thermoscientific

Underivatized amino acid analysis in wine by HILIC separation and mass detection

Authors

Soo Hyun Park, Katherine Lovejoy, Sylvia Grosse, Mauro De Pra, Stephan Meding, Frank Steiner

Thermo Fisher Scientific, Germering, Germany

Keywords

Underivatized amino acids, HILIC, single quadrupole mass detection, internal standard quantification, wine, ISQ EM, ISQ EC

Goal

• To develop a sensitive and reproducible method for analysis of 22 underivatized amino acids.

• To apply the method to quantify 17 amino acids in wine.

• To accurately quantify proline, using an isotopically labeled internal standard.

Application benefits

• Simple sample preparation without derivatization and further cleanup step reduces analyst´s labor and sources of error.

• Mass detection enables the quantification of amino acids not fully resolved by chromatography.

• The method reproducibly and sensitively detects amino acids in wine.

• An isotopically labeled standard allows highly accurate quantification of proline in wine.

ThermoFisher
SCIENTIFIC

Introduction

Amino acids in grapes serve as a source of nitrogen for yeast growth during wine fermentation and also influence the organoleptic properties of wine as precursors of alcohols, aldehydes, and esters.[1] Monitoring of levels of various amino acids in grapes, prior to yeast fermentation, can be of great importance in controlling the wine making process.[2] Specifically, the amount of nitrogen available to the wine yeast and the potential wine aroma are affected by the levels and types of amino acids. Tightly controlling amino acid availability can be beneficial for the production of high quality wine. The amino acid content in wine is influenced by yeast strain, treatments used during fermentation, the grape variety, and the terroir.[3] Numerous reports have successfully used the amino acid composition to differentiate wine products according to grape varieties, geographical origin, vintage year, and so on.[2-4] This can be useful for detecting wine adulteration. Therefore, there is increasing interest and need for the accurate analysis of both amino acid composition of wines and changes in amino acid content during wine manufacturing and aging. Proline is the most abundant amino acid in wine, reaching 30% to 85% of total amino acid content.[3] Since proline is not metabolized during yeast fermentation,[3] it can be used as a diagnostic marker for different grape varieties and terroirs. In addition, accurate proline quantification can be useful for quality control purposes.

Because most amino acids lack a chromophore or fluorophore, pre- or post-column derivatization is required to analyze them by liquid chromatography (LC) coupled with a UV or fluorescence detector. In addition to increasing detection sensitivity, the derivatization also enables retention and separation of even the most hydrophilic amino acids on reversed-phase columns by adding hydrophobic moieties.[5] However, the derivatization technique requires handling of toxic chemicals such as *o*-phthalaldehyde (OPA) and fluorenylmethyloxycarbonyl (FMOC). Derivatization reagents can produce peaks that interfere with analytes due to reaction byproducts, the reagent itself, impurities of the reagent, and degradation products. In addition, the derivatization technique requires analyst's additional labor, which can negatively affect method robustness and reproducibility due to systematic and random errors during sample handling step.

Hydrophilic interaction liquid chromatography (HILIC) combined with mass detection is an alternative for the analysis of amino acids because amino acids can be analyzed without derivatization. HILIC is a good option for the retention and separation of the amino acids, which are mostly highly polar compounds. The use of a mass detector allows accurate quantification of analytes in complex samples despite incomplete separation. The ISQ single quadrupole mass detectors are easy to operate and control even for analysts who do not have in-depth mass spectrometry expertise.

In this work, we developed a sensitive and reproducible HILIC method combined with single quadrupole mass detection for the analysis of 22 underivatized amino acids, applicable to a variety of samples such as food, beverages, and fermentation media. The developed method was then applied to a wine sample and 17 amino acids, previously reported,[3] were analyzed. Finally, proline, a possible diagnostic marker for wine, was quantified using an isotopically labeled internal standard.

Experimental
Chemicals

- Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System, deionized water, 18.2 MΩ·cm at 25 °C (P/N 50136149)

- Fisher Scientific™ acetonitrile, Optima™ LC-MS grade (P/N A955)

- Fisher Scientific™ formic acid, Optima™ LC-MS grade (P/N A117)

- Fisher Scientific™ ammonium formate, Optima™ LC-MS grade (P/N A115)

- Thermo Scientific™ Pierce™ Amino Acid Standard H (P/N PI20088)

- L-Amino acids, Sigma-Aldrich (P/N SIAL-LAA21-1KT)

- L-proline-2,5,5-d$_3$, Sigma-Aldrich (P/N 791261)

- Hydrochloric acid, fuming, 37%, Merck (P/N 1.00317.1000)

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™ Finnpipette™ 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)

- Sartorius™ 17761---K, Minisart® RC Syringe filter (15 mm, 0.2 µm pore size), purchased from Fisher Scientific (P/N 10712712)

Instrumentation

Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:

- Vanquish System Base Horizon/Flex (P/N VH-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-02)

- 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 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, Hyp and Trp) from Sigma-Aldrich, as listed in Table 1. The Pierce Amino Acid Standard H contains 2.5 mM of each of the amino acids except cystine (1.25 mM, oxidized cysteine dimer) in 0.1 N HCl. Stock solutions of the five additional amino acids were prepared at a concentration of 10 mM in 0.1 N HCl solution.

