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Review

Determination of amino acids in grape-derived products: A review

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ABSTRACT

The amino acids present in foods and beverages affect the quality of these products and they play an important role in enology. Amino acids are consumed by yeasts as a source of nitrogen during alcoholic fermentation and are precursors of aroma compounds. In this review various chromatographic methodologies for the determination of amino acids are described, and specific applications for the analysis of amino acid content are discussed. Amino acids usually need to be derivatized to make them more detectable. Several derivatizing reagents have been employed for the determination of amino acids in enological applications, and each has its advantages and disadvantages.

Contents

1. Applications of amino acid analysis in enology

Amino acids need to be analyzed in many fields of research, and one of the most important of these is concerned with assessing the nutritional value of food and drink products. The monitoring of fermentation and correlated flavour trends in the development of foods and drinks, and the assessing of levels of amino acid fortification also require these compounds to be analyzed [1]. The increasing demand for information relating to nutritive value means there is a need for increasingly accurate determination of amino acid content. Among the ever-increasing applications in which analysis of amino acid content is required are the detec-

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tion of adulteration of foods and beverages, and the detection of potentially toxic amino acids or peptides produced by new food-processing techniques [2].

In 1958, Stein and Moore applied liquid chromatography to determine amino acid content using an ion-exchange column and post-column reaction with ninhydrin. Since then the analysis of amino acids has become increasingly important, not only for food products but also in clinical and pharmaceutical contexts.

It is well known that the amino acids found in grape musts are important as nutrients for the growth of yeasts, since they are consumed as a nitrogen source during the alcoholic fermentation [3], and play a role as precursors of aroma compounds. Several amino acids undergo a series of biotransformations, yielding higher alcohols, aldehydes, esters and ketonic acids. Hence amino acids have a considerable impact on the organoleptic properties of wine [4]. Moreover, the amino acid composition of the grape must is closely related to the maturity of the grapes and the fertility of the soil in which they are cultivated, so many enology laboratories are interested in the analysis of these compounds [5]. Free amino acids represent the most important part of the total nitrogen content in grape must and wine: approximately 30–40% of the total [6,7]. These amino acids have a variety of origins. Apart from those that are present in the grape and that can be partially or totally metabolized by yeast during the growth phase, some are excreted by living yeasts at the end of fermentation [8], some are released by proteolysis during autolysis of dead yeasts, and other are produced by enzymatic degradation of the grape proteins. Furthermore, the amino acid content of grapes is dependent on soil fertility, climatic conditions and the length of time that the grape skin is macerated in the must [9].

Despite this wide range of factors influencing the amino acids present in wine, some researchers have successfully employed the finished wine's composition in amino acid for purposes of differentiating products [10]. Several different chemometric procedures have already been applied for this purpose, to establish the authenticity of wines and criteria for their differentiation: cluster analysis (CA), principal component analysis (PCA), and discriminant analysis (DA). Héberger et al. [7] showed that, on the basis of the results of chemometric analysis, free amino acid and biogenic amine contents seem to be useful for differentiating wines according to the wine-making technology employed and, to a less extent, according to geographic origin, grape variety, and year of vintage, too.

Soufleros et al. [4] studied the primary amino acid profiles of Greek white wines from seven grape varieties, six geographic regions and three different vintages, and found that they could be classified according to these variables by discriminant analysis.

Brescia et al. [11] differentiated wines from small production areas located within the same region by applying multivariate statistics to analytical and nuclear magnetic resonance (NMR) determinations. Hence, a useful method was developed for the protection of the denomination of controlled origin (DOC) against adulteration.

Balsamic vinegars have also been characterized by amino acid content using a multivariate statistical approach [12].

Since yeasts play an important role in the amino acid content of wine, another relevant application is to study the influence of different yeast strains on the changes of amino acids, peptides and proteins. One such study considered the production of sparkling wine by the traditional method [13]; in this work, the effect of five different yeast strains on the same wine was studied. From the results, it can be deduced that the changes taking place in the industrial process of sparkling wine production by the traditional method occur in at least four clearly differentiated stages. In addition, yeast strain influences the content of free amino acids and peptides.

Pérez-Coello et al. [14] analyzed the volatile composition and amino acid profile of 15 wines fermented with different strains of *Saccharomyces cerevisiae* isolated from the La Mancha region, in order to determine which strains would be more suitable for use as inocula in musts from this region.

In other research, authors have studied the influence of adding ammonium and amino acids during the fermentation of synthetic grape must, on fermentation kinetics and nitrogen consumption [15]. Ammonium and amino acids have also been added to musts to study their effect on the aromatic composition and sensory properties of the finished wines [16,17]. Although the yeast strain is known to be the determining factor in the volatile composition of wine, the addition of nitrogen to the must also has an influence: it decreases the content of β-phenylethanol, methionol and isoamyl alcohol, and increases that of propanoic acid. Moreover, samples from musts supplemented with ammonium had higher levels of ethyl lactate and *cis*-3-hexenol, while samples from must supplemented with amino acids had higher levels of γ-butyrolactone, isobutanol and isobutyric acid. In respect of sensorial properties, wines made from musts supplemented with ammonium became more citric and less "sulfury", independently of the yeast strain [16]. Other authors observed a decrease of H_2S development during fermentation after the addition of ammonium to the must. This fact illustrates the role of assimilable nitrogen (amino acids and ammonium) as the main controlling factor in the development of H_2S [18].

