

Article

Development and Validation of a Fast UHPLC–HRMS Method for the Analysis of Amino Acids and Biogenic Amines in Fermented Beverages

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Abstract

Considering the importance of free amino acids (FAAs) and biogenic amines (BAs) in the production of fermented beverages (FB), the interest in the quantification of these compounds has been growing. So far, most of the analytical methods developed entail a derivatization step. While this technique allows for the detection of several compounds, it is often associated with scarce accuracy and poor resolution. To counteract the drawbacks, in this study, we aimed to develop a fast, simple, and effective method that combines the use of ultra-high-performance liquid chromatography (UHPLC) and high-resolution mass spectrometry (HRMS) to quantify underivatized FAAs and BAs in FBs. The method was successfully developed and validated: it allowed for the accurate and precise quantification of 20 FAAs—including leucine and isoleucine—and 12 BAs in just 12 min. Its applicability was demonstrated on commercial samples of wines, beers, ciders, saké, and vinegars.

Keywords: underivatized; chromatography; food composition; fermentation

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1. Introduction

Fermented beverages (FBs) have played a central role in human societies since ancient times, not only as dietary staples but also as cultural and ritual elements. Their production offered clear advantages, including longer shelf life, improved safety, enhanced nutritional value, and more appealing sensory properties [1,2]. Today, the FB sector is evolving in response to modern consumer demands. Alongside traditional alcoholic drinks, there is increasing interest in FBs with potential health benefits, which now represent a rapidly expanding segment of the functional food and beverage market [3].

FBs are the result of microbial-driven biochemical transformations. During fermentation, microorganisms (m/os) convert sugars and other components of raw materials into a range of bioactive compounds, leading to changes in taste, aroma, texture, and nutritional content [4]. To perform these transformations, m/os require various nutrients, among which nitrogen (N) is essential. N can be assimilated in different forms, including ammonium, peptides, and free amino acids (FAAs) [5]. Among these, FAAs are particularly important because they not only sustain microbial growth but also influence key metabolic pathways that determine the sensory and functional properties of the final product [2,6,7]. In oenology, for example, the amino acid (AA) profile of grape must directly impacts yeast performance and the development of aromatic compounds [8]. FAAs also contribute to other sensory attributes such as color [9] and flavor [10,11].

From a nutritional standpoint, FAAs are bioavailable compounds with diverse physiological functions. They serve as precursors in biosynthetic pathways, act as neurotransmitters, and support energy metabolism [12]. Thus, their quantification not only provides insights into the fermentation process but also allows the assessment of the nutritional quality of FBs. In addition, FAA profiles can be used to trace food origin and authenticity, supporting food quality assurance [13,14].

Biogenic amines (BAs) are another important class of nitrogenous compounds that may form during fermentation. They are primarily produced by the microbial decarboxylation of FAAs or through reductive amination and transamination reactions involving aldehydes or ketones. Some BAs may also derive from enzymatic activity in raw materials [15,16]. While small amounts of BAs are common in fermented products, their excessive accumulation can pose health risks, including headaches, hypertension, and allergic responses. Ethanol—produced during fermentation—can inhibit the activity of amine-degrading enzymes such as monoamine oxidase and diamine oxidase, exacerbating the potential for BA build-up in alcoholic FBs [17].

Given the dual importance of FAAs and BAs in terms of nutritional value and food safety, their accurate and simultaneous quantification is of growing interest to researchers and industry alike [18–20]. Numerous analytical methods have been developed for this purpose, including high-performance liquid chromatography (HPLC) with fluorescence detection [21–24], mass spectrometry (MS and MS/MS) [25–28], gas chromatography (GC) [29,30], capillary electrophoresis (CE) [31,32], and ion-exchange chromatography (IEC) [33,34]. However, the inherent chemical properties of FAAs and BAs—such as high polarity, low volatility, and lack of chromophores—often necessitate derivatization prior to detection. Derivatization reagents such as o-phthalaldehyde (OPA), FMOC, dansyl chloride, and others are commonly used to improve chromatographic behavior and detector response [24–41].

Despite their utility, derivatization steps introduce notable challenges, including incomplete reactions, derivative instability, toxic reagents, and extended sample preparation times. These issues can compromise the accuracy, sensitivity, and repeatability of the methods [12,42]. To overcome these limitations, several researchers have explored LC–MS/MS techniques for the direct, underivatized detection of FAAs and BAs [23,43–47]. While these methods show promise, they often face constraints such as limited linear range, long run times, or an insufficient resolution of isomeric compounds [48–53].

Additionally, most studies have focused on individual compound classes (either FAAs or BAs) and on food matrices other than FBs. Only a few have addressed fermented beverages specifically [54,55], and the simultaneous quantification of underivatized FAAs and BAs remains extremely rare. One such attempt—by Nakano et al. [56]—required dual-column, dual-ionization setups to analyze cheese samples, underscoring the complexity of such an analysis. To our knowledge, no comparable method has been developed for FBs.

In this context, the aim of the present study was to develop a simple, rapid, and robust UHPLC–HRMS method for the direct quantification of underivatized FAAs and BAs in FBs. The method also includes glutathione (GSH), a yeast-derived tripeptide with antioxidant properties and increasing relevance in both oenology and functional beverage development [16,57–60]. The method was applied to six commercial FBs and grape must samples, chosen for their relevance to both fermentation science and practical industry applications.

2. Materials and Methods

2.1. Chemicals and Reagents

FAAs, Bas, and GSH reference standards and their purities are reported in the Supplementary Materials (Table S1). Methanol (MeOH; Honeywell, Charlotte, NC, USA) and acetonitrile (ACN; Honeywell, NC, USA) were LC–MS grade; deionized water was produced with an Arium® 98 Pro Lab Water System (Sartorius AG, Gottingen, Germany).