Working solutions and calibration standards were prepared by diluting the stock solution with an appropriate volume of 0.1 N HCl solution. Table 2 lists concentrations and volumes used to prepare 500 µM standard mixture for 22 amino acids. External calibration curves for all 22 amino acids were obtained by injections of calibration standards with three replicates at each concentration level, with a range from 0.1 to 500 µM. Internal calibration standard solutions for proline quantification were prepared by adding the same concentration of proline-2,2,5-d_3 (50 µM) to each concentration level for the calibration. A white wine (Riesling from the Palatinate, Germany) was purchased from a local supermarket. One milliliter of wine was filtered using a 0.20 µm syringe filter and was then diluted 5-fold or 8-fold with 0.1 N HCl prior to injection. For proline quantitation in wine using the internal calibration, 40 µL of (either 5-fold or 8-fold) filtered wine, 100 µL of pro-2,5,5-d_3 (100 µM), 60 µL of 0.1 N HCl solution were mixed, spun down and transferred into a sample vial for injection.

Table 1. Molecular weight, selected ion monitoring (SIM) mass, and SIM acquisition polarity for 22 amino acids. SIM mass is [M+H]+ for positive mode and [M-H]− for negative mode acquisitions.

Name	Acronym	Monoisotopic mass [M]	SIM mass	SIM acquisition polarity
Alanine	Ala	89.05	90.0	Positive
Arginine	Arg	174.11	175.1	Positive
Asparagine	Asn	132.05	133.1	Positive
Aspartic acid	Asp	133.04	132.0	Negative
Cystine	Not available	240.02	241.1	Positive
Glutamic acid	Glu	147.05	146.1	Negative
Glutamine	Gln	146.10	147.1	Positive
Glycine	Gly	75.03	76.0	Positive
Histidine	His	155.07	156.1	Positive
Hydroxyproline	Hyp	131.06	132.1	Positive
Isoleucine	Ile	131.09	132.1	Positive
Leucine	Leu	131.09	132.1	Positive
Lysine	Lys	146.11	147.1	Positive
Methionine	Met	149.05	150.1	Positive
Norvaline	Nva	117.08	118.1	Positive
Phenylalanine	Phe	165.08	166.1	Positive
Proline	Pro	115.06	116.1	Positive
Serine	Ser	105.04	106.0	Positive
Threonine	Thr	119.06	120.1	Positive
Tryptophan	Trp	204.23	205.1	Positive
Tyrosine	Tyr	181.19	182.1	Positive
Valine	Val	117.15	118.1	Positive

Table 2. Preparation of 500 µM standard mixture for 22 amino acids

Solution	Concentration	Volume (µL)
Pierce Amino Acid Standard H	2.5 mM	200
Asparagine	10 mM	50
Glutamine	10 mM	50
Tryptophan	10 mM	50
Norvaline	10 mM	50
Hydroxyproline	10 mM	50
Hydrochloric acid	0.1 N	550

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 phases was 20 mM ammonium formate.

Chromatographic conditions

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
	5	0.0
	15	15.6
	20	33.3
	30	33.3
	30.2	0.0
	40	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

Ionization mode:	ESI
Polarity (Spray voltage):	Positive (+2500 V) and Negative (-2000 V)
Full scan:	m/z 60–350
SIM scan:	SIM masses are listed in Table 1.
SIM width:	0.2 amu
Dwell time:	0.1 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
	Auxiliary gas, 7.3 psig
	Sweep gas, 2.0 psig

Software
Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.9, was used for data acquisition and analysis.