Regarding vinegars, the analysis of amino acid content has been applied to the characterization of wine vinegar [19], to study the chemical and biochemical transformations that take place in Sherry vinegar during ageing [20] and to explore the different requirements of acetic acid bacteria strains for these compounds, and differences in the pattern of amino acid consumption between surface and submerged acetification [21].

The fermentation process that leads to the formation of vinegar essentially involves the conversion of ethanol into acetic acid. The microbes responsible for this transformation are acetic acid bacteria from the genera *Acetobacter* and *Gluconobacter*. Free amino acids present in the medium are known to be the main sources of nitrogen for these bacteria. Because the initial substrate comes from a previous fermentation performed by yeasts, it is essential to ensure that enough nitrogen is available for the acetic acid fermentation to take place [22].

Several methods have been developed for the simultaneous determination of amino acids and other substances of interest, such as biogenic amines and polyamines, in grapes and wines [23–32] and in vinegars [19]. Biogenic amines are compounds that affect human health and may also be indicators of unhygienic production conditions. They can be directly formed from certain amino acids by decarboxylation [23]. However, these substances are not routinely quantified in the quality control of wines, mainly due to difficulties in their analysis. For this reason, the development of simple methods for the simultaneous determination of biogenic amines and amino acids in wines or vinegars is of primary importance, from the practical point of view. Polyamines have many functions in living organisms, acting as growth factors, antioxidants, stabilizers of DNA and RNA, metabolic regulators, nutrients and secondary messengers [25]. There are several reports in the literature describing the presence of polyamines in grapes and wines [25,26] and their role in grape berries has been studied.

Another topic which is important for amino acid analysis is racemization. L-Amino acids in food proteins are partially isomerized to their D-isomers by alkaline or heat treatment. Such treatment can affect the nutritive value and safety of foods, since most D-amino acids cannot be utilized by humans and some are

toxic. Isomers have identical chemical properties and therefore must be converted to diastereometric dipeptides by reaction with chiral reagents before chromatography [2].

Several methods have been proposed for the determination of the enantiomers of amino acids and chiral amines in wines, vinegars and food, and each method has its advantages and disadvantages [33–40].

2. Methods of analysis of amino acid

In recent years, the evolution of instrumental analysis has allowed the detection and quantification of more and more free amino acids with increasing sensitivity and accuracy. The determination of amino acids in musts, wines and vinegars must provide: first, the simultaneous detection of primary and secondary amino acids, such as proline, which is the predominant amino acid found in wines; second, the accurate analysis of arginine, due to the toxicological implications of this compound in subsequent reactions which lead to the formation of ethyl carbamate and urea; third, low detection limits for all the other amino acids; fourth, a short analysis time, for performing routine analyses for wine certification; and fifth, the ability to create a database for chemical characteristics of the product, to help in detecting adulteration [24].

Several different techniques are used in the analysis of amino acids in wines, and the three that are used most frequently are: ionic chromatography with post-column derivatization using ninhydrin as derivatizing agent and ultraviolet (UV) detection; separation of volatile amino acid derivatives by gas chromatography (GC) and detection by flame ionization detection (FID) and/or mass spectrometry (MS); and separation of amino acid derivatives by liquid chromatography (LC) and their detection by fluorescence.

2.1. Ion-exchange separation

Traditionally the analysis of amino acids was carried out using ion-exchange chromatography, where the amino acids were separated and then reacted with ninhydrin in a post-column derivatization system. Finally, they were detected by absorbance in the UV–vis region at one or two wavelengths.

The stationary phases of ion-exchange columns, also known as ion-exchange resins, are sulfonated polymers with a particle diameter of 5–10 μm. The separation mechanism is based on ionic interaction with a strongly acidic medium, which is the basis of the exchanger. First the acidic amino acids are eluted, followed by the hydroxylic, then neutral and finally the basic ones [5]. Nowadays, the columns are very efficient, so the analysis time is shorter, but they are quite expensive.

Although this method has shown good reliability and an excellent resolving power, the analysis times are too long, the sensitivity is limited, the peaks that appear may be too wide, and post-derivatization systems are difficult to manage and maintain. This method gives good results but requires lengthy sample preparation, and its use in wines does not allow the quantification of cysteine [6]. It has also presented other problems such as matrix interferences and high detection limits [24]. Even with these limitations, this technique has been used for the characterization of wines [41] and vinegars [12] based on the product's content of amino acids.

Despite its drawbacks, ion-exchange chromatography with post-column derivatization gives more repeatable results than pre-column derivatization followed by reverse-phase liquid chromatography, because the chromatography and derivatization are two separate events that can be individually optimized [1].

Recently the content of amino acids in table vinegar has been determined by high-performance anion-exchange chromatography with pulse amperometric detection. This method does not need pre-column or post-column derivatization [42].

2.2. Reverse-phase high-performance liquid chromatography

In most studies dealing with the analysis of amino acids by liquid chromatography (LC), this has been carried out using reverse-phase columns that have a silica stationary phase with C-8 or C-18 groups. These columns, packed with very small silica particles (3–10 μm) and small column diameter (2–5 mm), are subjected to high pressure and have a very controlled flow rate of mobile phases.