2.2. Standard and Sample Solutions Preparation

A stock solution containing all the analytes of interest was prepared by dissolving the standards in water/MeOH (90:10) with a few drops of 37% hydrochloric acid (HCl; Honeywell, NC, USA) to improve solubility. Matrix-matched calibration curves were built by adding 20 µL of sample to different dilutions of the stock solution in a final volume of 1 mL. A water/ACN (20:80) mixture containing 0.1 N HCl was used as diluent. In-solvent calibration curves were also created by diluting the stock solution with the same diluent. On average, concentrations of 25 mg/L were obtained for the stock. As for the calibration, solutions were prepared directly into HPLC vials at concentrations ranging from 0.001 to 10 µg/L. The working solution was prepared immediately before being used and then kept refrigerated. Calibration solutions were prepared freshly on the day of the analysis.

To study the potential applications of the method, the following FBs were purchased at a local supermarket: n° 3 apple ciders, n° 3 beers, n° 3 vinegars, and n° 3 saké. Three white and three red wines, as well as three must samples, were provided by the experimental micro-vinery of the Edmund Mach Foundation (San Michele all'Adige, TN, Italy). Regarding sample preparation, upon centrifugation ($1400 \times g$, 5 min) and filtration with 0.45 µm polytetrafluoroethylene (PTFE) filters, samples were diluted 50-fold in a water/ACN (20:80) solution containing 0.1 N HCl. A final volume of 5 mL was obtained for each diluted sample.

2.3. LC–MS Parameters

The LC–MS system consisted of an Ultimate R3000 UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an HRMS (Q-Exactive hybrid Q-Orbitrap, Thermo Fisher Scientific) equipped with a HESI-II interface working in positive ionization mode. The UHPLC system included a binary solvent delivery system, an autosampler, and a column oven. Data acquisition and processing were operated using Chromeleon 7.3 Chromatography Data System software (Dionex; Sunnyvale, CA, USA).

Chromatographic separation was obtained using a gradient elution on a Raptor Polar X column (2.1×100 mm, 2.7 µm), which integrates hydrophilic interaction liquid chromatography (HILIC) and ion-exchange retention mechanisms in a single ligand. The mobile phases consisted of (solvent A) water with 0.5% formic acid (FA; Honeywell, NC, USA) and (solvent B) acetonitrile (ACN; Honeywell, NC, USA). Gradient elution started with 17% A, 83% B for 1.5 min; then, it ramped to 80% A, 20% B within the next 5.5 min and was held for 2.4 min. Finally, eluents returned to the initial condition within 0.1 min and were held for 2.4 min for column re-equilibration. The column was operated at 30 °C throughout the total run time of 12 min. The flow was kept constant at a rate of 0.5 mL/min, and the injection volume was 5 µL.

2.4. Method Validation

The validation protocol followed the guidelines provided by the Food and Drug Administration (FDA), AOAC International, and Eurachem [61–63]. Linearity, limits of detection (LODs) and quantification (LOQs), precision (inter- and intra-day), and accuracy (recovery) were assessed. Linearity was evaluated using a calibration curve consisting

of 13 points (0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 $\mu\text{g/L}$) and plotting the areas of the quantifier ions against the relative concentrations. For each FB and grape must under evaluation, the validation procedure entailed the use of samples fortified with known amounts of analytes. Two concentration levels, “low” and “high,” were selected as spikes, corresponding to 30% and 60% of the linearity ranges, respectively. Recovery was calculated as the mean concentration found in spiked samples ($N = 4$) minus the original sample concentration divided by the spiked concentration. Repeatability and reproducibility were measured by recurrent injections of fortified samples performed on the same (intra-day) and different days (inter-day). Precision was determined by computing the relative standard deviations (RSDs) for each analyte. LODs and LOQs were evaluated by calculating the RSD of the peak areas of diluted standard solutions injected six times. The lowest detectable concentration that produced an RSD lower than 10% was used to determine the LOD, while the lowest amount associated with an RSD below 30% was defined as the LOQ. Method applicability was tested on six FBs and samples of grape must.

2.5. Data Processing, Statistical Analysis and Modelling

Data analysis was carried out using XLSTAT 2021.5 (Addinsoft, Long Island City, NY, USA) and Microsoft Excel 2013 (Redmond, Washington, DC, USA). An example of a possible method application was presented by analyzing a set of wine samples and modeling the data with a PLS-DA regression.

The factors considered in the two-component model were the FAA, GSH, and BA measurements (quantitative explanatory variables, X_s) and the vintage year (qualitative dependent variables, Y_s).

3. Results and Discussion

3.1. Gradient Elution Study

To achieve optimal chromatographic separation (resolution) and peak shapes, eluents were adjusted regarding their composition, timing, and magnitude of the ramping and re-equilibration phase. Indeed, not only does solvent composition dramatically affect the ionization capacity of the ESI-II source [64], but also, when using a HILIC column, the timing, magnitude, and shape of the ramping of the aqueous phase influence the elution of the analytes [65]. After several attempts (Figure S1, graphical representation of the elution study), it was found that the addition of FA to the aqueous phase (solvent B) at a final concentration of 0.5% (v/v) combined with the gradient reported above led to a suitable chromatography separation of 20 FAAs, 12 BAs, and GSH (Figure 1). Moreover, the obtained resolution allowed for the separation of Leu and Ile, two AAs deeply involved in flavor development and fermentation performance [66–68].

3.2. Optimization of the MS/MS Conditions

Nitrogen was used as the drying and collision gas for the HESI-II source. The tune parameters were optimized by directly infusing standard mixtures in an eluent solution (0.5% FA/ACN 50:50 v/v). The final operating conditions were as follows: the heated capillary temperature was set at 330 $^{\circ}\text{C}$, the sheath gas flow rate was 40 arbitrary units, the auxiliary gas flow rate was 20 arbitrary units, and the auxiliary gas heater temperature was adjusted to 300 $^{\circ}\text{C}$. Spray voltage was 3 kV, and the S-Lens RF level was fixed at 50 arbitrary units.

As for the fragmentation, the normalized collision energy (NCE) was set at 30, 35, 45, and 60 to assess which would work better for the analytes under investigation. Ultimately, it was found that an NCE of 35 was ideal as the obtained spectrum allowed us to observe the presence of both the molecular ion and at least one of the fragments reported in Table 1.