Results and discussion
Optimization of buffer salt content
Buffer salt content in the HILIC mobile phase is an important parameter to control both peak shape and retention of analytes because it influences secondary interaction such as the polar interaction between analytes and the stationary phase and the thickness of the water layer formed at the surface of the stationary phase. Ammonium formate or acetate are suitable salts used for mass detection because they are volatile. However, due to contamination problems for the ion source, a maximum salt concentration of 20 mM is recommended. Figure 1 illustrates the effect of ammonium formate salt content on peak shape and retention for ten early-eluting amino acids on the Accucore-150-Amide-HILIC column. The analytes eluted faster and produced better (and narrower) peak shapes with increasing salt content in the mobile phase. For example, methionine and tryptophan produced symmetric peaks using a salt concentration of 20 mM but showed peak-fronting (methionine) and peak-tailing (tryptophan) at 5 mM. Therefore, 20 mM was set as final salt concentration. The mobile phase flow rate of 0.9 mL/min and buffer pH 3 were used as initial conditions. Optimization yielded final conditions of 0.4 mL/min for the flow rate and mobile phase pH at 2.8. Signal sensitivity, peak shapes, and chromatographic selectivity were considered for the optimization. For example, overall signal responses for 22 amino acids analyzed in this work increased roughly 2.6 times when using a flow rate of 0.4 mL/min, compared to the flow rate of 0.9 mL/min. In addition, better peak shapes (especially for aspartic acid and histidine) and chromatographic selectivity were generally obtained at buffer pH 2.8 among five pH tested (i.e., pH 2.8, 3.3, 3.5, 4.3, and 4.8) (data not shown).
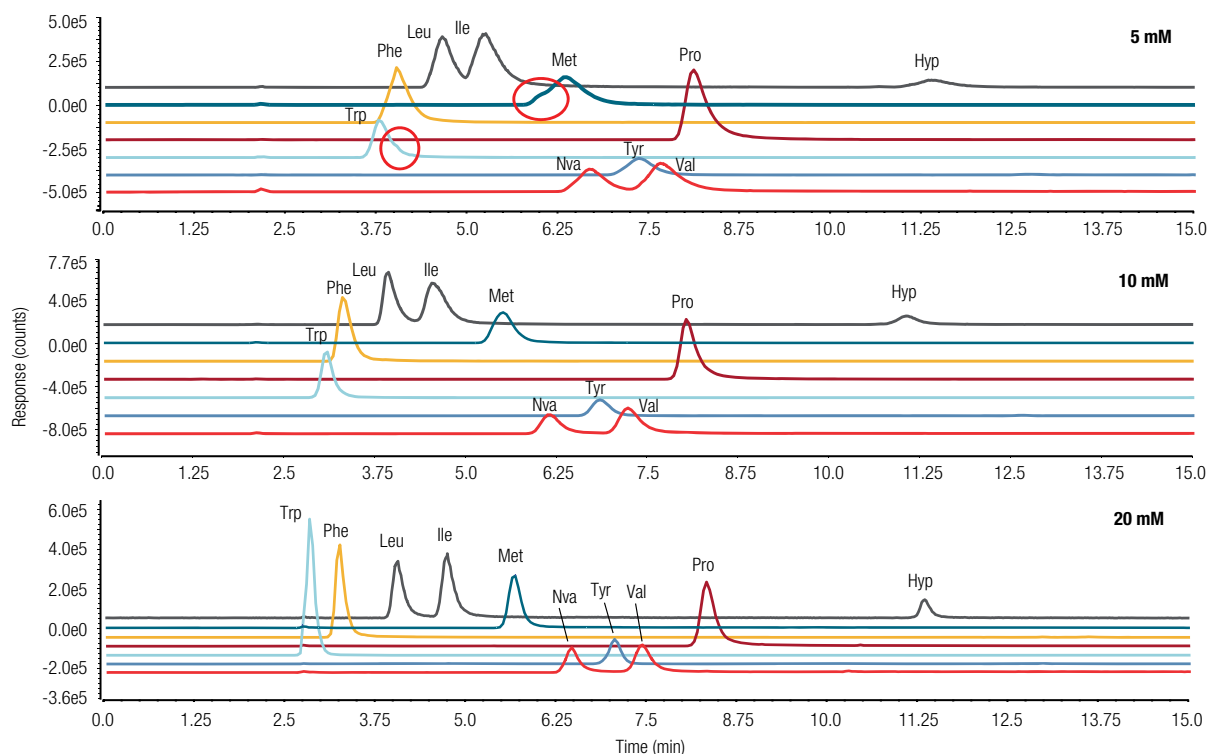
Figure 1. Optimization of buffer salt content on HILIC, showing the enhancement of peak shape and retention for ten selected amino acids at the optimal concentration of 20 mM. Ammonium formate buffer (of 5, 10, and 20 mM) at pH 3 was used in the mobile phase. The standard mixture of 250 µM was injected 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.

Optimization of ion source parameters of mass detector

Ion source parameters for aspartic acid and glycine were optimized since the two amino acids showed the lowest detection responses for 250 µM standard mixture under the test condition, resulting in the lowest signal response for the aspartic acid in 5-fold diluted wine and no detection for the glycine. Autospray intelligent source settings in the Chromeleon CDS software initially allowed rough and straightforward selection of ion source settings such as vaporization temperature, ion transfer tube temperature, and sheath, auxiliary, and sweep gas pressure. The default values for these ion source settings are 227 °C, 300 °C, 42.9, 4.8, and 0.5 psig, respectively, at the flow rate of 0.4 mL/min. In the Autospray ion source setting, three slider bars, related to basic parameters such as volatility of mobile phase, robustness/sensitivity, and thermal stability of analytes, can be easily moved up and down for the adjustment of the parameters.[5] The adjusted values of vaporization temperature, ion transfer tube temperature, and sheath, auxiliary, and sweep gas pressure were 427 °C, 300 °C, 72.9, 6.8, and 2 psig, respectively. The fine optimization of these parameters, along with additional two parameters (such as source voltage and source Collision

Table 3. Test points for fine optimization of ion source parameters

Test parameter	Test points
Vaporizer temperature (°C)	377, 425, 477
Sheath gas pressure (psig)	75, 77.5, 80
Auxillary gas pressure (psig)	6.8, 7.3, 7.8
Sweep gas pressure (psig)	1.5, 2
Ion transfer tube temp. (°C)	275, 300, 325
Positive source voltage (V)	2500, 3000, 3500
Negative source voltage (V)	1500, 2000, 2500
CID voltage (V)	0, 5, 10, 15, 20, 25, 30

Induced Dissociation [CID] voltage), followed, as shown in Table 3. The optimized values of these parameters were 477 °C, 300 °C, 80, 7.3, and 2 psig, respectively, as summarized in the table of MS detector settings.