The mobile phases used most frequently in reverse phase are mixtures of water and an organic solvent (methanol, acetonitrile, or oxalane, also named "tetrahydrofuran"). When the sample has substances containing acidic or basic groups such as amino acids, the pH of the mobile phase is controlled through a buffer [5].

Reverse-phase liquid chromatography uses the solubility properties of the sample to partition it between a hydrophilic and a lipophilic solvent. The partition of the sample components between the two phases will depend on their respective solubility characteristics. Less hydrophobic components will associate primarily with the hydrophilic phase, while more hydrophobic components will be found in the lipophilic phase. The whole process depends on the extractive power of the hydrophilic phase. In the reverse phase, silica particles coated with chemically bonded hydrocarbon chains represent the lipophilic phase, while the aqueous mixture of an organic solvent that surrounds the particles represents the hydrophilic phase.

Since all the amino acids show a wide range of polarities, to resolve them in a single chromatogram it is necessary to vary the composition of the mobile phase to increase its elution power. In this way, using a gradient of polarity, it is possible to achieve the separation of all the compounds in a reasonable time, which would be impossible to achieve with an isocratic elution [5].

Reverse-phase LC uses pre-column derivatization methods. This technique is simpler, faster, has greater sensitivity, and uses somewhat less expensive LC systems that operate at higher pressures compared to dedicated, ion-exchange-based amino acid analyzers [1].

Many applications of this technique have been described, including the characterization of wines [4] and differentiation of vinegars based on the composition of amino acids [22]; the study of the evolution of amino acids during the wine-making process [13,43] and vinegar-making process [21]; determining the relationship between the amino acid content and the volatile compounds in wine [44]; and the simultaneous determination of biogenic amines and amino acids in wines [25,45] and in vinegars [19]. In addition, liquid chromatography has been used to determine L- and D-isomers of amino acids in wines [33–36].

2.3. Gas chromatography

Gas chromatography (GC) is another alternative for the analysis of amino acids. This technique is fast and has a high power of resolution and high sensitivity, but its performance requires considerable experience. Since free amino acids are not sufficiently volatile, they have to be converted into volatile derivatives to be determined. The production of several different esters of amino acids has been used for the profiling and quantification of amino acids in food products by GC. Some authors have used ethyl carbonochloridate with pyridine catalyst for the rapid derivatization of amino acids to their N(O, S)-ethoxycarbonyl ethyl esters [46]. Other derivatization reagents also used in GC are 2,2,3,3,3-pentafluoropropanoate and 2,2-dimethylpropanoyl chloride, which convert amino acids into their pentafluoropropionyl-2-propyl and dimethylpropanoyl derivates, respectively [37,38].

The main disadvantage of GC seems to be the procedure of derivatization, due to the complexity of reactions and types of reagents used. The speed at which derivatization takes place dif-

fers from one amino acid to another, and strict reproduction of reaction conditions is essential for all samples. Moreover, most of the volatile derivatives of amino acids may be lost during the concentration of the sample. Furthermore, this technique requires a careful sample extraction and preconcentration, and these requirements must be taken in account when assessing the reliability of final results [24]. Despite these drawbacks, GC is considered selective, sensitive, precise, accurate, inexpensive, and versatile, relative to ion-exchange methodology, and its ability to interface with MS allows identifications based on more than just retention times [1].

Ion-exchange chromatography and GC have been compared in terms of accuracy and neither method was clearly superior [2]. GC proved to present less intra-laboratory variance, except for arginine and histidine. GC and LC have also been compared [47], and the method of choice is LC, considering many factors including simplicity of derivatization. Although GC has advantages (speed and sensitivity), most analyses of amino acids are still carried out by classical ion-exchange chromatography or reverse-phase LC.

However, GC coupled with mass spectrometry (GC/MS) can be a useful alternative to other methods of amino acid analysis, especially when the sample quantities are limited and high sensitivity is required [48]. GC/MS has been used for rapid profiling and screening of 17 protein and non-protein amino acids in different wines, and the study of the GC profiles obtained suggests that the method is potentially useful for the classification of wine brands [49].

GC, using a chiral stationary phase, is especially useful in the quantification of amino acids enantiomers in food matrices [50]. In fact, many authors have applied GC for the determination of D-amino acids and the separation of enantiomers in wines, using a capillary column coated with dimethyl(oxo)silane anchored to (S)-(−)-t-Leu-t-butyl-amide [37] and in vinegars, using a fused silica Chirasil-L-Val capillary column [38,39].

In recent years, the capacity of GC for the separation of analytes has been further improved with the emergence of "two-dimensional comprehensive" gas chromatography (GC × GC) [51], in which the sample is eluted in a first column and then introduced into a second one of a different nature. Usually, the first column has a low-polarity, such as a BPX5 phase, while the second has a polar nature such as the Solgel Wax column. All the analytes of the sample will thus be subjected to two separate mechanisms and their complete separation is achieved [52]. This technique has already been applied in wines for the determination of amino acids [53].