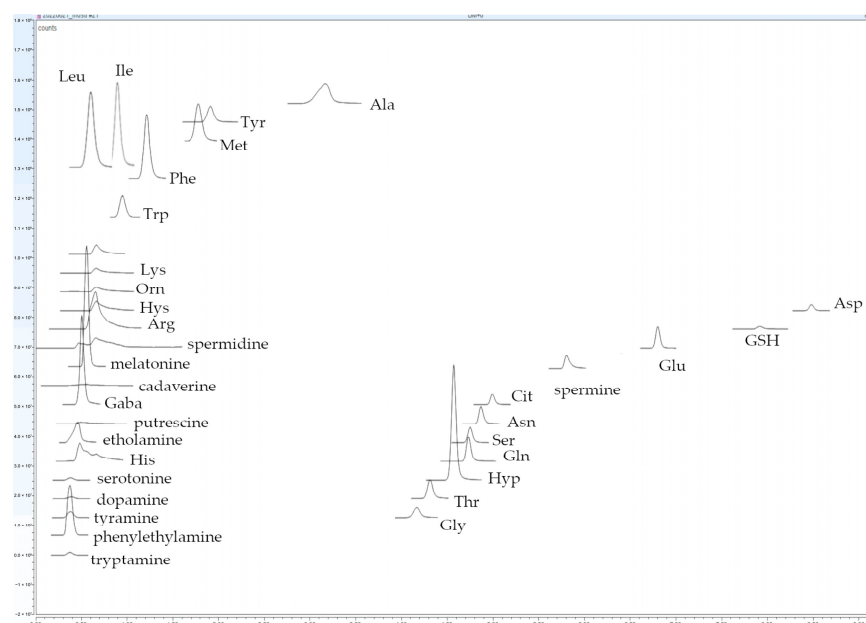


Figure 1. Chromatographic resolution of free amino acids (FAAs), biogenic amines (BAs), and glutathione (GSH) obtained with the reported method. Gaba, γ -aminobutyric acid; Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; Arg, arginine; Asn, asparagine; Cit, citrulline; Phe, phenylalanine; Gly, glycine; Hyp, hydroxyproline; Ile, isoleucine; Leu, leucine; His, histidine; Lys, lysine; Met, methionine; Orn, ornithine; Ser, serine; Tyr, tyrosine; Thr, threonine; Trp, tryptophan; GSH, glutathione.

Mass spectra were acquired in profile mode through a full MS-data-dependent analysis (full MS/dd-MS²). A resolution of 70,000 full-width at half-maximum (FWHM, calculated for m/z 200, 12 Hz) was set for recording full mass spectra. The automatic gain control (AGC) target was adjusted to 3×10^6 ions, and the maximum inject time (IT) was adjusted to 100 ms. dd-MS2 spectra were recorded at a resolution of 17,500 FWHM; AGC target was 1×10^5 ions and maximum IT was 50 ms.

Method characteristics were appraised using reference standards (Tables 1 and S1). As for the quantification, the protonated molecule $[M + H]^+$ corresponding to the precursor ion observed in the extracted ion chromatogram (EIC) was used. Targeted compounds were identified by comparing the m/z values with a mass tolerance lower than 5 ppm [69] and their retention times (RTs). Further, matching between the two dd-MS2 highest fragments obtained from the standards (Table 1) and the samples was operated to confirm the correct identification of the analytes. Thus, this method based on chromatographic separation and distinctive spectra can be employed as a reference for the quantification of targeted AAs and BAs in fermented beverages and the quality control of those products.

3.3. Matrix Effect and Sample Preparation

In LC–MS analysis, matrix interference can lead to ion enhancement or suppression, affecting quantification accuracy [70]. Therefore, sample preparation was optimized to minimize the matrix contribution to analyte ionization. Dilution was used to determine the optimal conditions for quantification, and it was found that a 50-fold dilution was optimal in terms of signal intensity, recovery, and the number of quantifiable analytes.

Table 1. Chemical formula, accurate mass $[M + H]^+$, difference between exact and accurate mass ($\Delta m/z$), retention time (RT), characteristic fragments, limit of detection (LOD), limit of quantification (LOQ), and linearity range of the free amino acids (FAAs), biogenic amines (BAs), and glutathione (GSH) quantified with the method.

