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 robust 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 5 to 500 µM standard amino acids. The developed method was successfully applied to detect 17 amino acids commonly found in wine [1]. The 15 amino acids except a peak-pair (*i.e.*, Ala/Hyp) were well separated. 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 [2]. The amino acid content of wine is influenced by yeast strain, treatments used during fermentation, the grape variety, and production area [1]. 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 is labor intensive and time-consuming. It also requires careful control to achieve method robustness and reproducibility with minimal reagent interference [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 robust HILIC method with SQMD to determine 22 native amino acids. The developed method was then applied to analyze 17 amino acid components in a white wine. Finally, proline, a possible and important marker for wine diagnostic, was quantified using an isotopically labelled internal standard (proline-2,2,5-d_3).

MATERIALS AND METHODS

Sample Preparation

Standard solutions were prepared with the Pierce amino acid standard H, containing 17 amino acids (Ala, Arg, Asp, Cys, 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 in 0.1N HCl. Cystine is present at a concentration of 1.25 mM as the oxidized dimer form of cystein. 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 calibration 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 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/20 mM ammonium formate pH 2.81	
Mobile phase B	90/10 (v/v) 20 mM ammonium formate pH 2.81/ACN	
Flow rate	0.4 mL/min	
Column temp.	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	Stop run

Table 3. MS conditions

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

Data Analysis

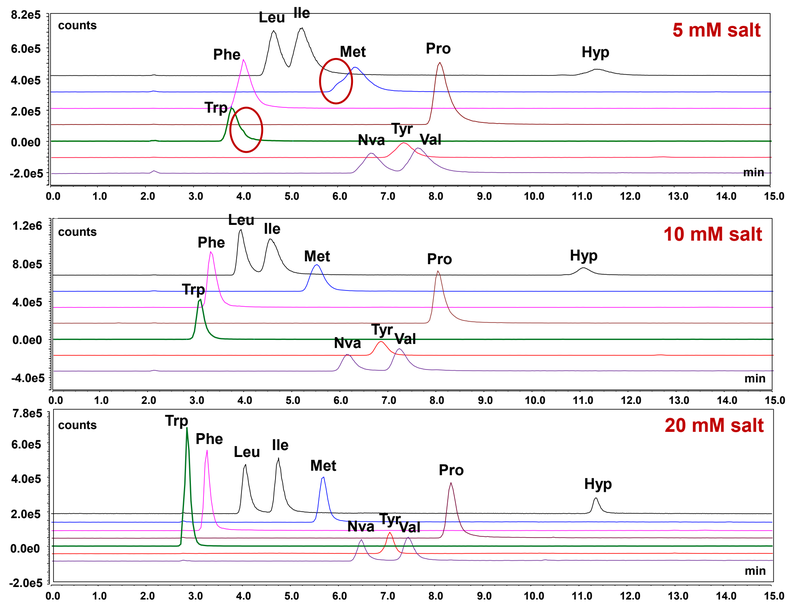
Thermo Scientific™ Chromeleon™ software (version 7.2.8) was used for instrument control and data acquisition and processing.

Results

Salt content optimization

Salt content is an important parameter to control the secondary interaction in HILIC, which influences both the peak shape and retention of analyte. According to the increase of the salt content (from 5 mM to 20 mM ammonium formate) in the mobile phase, analytes eluted faster and produced better peak shapes (Fig. 1). Ten selected amino acids of 250 µM were analyzed at a mobile phase flow-rate of 0.9 mL/min. Ammonium formate buffer at pH 3 was used as the mobile phase and the injection volume was 1 µL.

Figure 1. Effect of salt content on peak shape and retention for 10 selected amino acids in HILIC



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 on the Accucore™-150-Amide-HILIC column, within 22 minutes (Fig. 2). The *m/z* value for selected ion monitoring (SIM) scan for the detection of each amino acid component is listed in Table 4.

Figure 2. Chromatograms of SIM scan of 22 standard amino acids at a concentration of 100 µM. The LC and MS conditions are listed in Table 2 and 3