2.4. Capillary electrophoresis

Capillary electrophoresis (CE) has been used to separate and detect amino acids, peptides and proteins. This technique allows the analysis of extremely small sample volumes, and therefore, it requires detection of high sensitivity. Methods of detection used include laser-induced fluorescence, which has limits of detection of about 10^{-20} moles for the separation by CE of derivatized amino acids. One drawback of this technique is the length of time taken in sample preparation, since it requires a process of derivatization and preconcentration prior to analysis [54]. The derivatized analyte may have additional properties that enhance separation. Pre-column, post-column and on-column derivatizations have been used for CE separations, although on- and post-electrophoretic derivatizations are mostly employed.

In addition, CE can be applied with a laser-induced fluorescence detector for post-column reactions [55]. This technique has also been applied with electrochemical detection to determine amino acids in vinegar [56].

The analysis of amino acids by CE gives extremely low detection limits but, in practice, these low limits of detection are not required in routine analysis of amino acids in food and drink products. However, in future methods of food processing, new compounds may occur at levels sufficiently low to recommend the application of this technique.

Relative to LC methods for separation of amino acids, CE is cheaper, involves shorter analysis times without the need for gradient elution, uses less solvent (mL/day), produces less solvent waste, and its mass detection capability is 1000 times lower due to smaller injection volumes. It also offers different selectivity and higher efficiency compared to LC [57].

2.5. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy offers some outstanding advantages in the field of chemical analysis of food products because it is non-destructive, selective and capable of simultaneous detection of a great number of low molecular mass components, such as amino acids, in complex mixtures [54].

The popular and widely used LC and CE techniques are more sensitive than high-resolution NMR spectrometry, but they require time-consuming sample preparation before measurements can be made. Separation, derivatization, and preconcentration in the case of compounds in low concentration, are usually common steps in these procedures. However, sample preparation for NMR spectroscopy is simpler and less time-consuming. Another great advantage of high-resolution NMR is the possibility of detecting the magnetic resonance of different nuclei present in a molecule in different electronic and spatial environments.

This technique has proven to be useful for assessing wine quality; for example, it has been used in the verification of the origin and age of wine, and the effects of adulteration [58]. In recent years the use of high-resolution NMR techniques in the study of wine has attracted the interest of several groups, and, as result, one-dimensional and two-dimensional NMR experiments have been conducted to characterize and classify a wide variety of wines [3,11,59,60]. However, only a few researchers report using intensities from proton nuclear magnetic resonance spectroscopy (^{1}H NMR) for the quantification of various components in wine including amino acids [61]. Recently, ^{1}H NMR has been employed to monitor the levels of important metabolites of Rioja red wine during the alcoholic and malolactic fermentation processes [62]. This method allowed the simultaneous quantification of ethanol, acetic, malic, lactic and succinic acids and some amino acids. In addition, ^{1}H NMR spectroscopy with pattern recognition methods has been used to investigate the metabolic differences in grape pulp, skin, seed and wines from different regions [60].

In vinegar, this technique has also been applied for the identification and quantification of the main organic components such as carbohydrates, alcohols, organic acids, volatile compounds and amino acids [63].

2.6. High-performance liquid magneto-chromatography

High-performance liquid magneto-chromatography (HPLMC) is a new chromatography technique with two distinctive features: a high surface area stationary phase with paramagnetic properties (SiO_2/Fe_3O_4) and a magnetic field intensity (variable from 0 to 5.5 mT) that selectively retains paramagnetic substances in the stationary phase, depending on their magnetic susceptibility. Hence, the separation takes place due to the difference in the force of attraction exerted by the high gradients [64].

The separation of different compounds according to their magnetic properties is a common practice in the manufacturing and mining industries. However, in recent years, the use of this technique has expanded to other areas such as biotechnology and analytical chemistry. Given its benefits including speed, simplicity and selectivity, magnetic separation has promising applications in the field of separation techniques [64]. In fact, this technique has

been applied to identify and quantify amino acids in samples of Spanish red and white wines [65] and has required shorter sample processing and analysis times than other techniques. In addition, it also showed good signal reproducibility and repeatability.

3. Derivatization reagents of amino acids

Amino acids can be detected directly by ultraviolet (UV) or visible light detection since they absorb at a wavelength between 190 and 210 nm. However, the majority of solvents and other components of the samples also absorb in this region of the spectrum; hence, the amino acids have to be derivatized prior to analysis. Such derivatization can be undertaken either before (pre-column), or after (post-column) chromatographic separation of amino acids, and more rarely, on the column (on-column). Each way of obtaining the derivative has its own advantages and disadvantages (Table 1).

In post-column derivatization the separation of amino acids is carried out with a cation exchange resin and a gradient of acidic buffers. After separation, amino acids are converted into coloured ninhydrin derivatives for colorimetric detection, or into orthophtaldehyde (OPA) for fluorescence detection.

Although pre-column derivatization presents more advantages, traditional post-column methods have not been totally discarded.

Various different derivatizing agents have been employed for the determination of amino acids. Those most widely used are listed below and their corresponding advantages and disadvantages are shown in Table 2.

3.1. Derivatizing agents

3.1.1. Ninhydrin

Nindydrin-based monitoring systems remain among the most widely used methods for the quantitative determination of amino acids after they have been separated in their native form by ion-exchange chromatography. Hence, derivatization with ninhydrin is exclusively of the post-column type [2].