	FAAs/BAs	Chemical Formula	$[M + H]^+$ (m/z)	$\Delta m/z$ (ppm)	RT (min)	Fragments (m/z)	LOD (mg/L)	LOQ (mg/L)	Linearity (mg/L)	R ²
Gaba	γ -Aminobutyric acid	C ₄ H ₉ NO ₂	104.0706	0.5	0.53	87.0444, 69.0340	0.001	0.003	0.001–2	0.99
Asp	L-Aspartic acid	C ₄ H ₇ NO ₄	134.0448	0.0	8.49	88.0397, 74.0241	0.0015	0.005	0.005–5	0.99
Glu	L-Glutamic acid	C ₅ H ₉ NO ₄	148.0602	1.6	6.82	130.0498, 84.0448	0.0015	0.005	0.005–5	0.99
Ala	L-Alanine	C ₃ H ₇ NO ₂	90.0553	−4.1	3.16	72.0444	0.003	0.01	0.01–5	0.99
Arg	L-Arginine monohydrochloride	C ₆ H ₁₄ N ₄ O ₂	175.1186	2.2	0.69	116.0706, 70.0656	0.0006	0.002	0.002–5	0.99
Asn	L-Asparagine	C ₄ H ₈ N ₂ O ₃	133.0607	0.8	4.84	99.0063, 74.0242	0.003	0.01	0.01–5	0.99
Cit	L-Citrulline	C ₆ H ₁₃ N ₃ O ₃	176.1031	−0.7	5.00	159.0765, 113.0711	0.006	0.02	0.02–5	0.99
Phe	L-Phenylalanine	C ₉ H ₁₁ NO ₂	166.0862	0.2	1.19	120.0808, 95.0494	0.0003	0.001	0.001–1	0.99
Gly	Glycine	C ₂ H ₅ NO ₂	76.0394	−1.6	4.18	-	0.006	0.02	0.02–5	0.99
Hyp	trans-4-Hydroxy-L-proline	C ₅ H ₉ NO ₃	132.0654	1.1	4.57	100.0139, 86.0604	0.0015	0.005	0.005–5	0.99
Ile	L-Isoleucine	C ₆ H ₁₃ NO ₂	132.1018	1.1	0.87	86.0964, 69.0699	0.0003	0.001	0.001–2	0.99
Leu	L-Leucine	C ₆ H ₁₃ NO ₂	132.1018	1.2	0.59	86.0964	0.0001	0.001	0.001–5	0.99
His	L-Histidine monohydrochloride monohydrate	C ₆ H ₉ N ₃ O ₂	156.0766	0.1	0.61	110.0713, 95.0606	0.0015	0.05	0.05–5	0.99
Lys	L-Lysine monohydrochloride	C ₆ H ₁₄ N ₂ O ₂	147.1125	2.1	0.65	130.0862, 84.0811	0.015	0.05	0.05–2	0.99
Met	L-Methionine	C ₅ H ₁₁ NO ₂ S	150.0582	0.6	1.79	133.0316, 104.0530	0.0015	0.005	0.005–2	0.99
Orn	L-Ornithine dihydrochloride	C ₅ H ₁₂ N ₂ O ₂	133.0971	0.5	0.67	116.0706, 70.0656	0.015	0.05	0.05–5	0.99
Ser	L-Serine	C ₃ H ₇ NO ₃	106.0500	−1.3	4.64	88.0396, 60.0450	0.003	0.01	0.01–5	0.99
Tyr	L-Tyrosine	C ₉ H ₁₁ NO ₃	182.0809	1.4	1.87	136.0756, 123.0441	0.0015	0.005	0.005–1	0.99
Thr	L-Threonine	C ₄ H ₉ NO ₃	120.0654	0.9	4.29	102.0551, 74.0605	0.006	0.02	0.02–5	0.99
Trp	L-Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	205.0971	0.2	0.95	188.0702, 146.0599	0.0015	0.005	0.005–5	0.99
ethanolamine	Ethanolamine	C ₂ H ₇ NO	62.0603	−4.2	0.45	-	0.0015	0.005	0.005–1	0.99
phenylethylamine	2-Phenylethylamine hydrochloride	C ₈ H ₁₁ N	122.0958	4.8	0.39	105.0701, 79.0546	0.0003	0.001	0.001–0.1	0.95
putrescine	1,4-Diaminobutane dihydrochloride	C ₄ H ₁₂ N ₂	89.1073	0.7	0.53	72.0813	0.03	0.1	0.1–2	0.99
cadaverine	1,5-Diaminopentane dihydrochloride	C ₅ H ₁₄ N ₂	103.1233	−2.9	0.56	86.0968, 69.0704	0.015	0.05	0.05–1	0.99
histamine	Histamine dihydrochloride	C ₅ H ₉ N ₃	112.0869	0.3	0.45	95.0606, 83.0608	0.015	0.05	0.05–2	0.99
spermine	Spermine tetrahydrochloride	C ₁₀ H ₂₆ N ₄	203.2159	−3.4	5.81	112.1122, 84.0811	0.006	0.02	0.02–5	0.98
spermidine	Spermidine trihydrochloride	C ₇ H ₁₉ N ₃	146.1651	0.5	0.71	129.1386, 72.0813	0.003	0.01	0.01–5	0.96
tyramine	Tyramine hydrochloride	C ₈ H ₁₁ NO	138.0908	4.1	0.44	121.0648, 91.0547	0.0003	0.001	0.001–2	0.99
tryptamine	Tryptamine hydrochloride	C ₁₀ H ₁₂ N ₂	161.1066	4.7	0.37	144.0805, 117.0697	0.0015	0.005	0.005–1	0.99
melatonin	Melatonin	C ₁₃ H ₁₆ N ₂ O ₂	233.1283	−1.1	0.63	174.0912, 159.0677	0.0015	0.005	0.005–2	0.99
dopamine	Dopamine hydrochloride	C ₈ H ₁₁ NO ₂	154.0864	−3.0	0.39	137.0596, 91.0546	0.003	0.01	0.01–1	0.99
serotonin	Serotonin hydrochloride	C ₁₀ H ₁₂ N ₂ O	177.1018	0.2	0.39	160.0755, 132.0807	0.006	0.02	0.02–2	0.99
GSH	L-Glutathione reduced	C ₁₀ H ₁₇ N ₃ O ₆ S	308.0904	2.1	7.92	162.0217, 76.0220	0.0006	0.002	0.002–5	0.99

Gaba, γ -aminobutyric acid; Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; Arg, arginine; Asn, asparagine; Cit, citrulline; Phe, phenylalanine; Gly, glycine; Hyp, hydroxyproline; Ile, isoleucine; Leu, leucine; His, histidine; Lys, lysine; Met, methionine; Orn, ornithine; Ser, serine; Tyr, tyrosine; Thr, threonine; Trp, tryptophan; GSH, glutathione.

While developing the method, both solvent and matrix-matched calibration curves were prepared and analyzed. Considering that the slopes of the curves differed, the matrix-matched curves were used to quantify the analytes in the samples. Further, recovery studies were performed to assess the use of those curves on other samples belonging to the same matrix class. Recovery data for each analyte and matrix were obtained and are reported in Table 2. For most compounds, the recovery values were within the 60–120% range, indicating good matrix robustness and method applicability for the samples under examination. Some recovery values fell outside the validation limits for specific compounds, such as spermidine in red wine, tyrosine (Tyr) in beer, and alanine (Ala) in vinegar. Additionally, γ -aminobutyric acid (Gaba), Ala, and arginine (Arg) recovery values were unsatisfactory for the grape must samples, possibly due to the interference of sugars in the matrix. Nevertheless, as the recovery values for all the different types of FB tested were adequate, it can be concluded that this method allows for the accurate quantification of FAAs and BAs in samples of wine, vinegar, beer, saké, grape must, and cider.

Table 2. Recovery rates (%) of samples of wine, must, cider, beer, vinegar, and saké spiked with two known concentrations of a standard solution.