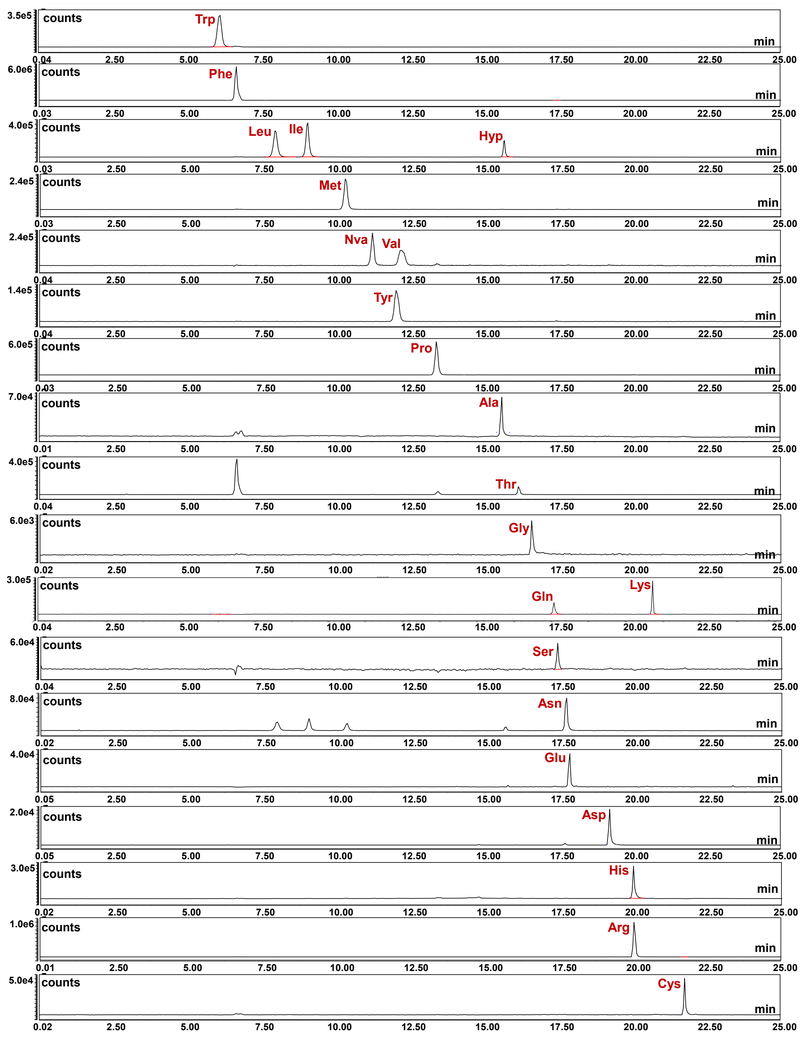


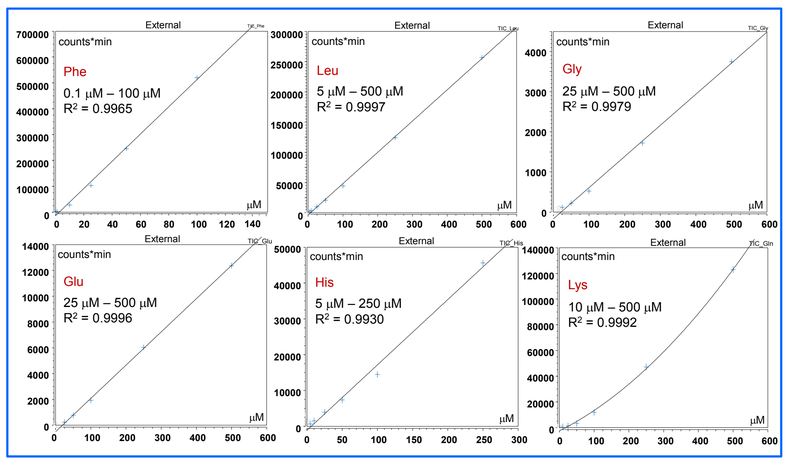
Table 4. Calibration parameters, retention time (RT), relative standard deviation (RSD) of RT and peak area for 22 standard amino acids, along with their *m/z* values (n = 10)

Name	*m/z*	RT	Calibration range (µM) & type	Regression	RT RSD (%)	Area RSD (%)
Trp	205.1	6.15	1 – 250 (Linear)	1.0000	0.39	2.15
Phe	166.1	6.64	0.1 – 100 (Linear)	0.9965	0.00	9.50
Leu	132.1	7.96	5 – 500 (Linear)	0.9997	0.00	2.80
Ile	132.1	9.07	5 – 500 (Linear)	0.9993	0.00	2.49
Met	150.1	10.31	5 – 250 (Linear)	0.9994	0.00	2.10
Nva	118.1	11.26	5 – 250 (Linear)	0.9983	0.00	2.06
Tyr	182.1	12.05	5 – 500 (Linear)	0.9991	0.00	1.85
Val	118.1	12.20	10 – 250 (Linear)	0.9997	0.16	2.43
Pro	116.1	13.42	1 – 500 (Linear)	0.9974	0.00	1.86
Ala	90.0	15.62	10 – 250 (Linear)	0.9984	0.00	1.89
Hyp	132.1	15.67	5 – 250 (Linear)	0.9968	0.04	2.00
Thr	120.1	16.17	5 – 250 (Linear)	0.9952	0.00	3.22
Gly	76.0	16.60	25 – 500 (Linear)	0.9979	0.00	6.44
Gln	147.1	17.36	10 – 250 (Quadratic)	0.9991	0.03	2.47
Ser	106.1	17.46	15 – 250 (Linear)	0.9984	0.02	3.98
Asn	133.1	17.74	5 – 250 (Linear)	0.9919	0.14	2.99
Glu	146.1	17.87	25 – 500 (Linear)	0.9996	0.07	4.05
Asp	132.0	19.24	10 – 500 (Linear)	0.9981	0.00	9.46
His	156.1	20.01	5 – 250 (Linear)	0.9930	0.00	1.85
Arg	175.1	20.05	5 – 500 (Linear)	1.0000	0.00	2.96
Lys	147.1	20.68	10 – 500 (Quadratic)	0.9992	0.00	3.81
Cys	241.1	21.78	15 – 500 (Quadratic)	0.9983	0.00	8.24

