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MINI REVIEW

Analysis of amino acids in food using High Performance Liquid Chromatography with derivatization techniques: a review

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Abstract

Amino acids (AAs) are essential components needed for human health. AAs are typically present as free or bound into protein backbones. Determination of AAs is important to evaluate the nutritional value of protein-containing foods and to authenticate food products from any adulteration practice. Due to its capability to provide the separation capacity, chromatographic-based methods are considered the method of choice for the analysis of AAs. The common detectors used for the analysis of food components are specific detectors including UV-Vis and fluorescence. Unfortunately, most AAs are non-chromophoric or fluorescence compounds, as a consequence, some derivatization processes are needed using derivatizing agents to make AAs detectable using UV-Vis or fluorescence detectors. This review highlighted the use of HPLC for the analysis of AAs in food products by applying pre-column and post-column derivatization techniques to be detectable by UV-Vis or fluorescence detectors.

1. Introduction

Amino acids (AAs) are groups of organic compounds, derivatives of hydrocarbons, which contain a primary amine group (-NH_2) or secondary amine (-NH), and carboxyl group (-COOH) along with a variable side chain, as shown in Figure 1. Despite being called acids, AAs are amphoteric due to the functional groups being able to donate or accept a proton. Under certain conditions, such as at physiological pH, AAs are capable of becoming zwitterions. AAs can be further classified into subgroups according to different criteria, namely polarity, chirality, biological function, and the position of the amine group in the side chain (Violi *et al*., 2020). Analysis of AAs can be found in many areas of research, and the most important purpose is to assess the nutritional value of food and drink products (Callejón *et al*., 2010). Recently, the profiles of AAs are used for the assessment of certain diseases including lung cancer by the differentiation between cancerous and adjacent normal tissues (Lu *et al*., 2021) or by investigating the amino acid biomarkers for the detection of sepsis (Ahn *et al*., 2021). The composition of AAs in certain foods can be used as a tool for the detection of adulteration. The amino acid profiles combined with chemometrics have been successfully applied for the authentication of orange fruit juice (Wistaff *et al*., 2021).

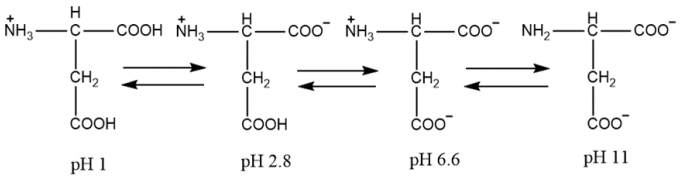

Figure 1. The ionization of aspartic acid (Asp) at different pH values. The isoelectric point of Asp was at pH 2.98.

Amino acids had the characteristics of zwitterion that the solubility of AAs around the isoelectric point is poor. The isoelectric point (pI) of an amino acid molecule is defined as the pH value, at which the molecule, in the solution, does not dispose of any charge (neutral charge). The value of pI of amino acids is a function of the pH values of the ionizable groups in the molecule. The conditions of the separation of the amino acids can be modified in a way that the isoelectric points, for all amino acids, are to be reached at various times. The following is an example of aspartic acid (Figure 1) which

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undergoes the different charges at different pH (Csapó *et al*., 2008).

At very acidic conditions (pH of approximately 1), aspartic acid (Asp) has one positive charge. If the pH value is increased, the larger number of molecules situated in the α-carboxyl group will have a negative charge up to the limit of pH of approximately 2.98 when all of them dispose of it. This situation (at pH 2.98) in which Asp do not dispose of any charge (neutral charge) is known as the isoelectric point of Asp. The carboxylic group in the side chains has less acid than the α-carboxylic acid, and the concentration of the hydrogen ions is sufficient enough to prevent its ionization. If the pH value rises to 6.6, the carboxylic group of the side chain will be ionized, and the molecule will get two negative and one positive charge, and if the pH rises to 11.0, the molecule will dispose only of two negative charges (Csapó *et al*., 2008). The example of chemical structures along with pI values was compiled in Table 1.