	% Recovery (n = 4)													
	White Wine		Red Wine		Must		Cider		Beer		Vinegar		Saké	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Gaba	118 ± 3	107 ± 4	69 ± 2	66 ± 3	-	117 ± 5	121 ± 5	114 ± 1	76 ± 6	112 ± 5	73 ± 3	102 ± 5	96 ± 4	107 ± 8
Asp	94 ± 5	99 ± 7	105 ± 2	100 ± 2	75 ± 7	93 ± 4	108 ± 3	107 ± 2	99 ± 2	96 ± 4	69 ± 2	100 ± 7	103 ± 4	105 ± 6
Glu	80 ± 3	79 ± 3	119 ± 2	100 ± 2	94 ± 5	65 ± 6	101 ± 6	104 ± 7	97 ± 4	104 ± 5	66 ± 7	103 ± 8	106 ± 6	111 ± 7
Ala	104 ± 6	100 ± 3	103 ± 4	101 ± 3	117 ± 7	-	83 ± 2	112 ± 2	84 ± 3	63 ± 7	-	95 ± 5	64 ± 7	107 ± 3
Arg	110 ± 3	92 ± 5	117 ± 7	102 ± 1	-	-	98 ± 1	105 ± 6	81 ± 3	110 ± 3	60 ± 2	107 ± 5	97 ± 6	116 ± 8
Asn	90 ± 3	103 ± 3	103 ± 4	98 ± 4	95 ± 7	99 ± 2	98 ± 6	105 ± 5	90 ± 8	99 ± 7	83 ± 8	98 ± 3	99 ± 4	110 ± 6
Cit	78 ± 2	94 ± 6	93 ± 4	98 ± 6	83 ± 3	97 ± 2	87 ± 1	96 ± 5	103 ± 1	94 ± 5	79 ± 8	96 ± 8	75 ± 7	99 ± 6
Phe	82 ± 1	94 ± 7	116 ± 3	113 ± 6	115 ± 6	109 ± 4	114 ± 4	111 ± 4	73 ± 5	64 ± 2	67 ± 8	98 ± 3	79 ± 3	112 ± 6
Gly	89 ± 6	95 ± 4	106 ± 5	108 ± 1	103 ± 4	101 ± 3	90 ± 7	107 ± 7	96 ± 3	92 ± 1	73 ± 7	97 ± 6	122 ± 7	100 ± 4
Hyp	91 ± 3	104 ± 3	109 ± 3	106 ± 2	97 ± 1	105 ± 1	103 ± 4	107 ± 3	104 ± 1	99 ± 1	118 ± 1	110 ± 2	93 ± 1	104 ± 1
Ile	63 ± 3	68 ± 7	102 ± 4	112 ± 4	68 ± 7	116 ± 4	129 ± 1	102 ± 8	88 ± 8	73 ± 6	111 ± 3	66 ± 4	87 ± 8	117 ± 4
Leu	123 ± 2	87 ± 8	122 ± 2	97 ± 6	101 ± 8	122 ± 2	120 ± 5	120 ± 8	105 ± 3	83 ± 6	133 ± 5	100 ± 6	83 ± 4	117 ± 1
His	113 ± 5	108 ± 2	102 ± 2	120 ± 1	133 ± 5	102 ± 7	97 ± 4	104 ± 5	96 ± 6	79 ± 4	96 ± 1	111 ± 6	104 ± 8	111 ± 8
Lys	87 ± 1	93 ± 3	100 ± 1	109 ± 1	113 ± 2	109 ± 6	98 ± 8	103 ± 2	78 ± 2	98 ± 2	73 ± 6	110 ± 2	98 ± 1	111 ± 4
Met	111 ± 1	100 ± 3	100 ± 6	100 ± 8	107 ± 7	119 ± 5	99 ± 2	100 ± 6	114 ± 7	86 ± 8	127 ± 5	130 ± 4	100 ± 6	120 ± 1
Orn	91 ± 5	113 ± 8	111 ± 3	119 ± 6	114 ± 8	114 ± 3	93 ± 8	96 ± 1	117 ± 5	87 ± 4	92 ± 5	103 ± 6	89 ± 5	106 ± 5
Ser	81 ± 8	95 ± 2	99 ± 4	103 ± 5	95 ± 5	101 ± 7	100 ± 1	103 ± 2	82 ± 7	92 ± 2	78 ± 3	104 ± 2	89 ± 6	106 ± 5
Tyr	60 ± 6	83 ± 8	116 ± 7	112 ± 3	108 ± 1	117 ± 8	103 ± 5	108 ± 8	-	94 ± 8	92 ± 7	120 ± 1	60 ± 3	120 ± 2
Thr	89 ± 4	89 ± 6	118 ± 6	103 ± 1	85 ± 6	79 ± 1	84 ± 6	101 ± 3	82 ± 3	93 ± 7	72 ± 7	100 ± 2	99 ± 5	108 ± 6
Trp	104 ± 7	111 ± 5	101 ± 3	113 ± 1	103 ± 8	126 ± 5	106 ± 8	104 ± 7	114 ± 3	100 ± 1	98 ± 1	110 ± 4	99 ± 1	113 ± 8
ethanolamine	105 ± 1	103 ± 8	112 ± 5	110 ± 5	103 ± 1	96 ± 4	86 ± 8	126 ± 2	80 ± 3	102 ± 4	106 ± 1	110 ± 1	120 ± 7	119 ± 1
phenylethylamine	113 ± 2	107 ± 5	109 ± 5	101 ± 1	105 ± 2	102 ± 1	114 ± 8	93 ± 1	110 ± 4	110 ± 1	124 ± 6	116 ± 6	124 ± 4	124 ± 8
putrescine	97 ± 2	104 ± 3	137 ± 4	121 ± 8	108 ± 5	114 ± 7	92 ± 5	122 ± 4	69 ± 2	98 ± 7	89 ± 3	116 ± 2	103 ± 5	110 ± 8
cadaverine	86 ± 2	98 ± 4	67 ± 7	103 ± 6	89 ± 3	101 ± 5	113 ± 5	110 ± 5	110 ± 4	95 ± 3	65 ± 2	97 ± 4	94 ± 7	111 ± 7
histamine	93 ± 8	103 ± 7	113 ± 6	120 ± 8	96 ± 8	110 ± 7	111 ± 1	97 ± 3	91 ± 5	97 ± 7	104 ± 2	109 ± 2	104 ± 7	115 ± 2
spermine	60 ± 6	95 ± 2	113 ± 4	91 ± 4	124 ± 5	98 ± 2	61 ± 3	69 ± 2	85 ± 2	121 ± 6	60 ± 2	83 ± 4	55 ± 5	79 ± 8
spermidine	142 ± 4	125 ± 2	-	61 ± 6	114 ± 8	116 ± 8	122 ± 2	117 ± 2	110 ± 2	132 ± 4	136 ± 2	120 ± 2	153 ± 3	132 ± 7
tyramine	92 ± 4	98 ± 2	122 ± 6	117 ± 4	115 ± 5	117 ± 3	131 ± 4	120 ± 2	88 ± 8	99 ± 5	79 ± 1	103 ± 3	101 ± 3	115 ± 8
tryptamine	80 ± 7	92 ± 8	103 ± 5	111 ± 3	93 ± 2	108 ± 1	129 ± 2	117 ± 7	89 ± 1	97 ± 1	85 ± 2	99 ± 1	86 ± 6	113 ± 2
melatonin	105 ± 4	102 ± 5	104 ± 2	131 ± 7	118 ± 8	118 ± 1	127 ± 3	96 ± 4	90 ± 4	93 ± 4	127 ± 7	130 ± 8	126 ± 7	116 ± 7
dopamine	81 ± 1	89 ± 7	117 ± 7	119 ± 2	100 ± 1	103 ± 8	122 ± 3	117 ± 7	87 ± 2	97 ± 6	110 ± 2	108 ± 6	89 ± 1	102 ± 5
serotonin	80 ± 5	92 ± 6	90 ± 2	109 ± 3	99 ± 1	104 ± 7	113 ± 2	108 ± 5	75 ± 2	102 ± 4	95 ± 8	94 ± 2	93 ± 8	107 ± 5
GSH	75 ± 7	79 ± 6	81 ± 1	87 ± 7	81 ± 8	96 ± 1	80 ± 6	90 ± 4	77 ± 1	72 ± 1	66 ± 4	82 ± 1	93 ± 3	102 ± 5