The signal (or area) responses for glycine and aspartic acid were increased by 5.1 times and 7.9 after the adjustment of Autospray ion source settings and by 6.1 times and 11.7 after the fine optimization (data not shown). The 22 SIM scans were performed simultaneously on the ISQ EM single quadrupole mass detector.
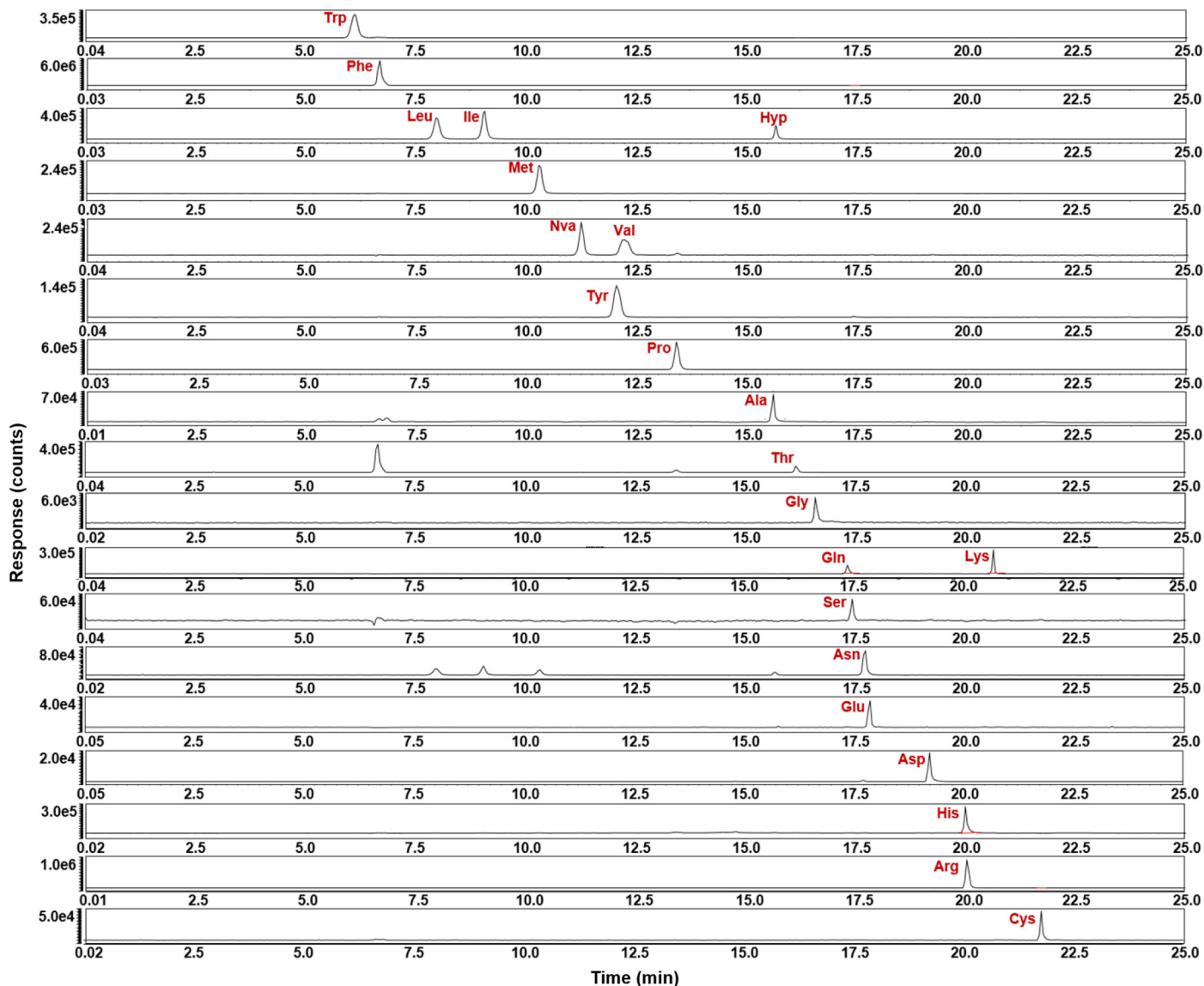
Figure 2. Chromatograms of SIM scan of 22 standard amino acids at a concentration of 100 µM. Amino acids with same *m/z* values (Leu/Ile/Hyp, Nva/Val, and Gln/Lys) are detected under the same SIM channels (*m/z* 132.1, 118.1, and 147.1).

Two amino acids (i.e., glutamic acid and aspartic acid) with carboxylic acid side chains were detected in negative ion mode in the same run as all the other 20 amino acids detected by positive ion mode. Figure 2 illustrates typical chromatograms of SIM scans of 22 standard amino acids. The *m/z* value for SIM scan for each amino acid component is listed in Table 1. All the amino acids except five peak pairs (i.e., Tyr/Val, Ala/Hyp, Gln/Ser, Asn/Glu, His/Arg) were baseline-separated on the Accucore-150-Amide-HILIC column within 22 minutes. In addition, two isomer peak pairs (i.e., Leu/Ile and Val/Nva) were well resolved with values of peak resolution R_s of 5.37 and 3.20, respectively.

Method linearity, sensitivity, and reproducibility

The calibration curves were constructed with either linear or quadratic fits, based on the averaged peak areas of the corresponding SIM scans. Figure 3 shows examples of the calibration curves of six selected amino acids. Calibration results for all 22 amino acids were listed in Table 4. Method linearity and curve fits were found to be excellent, with the coefficient of determination R^2 greater than 0.991 for all 22 amino acids and 0.999 for 10 of the 22.