The sensitivity of the reaction with ninhydrin is below 1 pmol but it is rarely reproducible below 100 pmol. This limitation makes it unsuitable for many of the more demanding applications. Ninhydrin can easily deteriorate on exposure to light, atmospheric oxygen and changes in pH and temperature. Therefore, these factors must be considered when this agent is used.

Ninhydrin decarboxylates and deaminates the primary amino acids, forming the purple complex known as Ruhemann's Purple, which absorbs maximally at 570 nm. A yellow-coloured product, which can be monitored at 440 nm, is formed upon reacting ninhydrin with the secondary amino acids, proline and hydroxyproline. When ninhydrin becomes oxidized, its colour does not develop well at 570 nm, but absorption at 440 nm remains fairly constant. A good indicator of reagent degradation is when the height of the proline peak at 440 nm approaches the height of the glutamic acid peak at 570 nm for equal amounts of each.

Several authors have applied this technique for the characterization of wines based on the content of amino acids and biogenic amines [7].

3.1.2. Dansyl chloride

Dansyl chloride (5-N,N-dimethylamino-naphthalene-1-sulfonyl chloride) (Dns-Cl) is a well-known fluorogenic pre-column derivatization reagent for the determination of primary and secondary amines.

Dansylation has been used mainly as a method for determining free amino acids, as well as protein hydrolysate amino acids and terminal amino acids from proteins and peptides. Dansyl amino acids are detected by fluorescence (λ_{ex} 360 nm; λ_{em} 470 nm) and UV (λ_{max} 250 nm). Detection levels are in the pmol or fmol range, depending on the sensitivity of the detector [2].

Dansyl amino acids are formed under optimal conditions within 35–60 min in the dark. On the other hand, any excess of Dns-Cl reacts with dansyl amino acids producing dansyl amide. Dansyl amide formation is an unavoidable limitation of the method and the quantity formed depends on the amino acid concentration and the excess of dansyl chloride. Hence, it is convenient to minimize the emergence of such secondary products, as far as possible.

Dansyl derivatives are relatively stable against hydrolysis, which does not occur with the reagent. However, the exposure of the derivatives to light should be avoided because they are photosensitive. Dansyl amino acids are stable for at least 7 days if they are kept at 4 °C.

This technique is generally used in pre-column derivatization. Many authors have applied it for the determination of amino acids in grape musts, wines and vinegars [22,68]. It has also been widely used for the determination of biogenic amines in wines [67].

Under certain derivatization conditions, histidine can form multiple peaks. Most amino acids give mono-dansyl derivatives; however, lysine, ornithine, histidine, tyrosine and cysteine form didansyl derivatives. This Dns-Cl method gives a good reproducibility for most amino acids except histidine due to the low relative fluorescence response of the didansylated adduct and the formation of two dansyl derivatives [2].

Table 1
Advantages and disadvantages of pre-column and post-column derivatization.

Derivatization	Advantages	Disadvantages
Post-column	It is possible to use different detection systems and elute the compounds detecting them by non-destructive methods prior to derivatization. The reaction is reproducible without the need to form a single derivative.	Presence of interferences due to excess of reagent or degradation products. Loss of resolution caused by the widening of the chromatographic bands in the reactor where the reaction is performed. It is not allowed to use very long retention times. This method can be expensive due to the changes that have to be performed to carry out the reaction.
Pre-column	The only limitation to the conditions of the reaction is that it must be completed in a reasonable time and quantitative. The reaction can be performed in a solvent not compatible with the mobile phase used in chromatographic separation. The secondary product formed in the column or before chromatographic separation can be separated.	Presence of interfering peaks in the chromatograms due to the reagent, reaction or degradation products or impurities of the reagents. Hence, it is convenient to remove the excess of reagent, solvents or other components of the reaction mixture prior to injection into the chromatograph. A substantial part of all derivatives will be identical. Minor differences in side chain of amino acids will have less effect on the chromatographic behaviour of the derivatives, making the separation more difficult.

Table 2
Agents employed in derivatization of amino acids.

Reagent	Derivatization			Separation	Run time (min)	Detection	Advantages	Disadvantages	Ref.
	Type	Conditions	Time (min)						
Ninhydrin[a]	Post-column	High temperature (~110 °C)	~10	Ion exchange	60–120	UV	Reacts with primary and secondary amino acids. Able to detect pmol.	Rarely reproducible below 100 pmol. Problems of interferences with matrix. Sensitive to light, O_2, temperature changes and pH.	[7,2]
Dns-Cl[a,b]	Pre- and post-column	25–40 °C in the dark	35–60	Reverse phase: Phenomenex® Luna C_{18} Spherisorb ODS-2	30–80	Fluorescence UV	Reacts with primary and secondary amino acids. Detection levels are in the pmol or fmol range. Derivatives are stable to hydrolysis. Good reproducibility for most amino acids except hystine Adequate to determine cysteine.	Reagent excess interferes with amino acid chromatogram peaks. Derivatives are photosensitive. Slow reaction. Not specific and reacts with other compounds.	[22,66,67]
DABS-Cl	Pre-column	~70 °C	10–20	Reverse phase: Spherisorb ODS-2 Lichrospher 100 RP-18	50–90	Visible	Reacts with primary and secondary amino acids. Derivatives are stable during four weeks at room temperature. Able to detect pmol.	Reagent excess interferes with amino acid chromatogram peaks. Presence of an excess of salt and detergents interferes with the reaction. It produces multiple derivatives.	[45,68]
DNFB	Pre-column	50–60 °C	30 min in the dark	Reverse phase: Catridge C_{18}	10–30	Fluorescence	Reacts with primary and secondary amino acids. Detection levels are in the pmol range. Determines amino acids that are poorly resolved by other agents.	Derivatives are photosensitive. Slow reaction. Destructive method for peptides.	[5]
PITC[a,b]	Pre-column	Room temperature	10–20	Reverse phase: Spherisorb ODS-2 Pico Tag Column	10–70	UV	Reacts with primary and secondary amino acids. High sensitivity for proline and hydroxyproline. Fast reaction. Derivatives are more stable than others. Reagent excess does not interfere.	Problems of interferences with the matrix in analysis of grape musts. Not suitable for automation. Less sensitivity than other methods. The procedure consists of several manual steps.	[20,71]