n = number of replicates. Gaba, γ -aminobutyric acid; Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; Arg, arginine; Asn, asparagine; Cit, citrulline; Phe, phenylalanine; Gly, glycine; Hyp, hydroxyproline; Ile, isoleucine; Leu, leucine; His, histidine; Lys, lysine; Met, methionine; Orn, ornithine; Ser, serine; Tyr, tyrosine; Thr, threonine; Trp, tryptophan; GSH, glutathione. Low and high represent spikes of 30% and 60% of the linearity range, respectively. Data presented as mean $\pm$ standard deviation (SD).

3.4. Precision

Precision was assessed by measuring four replicates of FBs and must samples previously fortified with two concentration levels corresponding to 30 and 60% of the linearity range.

Intra- and inter-day precisions were evaluated under repeatable and reproducible conditions. Fortified samples were prepared on the same day by the same analyst and on different days by different analysts in a random order over five months. Samples were injected four times, and the RSD of the obtained concentrations and RTs were calculated for all the analytes and matrices under assessment. Tables S2 and S3 report the intra- and inter-day precision RSD, respectively. The method proved to be robust both in terms of repeatability and reproducibility. Except for the lower spike in red wine and cider, where acceptable RSDs were not obtained for spermine and cadaverine, the overall values were below the 10% threshold. Hence, the method represents a valid procedure to assess the FAA and BA content of fermented beverages with great efficiency and precision.

3.5. Application of the Validated Method to FBs and Must Samples

The validated method allowed for quantifying 20 FAAs, 12 BAs, and GSH in six FBs and grape must samples. Table 3 reports the concentration values (mg/L) obtained for the samples under evaluation. Compared to what has been reported by other researchers [50,71–73], analogous results were obtained in our study. From a compositional perspective, cider was observed as the FB containing the lowest amounts of FAAs. Regarding BAs, significant amounts of ethanolamine were detected in all samples, while tyramine was present in all samples except for red wine and must. Finally, as reported in Table 3, GSH was found in significant amounts in wine and grape must samples.

Table 3. Average (n = 3) free amino acids (FAAs), biogenic amines (BAs), and glutathione (GSH) [mg/L] quantified in popular fermented beverages.