In addition to the calibration parameters (such as calibration range, fit type, R^2), values of limit of quantification (LOQ) and limit of detection (LOD) are listed in Table 4. The LOQ and LOD values for each analyte were estimated by extrapolation to signal-to-noise ratios (S/N) of 10 and 3, respectively, from actual measurements of samples diluted until their S/N was close to 10 for LOQ and 3 for LOD.
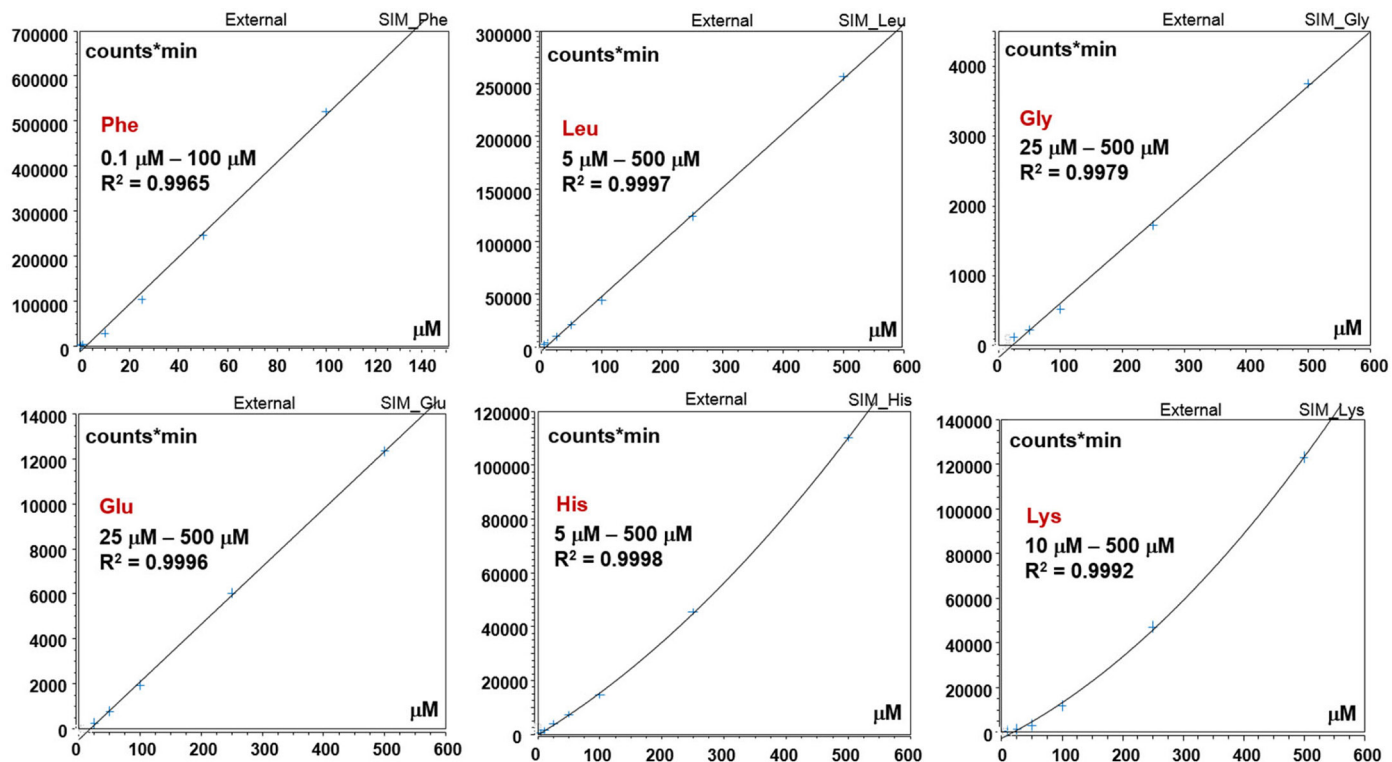
Figure 3. Examples of calibration curves of six selected amino acids (Phe, Leu, Gly, Glu, His, and Lys). The calibration curves were obtained based on the average peak area of each SIM scan (n=3). Phe, Leu, Gly, and Glu have linear fits, and His and Lys have quadratic fits.

Table 4. Calibration parameters, LOQ, and LOD values

Name	Concentration range (µM)	Curve fit type	R^2	LOQ (µM)	LOD (µM)
Trp	1–50	Linear	1.0000	0.66	0.20
Phe	0.1–100	Linear	0.9965	0.08	0.02
Leu	5–500	Linear	0.9997	1.56	0.47
Ile	5–500	Linear	0.9993	1.18	0.35
Met	5–250	Linear	0.9994	1.06	0.32
Nva	5–250	Linear	0.9983	2.34	0.70
Tyr	5–500	Linear	0.9991	2.42	0.73
Val	10–250	Linear	0.9997	6.21	1.86
Pro	1–500	Linear	0.9974	0.44	0.13
Ala	10–250	Linear	0.9984	5.50	2.50
Hyp	5–250	Linear	0.9968	2.95	0.88
Thr	5–250	Linear	0.9952	2.42	0.72
Gly	25–500	Linear	0.9979	18.12	5.67
Gln	10–250	Quadratic	0.9991	6.15	1.84
Ser	25–500	Linear	0.9971	17.73	5.32
Asn	5–250	Linear	0.9919	2.49	0.75
Glu	25–500	Linear	0.9996	17.56	5.27
Asp	10–500	Linear	0.9981	7.98	2.39
His	5–500	Quadratic	0.9998	2.08	0.62
Arg	5–500	Linear	1.0000	1.01	0.30
Lys	10–500	Quadratic	0.9992	10.00	3.10
Cystine	5–250	Quadratic	0.9982	9.65	2.89

Repeatability tests were performed by ten injections of a 100 µM standard mixture for 21 amino acids, except phenylalanine for which 25 µM solution was used. The method was found to be highly reproducible, showing low values of relative standard deviation (RSD) for retention time and peak area (Table 5) for most of the amino acids. The RSD of retention time were less than 0.1% for all the amino acids except valine (RSD of 0.18%) and asparagine (RSD of 0.12%). In addition, an extended column washing step for 10 minutes with 33.3%B was found to be effective in keeping column performance stable throughout the consecutive 20 injections.