Method validation - Linearity and reproducibility

Calibration curves for standard amino acids were obtained with a range from 0.1 to 500 µM, along with R^2 values greater than 0.991. Excellent reproducibility, having average RSDs for RT and area of 0.04% and 3.66%,respectively, was achieved by 10 injections of a 100 µM standard mixture for 21 amino acids, except phenylalanine for which 25 µM solution was used due to its lower linear range from 0.1 to 100 µM (Table 4). Examples of six calibration curves were shown in Figure 3.

Figure 3. Calibration curves of selected amino acids



Analysis of amino acids in white 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 [2]. An example SIM chromatogram (in Fig. 4) illustrates the separation of three amino acids (Leu, Ile, Hyp) in wine.

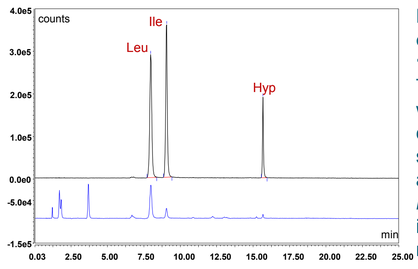
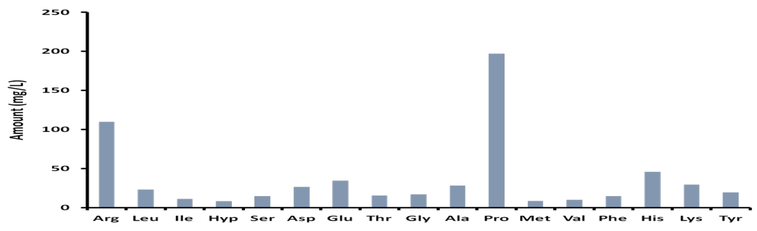


Figure 4. Example SIM chromatograms of amino acids recorded for the *m/z* 132.1
The chromatogram of wine sample (blue) was overlaid with that of the corresponding amino acids in 100 µM standard solution (black). Three amino acids (Leu, Ile, and Hyp) with the same *m/z* value could be separated and their identities were assigned based on their retention times.

The amounts of all 17 amino acids in the wine were determined by using external calibration listed in Table 4. Proline showed the highest amino acid amount with 197.2 mg/L which accounts for 32% of the total amino acid content [2]. The next most abundant amino acid was arginine which is usually not present in wine in significant amounts due to the intake by wine yeast. This result possibly implies less fermentation, resulting in the cheapest wine in the supermarket.

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



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). Figure 6 shows a calibration curve of proline, with excellent linearity (R^2 of 0.9999) over a range from 1 µM to 500 µM. The proline amount in wine was determined to be 203.6 mg/L (for 8-fold diluted wine) and was similar to that obtained by external calibration (*i.e.*, 197.2 mg/L), as discussed above. In addition, proline levels of 204.3 mg and 228.9 mg/L were obtained by internal and external calibration, respectively, when using 5-fold diluted wine (data not shown). 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.

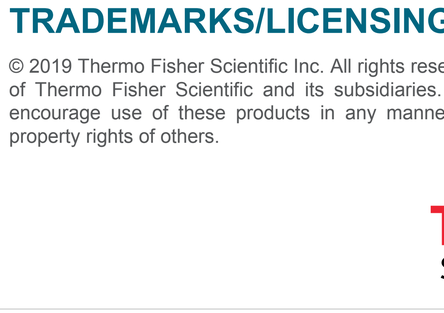


Figure 6. Internal calibration curve of proline
Y-axis represents %ISTD, calculated by the percentage of area ratio of analyte (*i.e.*, proline) and internal standard (*i.e.*, proline--2,2,5-d_3). X-axis represents the concentration of the proline.

CONCLUSIONS

- A straightforward and robust method for the analysis of 22 native amino acids was developed on the Accucore Amide HILIC column by LC-SQMD.
- The method saves analysis time in the laboratory relative to methods requiring pre- or post column derivatization.
- The method was successfully applied to detect 17 amino acids in a white wine.
- Proline quantification using internal calibration was more precise compared to external calibration, by eliminating the influence of sample matrix.

REFERENCES

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TRADEMARKS/LICENSING