	White Wine	Red Wine	Must	Cider	Beer	Vinegar	Saké	LOD	LOQ
Gaba	78.6 ± 3.8	89.2 ± 8.4	54.3 ± 9.7	<LOD	302 ± 13	138 ± 16	99.6 ± 22	0.05	0.15
Asp	15.3 ± 1.9	18.5 ± 3.6	16.5 ± 6.5	1.46 ± 0.93	9.78 ± 2.1	17.6 ± 3.4	7.69 ± 1.5	0.075	0.25
Glu	27.1 ± 4.6	34.3 ± 1.9	47.5 ± 4.5	2.53 ± 0.23	14.2 ± 3.7	15.4 ± 4.9	13.8 ± 2.8	0.075	0.25
Ala	95.4 ± 7.8	86.7 ± 4.8	29.6 ± 3.8	1.46 ± 0.34	93.6 ± 5.2	53.6 ± 12	55.1 ± 3.6	0.15	0.5
Arg	269 ± 25	326 ± 33	35.1 ± 8.2	<LOQ	57.6 ± 4.6	56.3 ± 3.7	125 ± 15	0.03	0.1
Asn	24.1 ± 3.4	15.6 ± 4.8	11.3 ± 2.6	0.862 ± 0.07	5.94 ± 1.9	4.03 ± 1.8	21.4 ± 3.2	0.15	0.5
Cit	1.29 ± 0.3	1.97 ± 0.53	4.28 ± 1.3	<LOD	7.12 ± 2.7	<LOD	<LOD	0.3	1
Phe	15.3 ± 2.1	17.3 ± 3.5	33.2 ± 3.8	2.36 ± 0.79	51.8 ± 3.2	21.8 ± 3.2	19.8 ± 3.7	0.015	0.05
Gly	33.9 ± 5.8	94.1 ± 6.3	23.8 ± 1.7	<LOD	66.8 ± 2.7	39.4 ± 7.2	77.5 ± 6.8	0.3	1
Hyp	6.18 ± 4.3	25.8 ± 1.5	16.5 ± 3.4	1.58 ± 0.42	1.67 ± 0.2	5.01 ± 1.6	<LOD	0.075	0.25
Ile	14.8 ± 1.6	9.31 ± 2.7	25.8 ± 6.2	0.561 ± 0.21	15.2 ± 3.7	32.6 ± 4.2	12.7 ± 1.7	0.015	0.05
Leu	18.6 ± 2.4	10.1 ± 2.8	23.4 ± 3.9	<LOQ	7.96 ± 1.8	23.4 ± 2.8	13.4 ± 4.1	0.005	0.05
His	15.7 ± 5.8	18.6 ± 1.8	115 ± 14	<LOQ	53.6 ± 5.7	15.3 ± 1.9	22.7 ± 3.9	0.075	2.5
Lys	56.1 ± 7.8	34.9 ± 3.4	<LOD	0.962 ± 0.15	6.73 ± 1.47	49.2 ± 5.1	26.9 ± 2.6	0.75	2.5
Met	4.74 ± 1.7	3.44 ± 1.7	7.62 ± 2.9	<LOD	16.9 ± 3.1	7.21 ± 1.5	<LOQ	0.075	0.25
Orn	11.9 ± 3.5	23.6 ± 2.6	6.86 ± 1.5	<LOD	<LOD	<LOQ	<LOD	0.75	2.5
Ser	19.8 ± 4.1	18.2 ± 2.5	148 ± 39	1.25 ± 0.68	7.52 ± 1.5	15.9 ± 3.2	16.8 ± 3.7	0.15	0.5
Tyr	8.56 ± 3.5	15.4 ± 1.7	22.4 ± 3.5	0.843 ± 0.32	42.3 ± 6.9	5.63 ± 2.7	11.8 ± 2.6	0.075	0.25
Thr	15.2 ± 1.9	17.9 ± 5.8	128 ± 32	2.5 ± 0.97	6.02 ± 1.08	14.7 ± 4.2	7.54 ± 2.6	0.3	1
Trp	6.72 ± 2.8	6.96 ± 5.2	33.9 ± 5.8	<LOD	7.39 ± 0.47	<LOD	0.984 ± 0.06	0.075	0.25
ethanolamine	40.8 ± 4.9	47.3 ± 4.7	35.6 ± 6.2	8.56 ± 1.6	26.9 ± 2.6	13.7 ± 1.7	33.1 ± 1.4	0.075	0.25
phenylethylamine	0.468 ± 0.15	<LOQ	<LOD	<LOD	<LOQ	1.47 ± 0.4	0.864 ± 0.29	0.015	0.05
putrescine	<LOQ	16.7 ± 5.2	11.6 ± 3.8	<LOD	<LOD	11.7 ± 2.6	<LOD	1.5	5
cadaverine	<LOD	<LOD	<LOD	<LOD	<LOD	12.5 ± 1.5	<LOD	0.75	2.5
histamine	<LOD	9.51 ± 4.7	<LOQ	<LOD	<LOD	<LOQ	<LOD	0.75	2.5
spermine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.3	1
spermidine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.15	0.5
tyramine	<LOQ	13.9 ± 4.1	<LOD	0.487 ± 0.12	1.45 ± 0.79	19.7 ± 1.8	1.71 ± 0.69	0.015	0.05
tryptamine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.075	0.25
melatonin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.075	0.25
dopamine	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD	0.15	0.5
serotonin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.3	1
GSH	2.85 ± 1.6	6.16 ± 3.5	3.92 ± 1.4	<LOD	<LOD	<LOD	<LOD	0.03	0.1

n = number of replicates. Gaba, γ -aminobutyric acid; Asp, aspartic acid; Glu, Glutamic acid; Ala, alanine; Arg, arginine; Asn, asparagine; Cit, citrulline; Phe, phenylalanine; Gly, glycine; Hyp, hydroxyproline; Ile, isoleucine; Leu, leucine; His, histidine; Lys, lysine; Met, methionine; Orn, ornitine; Ser, serine; Tyr, tyrosine; Thr, threonine; Trp, tryptophan; GSH, glutathione. LOD, limit of detection; LOQ, limit of quantification. Data presented as mean ± standard deviation (SD).

3.6. Modelling Based on Data Obtained from the Validated Method

The acquisition of high-dimensional datasets combined with statistical analyses makes it a valuable tool not only for researchers but also in the areas of food safety, quality, and authentication. Indeed, dealing with high data dimensionality increases the informational content of the dataset and opens up new opportunities for exploiting the data with advanced modelling techniques.

As an example of the method's application, a screening of 56 wines from three different vintages (2018, $n = 20$; 2019, $n = 17$; 2020, $n = 19$) was carried out. The obtained FAA, GSH, and BA profiles were used to perform a partial least squares discriminant analysis (PLS-DA), a covariance-based regression model commonly used for classification or prediction purposes [74].

Figure 2 shows the map of the PLS-DA model presenting the global relationship between variables: Ys are displayed on the components' vectors and Xs on the w^* vectors. The distance from the center represents the relative weight of the variable, and the model successfully discriminated the wine samples based on the vintage year. Compared to the other years, 2020 was characterized by wines displaying higher contents of γ -aminobutyric acid (Gaba), aspartic acid (Asp), phenylalanine (Phe), Leu, Ile, methionine (Met), spermine, spermidine, melatonin, and GSH. Phenethylamine and histidine (His) amounts were the highest in the wines produced in 2019. Finally, regarding 2018, the average values obtained for glutamic acid (Glu), alanine (Ala), arginine (Arg), asparagine (Asn), citrulline (Cit), lysine (Lys), ornithine (Orn), serine (Ser), tyrosine (Tyr), threonine (Thr), tryptophan (Trp), putrescine, and serotonin were greater than the ones detected in the other vintages. A VIP score quantifies the importance of a variable in the PLS-DA model, encapsulating the variable's contribution to the model. As for the PLS-DA model reported in Figure 2, the most important variables (characterized by a $VIP > 1$) are gly, thr, tyr, GSH, spermine, melatonin, and histamine.