Quantification of amino acids in wine

Seventeen amino acids, commonly found in wine[3], were quantified by external calibration. Five-fold and 8-fold dilutions of white wine were injected three times each and the averaged values were used for the quantification. An example SIM chromatogram of amino acids in wine is shown in Figure 4, which depicts excellent chromatographic resolution of three isobaric amino acids (Leu, Ile, and Hyp). The three amino acids, detected at the same m/z 132.1, were well resolved on the Accucore-150-Amide-HILIC column, including a challenging isomer peak pair Leu/Ile (Rs of 5.37). The peaks were identified and/or assigned by comparing retention times on the chromatogram of a wine sample (lower) with those on the overlaid chromatogram (upper), obtained for the 100 µM standard mixture.

Table 5. Reproducibility of retention time (RT) and peak area for standard amino acids (n = 10), along with the corresponding averaged RT

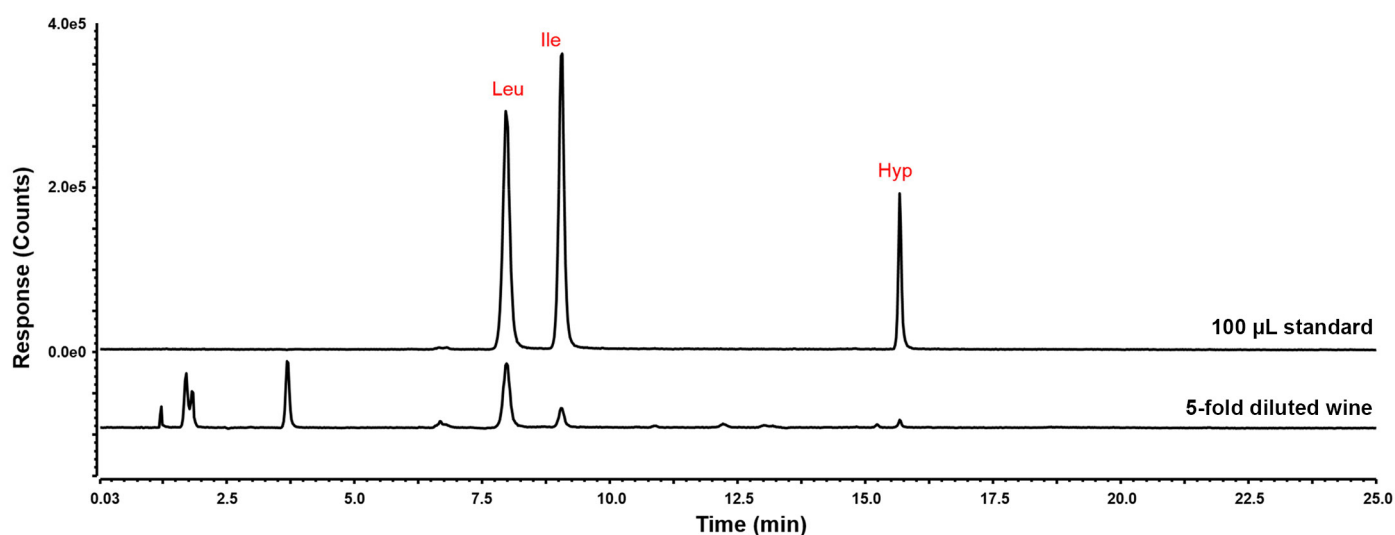| Name	Average RT (min)	RT RSD (%)	Area RSD (%)
Trp	6.11	0.008	2.15
Phe	6.64	0.005	9.50
Leu	7.96	0.012	2.80
Ile	9.07	0.008	2.49
Met	10.31	0.000	2.10
Nva	11.26	0.007	2.06
Tyr	12.05	0.000	1.85
Val	12.20	0.177	2.43
Pro	13.42	0.000	1.86
Ala	15.62	0.000	1.89
Hyp	15.68	0.041	2.00
Thr	16.17	0.003	3.22
Gly	16.60	0.000	6.44
Gln	17.36	0.025	2.47
Ser	17.46	0.000	3.98
Asn	17.73	0.120	2.99
Glu	17.87	0.078	4.05
Asp	19.24	0.003	9.46
His	20.01	0.000	1.85
Arg	20.05	0.002	2.96
Lys	20.68	0.000	3.81
Cystine	21.78	0.002	8.24