OPA[a,b]	Pre- and post-column	Room temperature	1–2	Ion exchange and Reverse phase: Nova Pack C_{18} Hypersil ODS	6–40	Fluorescence UV	Reagent excess does not interfere. Fast reaction. Derivatives are highly fluorescent. Ten times more sensitive than reaction with ninhydrin.	Unstable derivatives. Does not react with secondary amino acids. Needs a complete automation of the reaction. Low response for Lys, hydroxylysine and cysteine.	[4,6,19,24]
FMOC-Cl[a]	Pre-column	Room temperature	6–10	Reverse phase: Nucleodour C_{18} Hypersil ODS	30–40	Fluorescence	Reacts with primary and secondary amino acids. Fast reaction. Derivatives are stable and highly fluorescent. Very sensitive.	Produces multiple derivatives. Highly fluorescent and interferes with amino acid chromatogram peaks. Derivatization process is slow since the reagent has to be eliminated.	[25,83]
DEEMM	Pre-column	25–50 °C in a methanolic medium	30–50	Reverse phase: Nova Pack C_{18}	32–35	UV–vis	Reacts with primary and secondary amino acids Direct derivatization without prior preparation. Reagent excess does not interfere. Sensitivity of pmol.	Slow derivatization process Derivatives of proline and hydroxyproline are unstable Not sensitive below pmol level.	[75,76]
AQC[a,b]	Pre-column	Room temperature	10	Reverse phase: Nova Pack C_{18} Phenomenex® Luna C_{18}	70–80	Fluorescence UV	Reagent excess does not interfere. Reacts with primary and secondary amino acids. Derivatives are stable and highly fluorescent. Very sensitive (50–300 fmol). Fast reaction. Direct derivatization without prior preparation. Salts and detergents present in samples do not interfere.	Lys and Trp show a lower fluorescence. If ammonia is not completely derivatized, the excess may distort the analysis of Arg and Thr.	[77,80]

[a] Applied in wine.
[b] Applied in vinegar.

3.1.3. Dabsyl chloride

Another derivatizing agent used for the determination of amino acids is dabsyl chloride (4-dimethylaminoazobenzene-4-sulfonyl chloride) (DABS-Cl).

Dabsyl derivatives present maximum absorption at 420 nm (visible region), are highly stable and are readily separated by LC and detected at the pmol level. These derivatives remain quite stable for four weeks at room temperature.

A limitation of the DABS-Cl method is that the presence of excess amounts of salt, urea, SDS, phosphate, or ammonium bicarbonate will alter the pH of the buffer and interfere with the dabsylation reaction.

For the quantitative analysis of unknown samples, the DABS amino acid standards have to be obtained from an amino acid standard mixture that has been hydrolyzed and dabsylated in parallel with the unknown samples under identical conditions [2].

Krause et al. [45] proposed the use of dabsyl chloride as an alternative method to conventional analysis of amino acids and biogenic amines in food and biological samples, and achieved the separation of more than 40 compounds simultaneously. In enology, this agent has been also employed for the characterization of wines according to their biogenic amine content [68].

3.1.4. 1-Fluoro-2,4-dinitrobenzene

1-Fluoro-2,4-dinitrobenzene (FDNB) is a pre-column derivatizing agent used for the characterization of terminal amino acids of peptide chains.

This agent reacts with primary and secondary amino acids to form compounds that are fluorescent at 365 nm. The reaction is carried out at 50 °C for 30 min. Dinitrophenyl amino acids are known to be photosensitive but, when shielded from light, are stable for 48 h.

FDNB can detect small amounts of amino acids, in the low pmol range, and is suitable for determining amino acids that are poorly resolved in other systems [2].

3.1.5. Phenylisothiocyanate

The derivatizing agent isothiocyanatobenzene, also known as phenylisothiocyanate (PITC), has been used for more than 30 years in the Edman degradation method for sequencing peptides and proteins. PITC reacts with free amino acids to yield phenylthiocarbamyl (PTC) amino acids. This agent has been applied to determine amino acids in orange juices [69], wines [66,70,71] and vinegars [20].