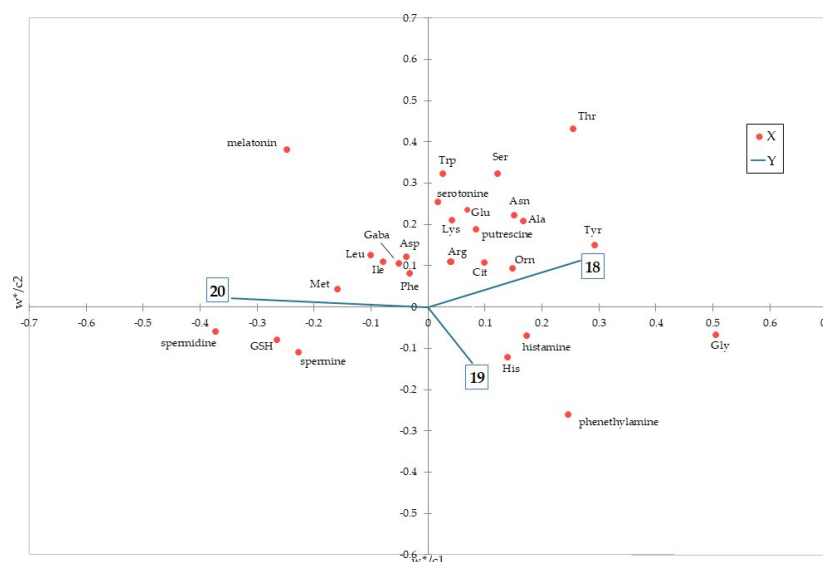


Figure 2. PLS-DA model of 56 wines from 3 vintages (2018, 2019, 2020) and relative FAA (free amino acid), BA (biogenic amine), and GSH (glutathione) content. Gaba, γ -aminobutyric acid; Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; Arg, arginine; Asn, asparagine; Cit, citrulline; Phe, phenylalanine; Gly, glycine; hydroxyproline; Ile, isoleucine; Leu, leucine; His, histidine; Lys, lysine; Met, methionine; Orn, ornithine; Ser, serine; Tyr, tyrosine; Thr, threonine; Trp, tryptophan; GSH, glutathione.

In this study, the PLS-DA model was used to investigate the differences in the FAA, BA, and GSH content of wines from three different harvest seasons. This analysis not only

provides accurate classification but also offers insight into the chemical variables that most influence the differentiation between different years of wine production. This information can be useful for winemakers and researchers interested in understanding the chemical composition of wines and how it may vary over different harvests.

4. Conclusions

The present study outlines a fast, simple, and sensitive LC–MS/MS method for the direct underivatized quantification of FAAs, BAs, and GSH in several FBs and grape musts. The excellent peak resolution allows for separating some of the most troublesome isomers, such as Ile and Leu. Furthermore, the use of an HRMS system prevents the occurrence of false positive results by detecting ions with precise mass measurements.

The method was successfully validated in terms of accuracy and reproducibility and complies with the requirements reported in the method validation guidelines of the FDA, AOAC International, and Eurachem. It provides an effective and reliable tool for evaluating the quality and chemical composition of some common FBs and must samples. Additionally, by looking at the recovery values obtained for the beverages under assessment (Table 2), applicability might be extended to other FBs. The application of the PLS-DA model to the FAA, BA, and GSH profiles of wines from three different vintages exemplifies how this method can help to identify specific analytes contributing to the differentiation of samples. Overall, this method has the potential to open up new avenues for scientific research in beverage analysis, paving the way for further investigations concerning the profiling of FAAs and BAs, food authenticity control, and safety assessment.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/beverages11050124/s1>, Table S1: Manufacturing details of the standard used; Table S2: Average relative standard deviations (% RSD) of the peak areas (A) and retention times (T) of spiked samples injected four times consecutively; Table S3: Average relative standard deviations (% RSD) of the peak areas (A) and retention times (T) of spiked samples randomly injected by different analysts four times on different days; Figure S1: Gradient elution study.

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Abbreviations

The following abbreviations are used in this manuscript:

FAAs	Free amino acids
BAs	Biogenic amines
FB	Fermented beverages
UHPLC	Ultra-high-performance liquid chromatography
HRMS	High-resolution mass spectrometry
N	Nitrogen
AA	Amino acid

HPLC	High-performance liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
GC	Gas chromatography
Fmoc	Fluorenylmethyloxycarbonyl chloride
DEMM	Diethyl ethoxymethylene malonate
LC–MS/MS	Liquid chromatography coupled to mass spectrometry
Leu	Leucine
Ile	Isoleucine
GSH	Glutathione
Cys	Cysteine
Gly	Glycine
MeOH	Methanol
HCl	Hydrochloric acid
PTFE	Polytetrafluoroethylene
CAN	Acetonitrile
FA	Formic acid
FDA	Food and Drug Administration
LOD	Limit of detection
LOQ	Limit of quantification
RSD	Relative standard deviation
HILIC	Hydrophilic interaction liquid chromatography
Gaba	γ -aminobutyric acid
Asp	Aspartic acid
Glu	Glutamic acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Cit	Citrulline
Phe	Phenylalanine
Gly	Glycine
Hyp	Hydroxyproline
His	Histidine
Lys	Lysine
Met	Methionine
Orn	Ornithine
Ser	Serine
Tyr	Tyrosine
Thr	Threonine
Trp	Tryptophan
NCE	Normalized collision energy
FWHM	Full-width at half-maximum
AGC	Automatic gain control
EIC	Extracted ion chromatogram
RT	Retention time
SD	Standard deviation
PLS-DA	Partial least squares discriminant analysis

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