Figure 4. Example SIM chromatograms of amino acids in wine, recorded at m/z 132.1. The chromatogram of (5-fold diluted) wine sample (lower) was overlaid with that of the corresponding amino acids in 100 µM standard solution (upper). 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 shows the amount of all 17 amino acids in the wine sample, which include Ala, Arg, Asp, Glu, Gly, His, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val. Slightly higher (from 1.02 to 1.5 times) amounts were detected for all amino acids except proline and valine using the 8-fold diluted wine than the 5-fold diluted wine, possibly due to less matrix-induced signal suppression. Levels of the outliers, proline and valine, were 1.2 and 1.02 times higher in the 5-fold diluted wine. The amounts of amino acids were within the range of values reported for those obtained from 42 Greek white wines.[6] Three amino acids (Gly, Hyp, Ser) for 5-fold diluted wine and six amino acids (Gly, Hyp, Ile, Met, Ser, Val) for 8-fold diluted wine were detected at the levels between LOQ and LOD. In addition,
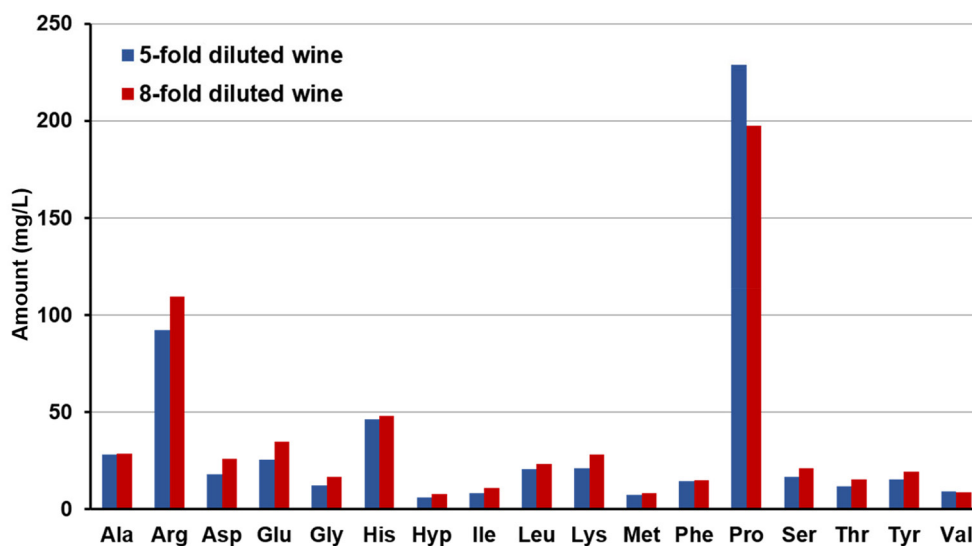

Figure 5. Amount of all 17 amino acids in wine. The amino acids content (mg/L) were quantified by external calibration. White wines 5-fold and 8-fold diluted with 0.1 N HCl were injected three times each, with the injection volume of 0.5 µL and average concentrations of the amino acids are presented.

Table 6. Quantification results of 17 amino acids in white wine. The unit of amount is mg/L and the standard deviations for three replicate injections are given in parentheses.

Name	5-fold diluted wine	8-fold diluted wine	Minimum amount in Greek wine[6]	Maximum amount in Greek wine[6]
Ala	28.0 (± 1.25)	28.6 (± 1.19)	3.79	238
Arg	92.1 (± 5.44)	109.6 (± 4.36)	4.05	1075
Asp	17.9 (± 0.51)	26.0 (± 0.46)	3.90	74.8
Glu	25.5 (± 0.25)	34.8 (± 2.88)	6.74	140.2
Gly	12.4 (± 0.27)	16.9 (± 0.79)	2.43	38.4
His	46.3 (± 2.54)	48.3 (± 2.01)	1.02	79.4
Hyp	5.9 (± 0.37)	8.0 (± 0.25)	NA	NA
Ile	8.2 (± 0.04)	11.2 (± 0.11)	0.00	18.0
Leu	20.6 (± 0.11)	23.2 (± 0.67)	3.92	44.5
Lys	20.9 (± 0.22)	28.0 (± 0.77)	5.42	78.8
Met	7.2 (± 0.13)	8.4 (± 0.22)	0.38	14.8
Phe	14.4 (± 0.11)	14.9 (± 0.02)	2.75	52.5
Pro	228.9 (± 3.95)	197.2 (± 4.28)	NA	NA
Ser	16.7 (± 0.38)	20.9 (± 0.79)	1.14	47.4
Thr	11.8 (± 0.23)	15.5 (± 0.22)	4.88	62.6
Tyr	15.5 (± 0.67)	19.3 (± 0.87)	1.86	36.2
Val	9.0 (± 0.37)	8.9 (± 0.57)	0.00	37.5

proline showed the highest amino acid amount with 228.9 mg/L for the 5-fold diluted wine, which accounts for 39% of total amino acid content. The next most abundant amino acid was arginine, which is present in various amounts in wine, depending on amounts of other amino acids preferably utilized by wine yeast.[6]

Quantification of proline in white wine by internal calibration

The quantification of proline was additionally performed using an isotopically labeled internal standard (i.e., proline-2,5,5-d_3). The proline-2,5,5-d_3 concentration of 50 µM was added to each of seven calibration standard levels (1, 10, 25, 50, 100, 250, and 500 µM). As shown in Figure 6a, excellent linearity (R^2 of 0.9999) was obtained over a calibration range from 1 µM to 500 µM. Results for the quantitation of proline are summarized in Table 7. The proline amount in wine was determined to be 204.3 mg/L and 203.5 mg/L for 5-fold and 8-fold diluted wines, respectively. The result was compared with that using external calibration to examine if external calibration can be properly applied for quantification of proline in wine. The external calibration curve is illustrated in Figure 6b, showing slightly lower linearity (R^2 of 0.9973) over the same calibration range. The proline amount in wine was determined to be 228.9 mg/L and 197.2 mg/L for 5-fold and 8-fold diluted wines, respectively. The use of internal calibration (in contrast to external calibration) for proline quantification produced less variation regardless of the dilution factor, implying the reduction of a variation in signal response in wine matrix (i.e., the elimination of matrix effects). In addition, fewer variations in proline amounts between injections were found using the internal calibration, showing improved precision of quantitation. The standard deviation values shown in Table 7 reflect this improvement.

Table 7. Quantification results of proline in white wine. The unit of amount is mg/L and the standard deviations for three replicate injections are given in parentheses.