One important feature of the analysis with PITC is the quantification of the secondary amino acids such as proline and hydroxyproline. Unlike the situation with post-column ninhydrin detection or ortho-phthalaldehyde derivatization, the proline and hydroxyproline PTC derivatives have the same chromophore and approximately the same molar response as other amino acids.

The specificity of PITC is well characterized, and there is no evidence for the formation of any di-substituted derivatives with tyrosine (Tyr) or histidine (His). Only cystine (Cys_2) and lysine (Lys) react with two PITC molecules. Under mild conditions, the reaction is essentially complete between 10 and 20 min and, because the reagent is volatile, a large excess can be used. This excess can be readily removed under reduced pressure. The dried samples may then be stored at −20 °C, at which temperature they should be stable for four weeks.

Since the derivatives do not fluoresce, the technique is limited to UV detection, which normally takes place at 254 nm (λ_{max} = 269 nm). Although this technique is not as sensitive as some of the fluorescent reagents, the highly UV-absorbing PTC derivatives can be detected at the low pmol level, which represents the practical level of sensitivity for real samples. The limit of sensitivity is 50 pmol at a signal-to-noise ratio of 2.5, which is ca. 50 times less sensitive than detection of OPA or FMOC adducts.

3.1.6. Ortho-phtaldehyde

Ortho-phtaldehyde (OPA) was introduced in 1971 and is probably the most commonly used derivatization agent in reverse-phase LC for the determination of free amino acids. It has been applied in wines [4,43,44,70,72] and vinegars [19]. OPA can also be used in CE and ion-exchange chromatography. Another application of this reagent is the determination of enantiomeric amino acids in foods and beverages [33].

OPA is a reagent that does not have a natural fluorescence, but this develops when OPA reacts with primary amino functions. This reagent may be applied as either a pre-column or a post-column derivatizing agent.

The reaction between OPA and amino acids takes place in an aqueous medium at a strongly alkaline pH in the presence of a thiol such as 2-mercapto-ethanol, 3-mercapto-1-propanol or ethanothiol to form highly fluorescent derivatives). The isoindol derivative formed is unstable and must be stabilized by acidification. Reaction is fully completed in 1 or 2 min at room temperature [5].

There is no need to remove excess OPA prior to sample injection since OPA itself will not interfere with separation or detection. However, since OPA-amino acid derivatives are unstable, complete automation of the pre-column reaction, with accurate control of reaction time, is essential for reproducible results. The limit of detection of OPA is around the fmol range; hence, this technique is 10 times more sensitive than the ninhydrin reaction.

On the other hand, OPA does not react with secondary amino acids. However, secondary amino acids, such as proline or hydroxyproline, can be determined if they are first oxidized with sodium hypochlorite, under alkaline conditions, which converts the secondary amino acids to primary amines. Another alternative is to use OPA with another agent that reacts with secondary amino acids. Several authors have used a double derivatization with OPA and 9H-fluoren-9-ylmethyl chloroformate (FMOC) to determine primary and secondary amino acids in musts and wines [13,15,24,73]. Pripis-Nicolau et al. [6] developed a method to determine cysteine and other amino acids in musts and wines using a first derivatization with iodoacetic acid followed by another derivatization with OPA.

Another inconvenient feature of this reagent is the low response of lysine, which can be due to the presence of two fluorescent isoindolic structures. Cysteine is another amino acid that presents a low response since the derivative decomposes readily. The solution proposed by some authors is to perform a prior oxidation with peroxyformic acid or a carboxymethylation and detect the amino acid as cysteic acid or carboxymethylcysteine, respectively [5].

The resulting derivatives can be also detected by UV monitoring at 330 nm, but for higher sensitivity, fluorescence detection is often chosen and emission is measured at wavelengths above 430 nm.

3.1.7. 9H-fluoren-9-ylmethyl chloroformate

9H-fluoren-9-ylmethyl chloroformate (FMOC-Cl) has recently been shown to be a suitable pre-column derivatizing reagent for the determination of primary and secondary amino acids, for which similar responses are given. This reagent results in highly fluorescent and stable derivatives [23].

In contrast to the other pre-column derivatization reagents that yield fluorescent derivatives (i.e. Dns-Cl and OPA), the reagent FMOC-Cl is fluorescent itself. Both FMOC and its hydrolysis products have absorption and fluorescence spectra that are similar to those of FMOC-amino acids, hence, they can interfere in the quantification of amino acid derivatives. However, this property need not be a limiting factor, since the reagent excess and fluorescent side-products can be eliminated without loss of the amino acid derivatives by liquid–liquid extraction. Excess FMOC remaining after derivatization reacts with water to form 9-fluorenemethanol (FMOC-OH). An alternative method of preventing interference of

FMOC-OH is to react FMOC with a very hydrophobic amine to form a derivative that elutes after the peaks of interest [2].

This derivatizing agent has been used to determine biogenic amines and their amino acid precursors in wines [23,70] and foods [74], and has also been applied to determine polyamines such as putrescine, spermine and spermidine simultaneously with other amines in Grenache Noir and Syrah grapes and wines of the Rhone Valley [23].

3.1.8. Diethyl 2(ethoxymethylidene)propanedioate

Diethyl 2(ethoxymethylidene)propanedioate, also known as diethyl ethoxymethylene malonate (DEEMM) is another pre-column derivatizing agent which gives amino acid derivatives detectable in the ultraviolet region. The derivatives are easily obtained and are very stable.

