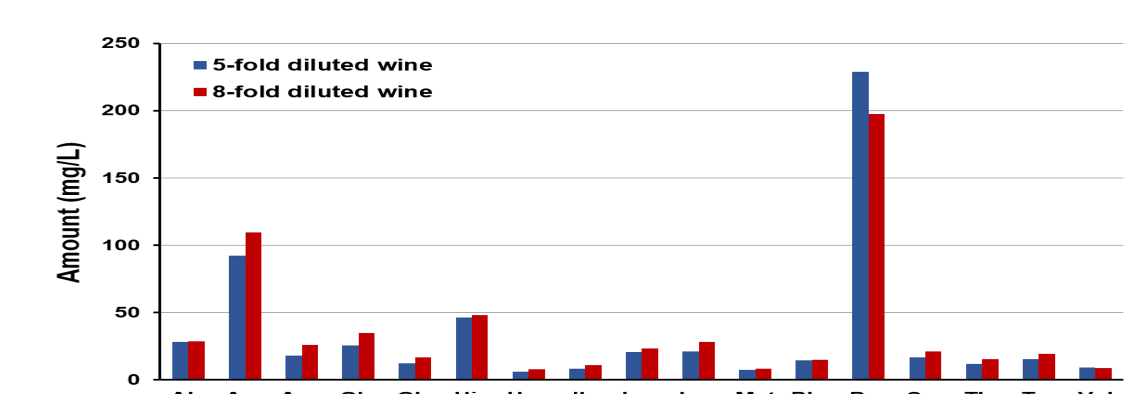


Figure 4. Example SIM chromatograms of amino acids in wine, recorded at *m/z* 132.1. Three amino acids (Leu, Ile, and Hyp) with the same *m/z* value could be separated and their identities were assigned based on their retention times.

Figure 5. Amount of all 17 amino acids in the white wine.



The amino acids content (mg/L) were quantified by external calibration. White wines 5-fold and 8-fold diluted with 0.1 N HCl were injected three times each, with the injection volume of 0.5 µL and average concentrations of the amino acids are presented.

Quantification of proline in wine by internal calibration

A Pro-2,2,5-d_3 concentration of 50 µM was added to each of seven calibration standard levels (1, 10, 25, 50, 100, 250, and 500 µM). The use of internal calibration (in contrast to external calibration) for proline quantification produced less variation regardless of the dilution factor, due to elimination of the matrix effect.

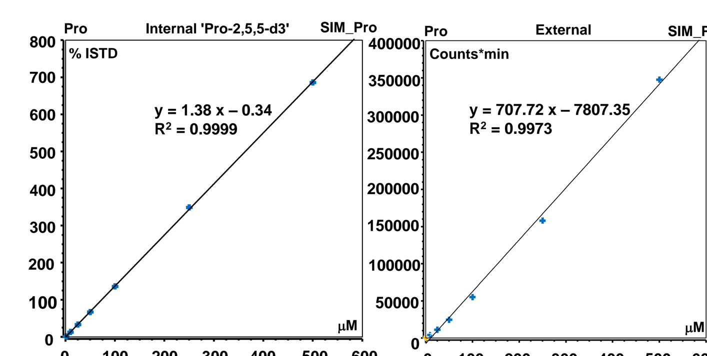


Figure 6. (a) Internal- and (b) external calibration curves of proline. Y-axis represents %ISTD, calculated by the percentage of area ratio of analyte (*i.e.*, proline) and internal standard (*i.e.*, proline--2,2,5-d_3). X-axis represents the concentration of the proline.

Table 5. Quantification results of proline in white wine (n = 3). The standard deviations for three replicate injections are given in parentheses.

	5-fold diluted wine (mg/L)	8-fold diluted wine (mg/L)
Internal calibration	204.3 (± 2.14)	203.5 (± 3.32)
External calibration	228.9 (± 3.95)	197.2 (± 4.28)

CONCLUSIONS

- A straightforward and reproducible method for the analysis of 22 native amino acids was developed on the Accucore Amide HILIC column by LC-SQMD.
- Mass detection enables the quantification of amino acids not fully resolved by chromatography..
- Proline quantification was more consistent and precise with internal calibration than external calibration, due to the elimination of the influence of sample matrix.
- The method reduces systematic and random error with less sample handing, resulting in method robustness.

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TRADEMARKS/LICENSING

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