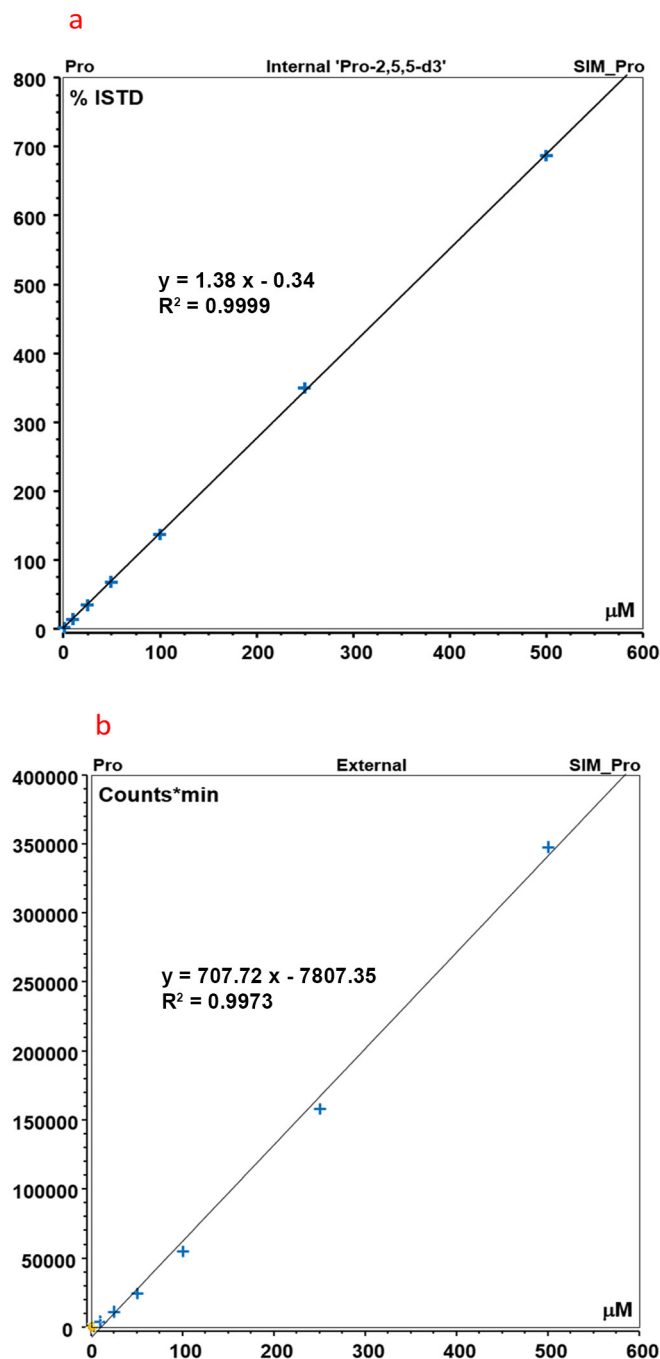|	5-fold diluted wine	8-fold diluted wine
Internal calibration	204.3 (± 2.14)	203.5 (± 3.32)
External calibration	228.9 (± 3.95)	197.2 (± 4.28)

Figure 6. (a) Internal- and (b) external calibration curves of proline. Seven calibration levels (1, 10, 25, 50, 100, 250, and 500 µM) were used for both calibrations. For the internal calibration, a proline-2,5,5-d_3 concentration of 50 µM was added to each of seven calibration standards. In the internal calibration curve, y-axis represents %ISTD calculated by the percentage of area ratio of analyte (i.e., proline) and internal standard (i.e., proline-2,5,5-d_3). X-axis represents the concentration of the proline. Y-axis in the external calibration curve represents peak area.

thermoscientific

Conclusion

- A straightforward and reproducible method for the analysis of 22 underivatized amino acids was developed on the Accucore-150-Amide-HILIC column with ISQ EM mass detector.

- ISQ EM provides accurate quantification while acquiring multiple SIM scans in both polarities in parallel.

- 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 handling, resulting in method robustness.

- The method here described is fully portable to the ISQ EC.

References

1. Belda, I.; Ruiz, J.; Esteban-Fernández, A.; Navascués, E.; Marquina, D.; Santos, A.; Moreno-Arribas, M.V. Microbial contribution to wine aroma and its intended use for wine quality improvement, *Molecules* **2017**, *22,* 189–217.

2. Callejón, R.M.; Troncoso, A.M.; Morales, M.L. Determination of amino acids in grape-derived products: A review, *Talanta* **2010**, *81,* 1143–1152.

3. Lehtonen, P., Determination of amines and amino acids in wine – A review, *Am. J. Enol. Vitic.* **1996,** *47,* 127–133.

4. Soufleros, E.H.; Bouloumpasi, E.; Tsarchopoulos, C.; Biliaderis, C.G. Primary amino acid profiles of Greek white wines and their use in classification according to variety, origin and vintage, *Food Chemistry* **2003,** *80,* 261–273.

5. Gokmen, V.; Serpen, A.; Mogol, B.A., Rapid determination of amino acids in foods by hydrophilic interaction liquid chromatography coupled to high-resolution mass spectrometry, *Anal. Bioanal. Chem.* **2012,** *403,* 2915–2922.

6. Thermo Fisher Scientific Application Note 72976: Identity confirmation and accurate quantitation of a genotoxic impurity in an active pharmaceutical ingredient by UHPLC-UV coupled to a single quadrupole mass detector. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-72976-lc-ms-genotoxic-impurity-pharmaceutical-an72976-en.pdf

Find out more at **thermofisher.com**

Thermo Fisher
SCIENTIFIC