This agent has been employed to determine the amino acid content in wines and in different types of Spanish honey [75]. In this last-cited research work, the information obtained was examined in order to establish whether free amino acid composition of a honey can explain its botanical origin.

The derivatization reaction is carried out in a methanolic medium for 30–50 min. Then, the sample is heated for 2 h at 70 °C for the complete degradation of excess reagent and its side-products. Most of the derivatives are perfectly stable, at least during the first week. Hence, for quantification purposes, the analysis should be performed in the 24 h following the derivatization reaction. This is one of the disadvantages of the method. Moreover, limits of detection are under 0.4 mg/L for amino acids and 0.07 mg/L for biogenic amines; hence, this reagent should be used when a sensitivity of below pmol is not required. Another disadvantage is the time required for the derivatization, since other agents give derivatization in shorter periods of time [76].

However, this reagent presents some advantages such as direct derivatization without previous preparation; simultaneous quantification of 24 amino acids (even proline), biogenic amines and the ammonium ion; the UV detector, which is the type available in most laboratories, can be employed, and there is no interference from the reagent excess.

3.1.9. 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate

6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) is a derivatizing agent specifically designed for the analysis of amino acids with the object of simplifying the derivatization reaction, increasing the yields of the reaction, and increasing the sensitivity and selectivity of the derivatives formed when fluorescence detection is used [77–79].

This compound reacts quickly with primary and secondary amino acids forming stable and highly fluorescent products at 395 nm. The derivatives are stable at room temperature for at least one week, and they are easily separated by reverse-phase LC using a C-18 column.

The excess reagent is hydrolyzed during the reaction to form 6-aminoquinoline (AMQ), whose spectral characteristics are different from any of the amino acids derivatized. It allows a wavelength to be selected that maximizes the emission response of derivatives and minimizes the response of the AMQ. During the hydrolysis of the reagent N-hydroxysuccinimide and carbon dioxide are also formed, but they do not interfere in the chromatographic analysis. Destruction of excess reagent is completed in less than 1 min.

The protocol for derivatization is simple and direct. The reagent is added to the sample pre-buffered and then is heated to carry out the reaction. Derivatives are injected without additional preparation of the sample, since salts present in the sample do not interfere in the reaction or in the reproducibility of results [79].

This method has been optimized for use with fluorescence detection to achieve detection limits of 50–300 fmol for existing amino acids in peptides and protein hydrolysates [80].

AMQ absorbs about 200 times more than any of the derivatized amino acids using a UV detector at 250 nm. This can cause difficulties in the quantification of aspartic acid, which is the first amino acid that elutes. This does not happen when the detection is performed by fluorescence, because the signal from the AMQ is much smaller than that obtained in UV detection. Another characteristic of AMQ is that its retention time can vary depending on the pH of the mobile phase.

This reagent has been used as an alternative to the most common derivatization agent, OPA, to determine biogenic amines and amino acids in wines [17,27,80,81] and to study the evolution of amino acids and peptides during alcoholic fermentation and autolysis of wines [82]. In addition, AQC has been applied to determine amino acids in vinegars in submerged and surface acetifications [21]. Recently, this compound has been used for the enantiomeric separation of ornithine in complex mixtures of amino acids such as those found in fermented food and drinks (wine and beer) by CE [40].

4. Conclusions

Amino acids are compounds present in food and beverages which affect the quality of these products in respect of taste, aroma and colour. There has always been interest in the development of reliable, rapid and accurate methods of analysis for assessing the quality of food for nutritional and regulatory purposes. The importance of amino acids in enology is well known, and they are present in grape must, where they act as nutrients for yeast growth; they are consumed as a source of nitrogen during alcoholic fermentation and are precursors of biogenic amines and aroma compounds such as high alcohols, aldehydes, esters and ketonic acids.

Different chromatographic methodologies for the analysis of amino acids have been outlined, and specific applications in enology for the analysis of amino acid content are discussed. The original method of post-column ninhydrin derivatization, followed by ion-exchange chromatography and UV detection using amino acid analyzers, has gradually been superseded by faster, more sensitive and versatile LC methods using pre-column derivatization. Derivatization is usually required to convert amino acids into more detectable forms. The reagents most widely used for pre-column derivatization are: Dns-Cl, DABS-Cl, DNFB, PITC, FMOC-Cl, OPA, EMMDE and AQC.

Although pre-column derivatization offers more advantages, none of the pre-derivatizing agents is considered “the universal agent” since none of them meets all the requirements:

- It must react rapidly under mild conditions to provide quantitative yields of derivative.
- It should react with both primary and secondary amino acids.
- The derivatives should remain stable for several days, preferably at room temperature, to permit automated analysis of multiple samples.
- There should be no interference from the reagent, breakdown products, or side reactions.
- Response should be linear over the concentration ranges typical of most applications.
- The derivatives should have reasonably similar molar response factors.

Each of the methods has its own advantages and disadvantages. Therefore, the predominant criteria that will influence selection of the most suitable type of derivatization and chromatographic pro-

cedure are the degree of resolution, sensitivity and speed required in the analysis, in each case.

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