The quantitative analysis of amino acids using modern and sensitive analytical methods is most commonly found in most food products, either in the form of free AAs form or as the building blocks of proteins (Otter, 2012). Compositional analysis of AAs can provide an excellent way of monitoring the nutritional value of foods. This is based on the fact that AAs are a part of proteins that are important in nutritional aspects (Gałęzowska *et al*., 2021). Because of its capability for the separation of AAs in food samples, chromatography techniques, especially high-performance liquid chromatography (HPLC) are widely used for the quantitative analysis of AAs.

The main obstacles to the separation and quantitative analysis of AAs in food products using HPLC are the fact that some AAs are polar, therefore, AAs are not retained enough in HPLC reversed column. In addition, most AAs are non-chromophoric in nature which is not suitable to be detected using UV-Vis and fluorescence detectors (Ferré *et al*., 2019). As a consequence, some derivatization agents are introduced and used during AAs analysis with HPLC to be detected using UV-Vis and fluorescence detectors, the common HPLC detectors available in the analytical laboratory (Callejón *et al*., 2010). Figure 1 revealed the chemical structures of AAs in which only Cysteine (Cys) and aromatic AAs of Phenylalanine (Phe), Tyrosine (Tyr), Tryptophan (Trp), and Histidine (His) can be easily detected using a UV detector. Basically, AAs can be detected directly using a UV-Vis detector because AAs absorb UV radiation at a wavelength of 190-210 nm. Unfortunately, the majority of solvents and other components of the samples are also absorbed in this region making the separation between AAs and solvents difficult, therefore the derivatization process is an excellent approach for analysis of AAs using HPLC combined with UV-Vis or fluorescence detectors (Ta *et al*., 2021).

The objective of this review was to give an overview of the application of HPLC using different types of detectors for the analysis of AAs in food samples. Special emphasis was given to the application of UV-Vis detectors, as a consequence, some derivatizing agents typically used for AAs derivatization to be detected by UV-Vis or fluorescence detectors were highlighted. In the last section, the application of HPLC for analysis of AAs derivatized either using pre-column or post-column derivatization was also presented.

2. Analysis of amino acids using HPLC

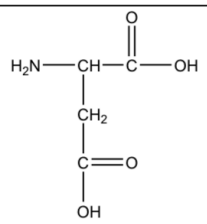
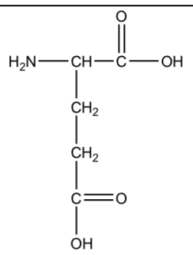
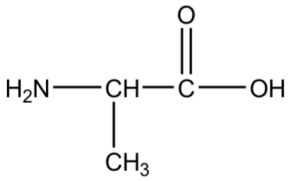
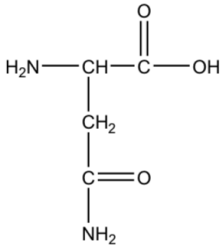
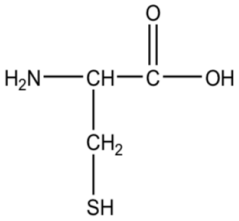
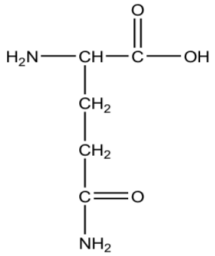
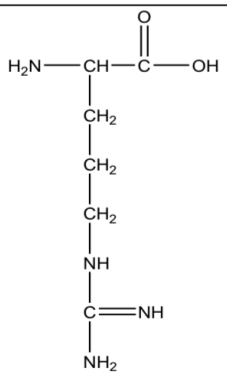
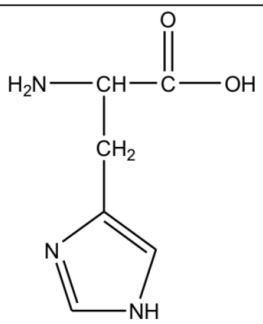
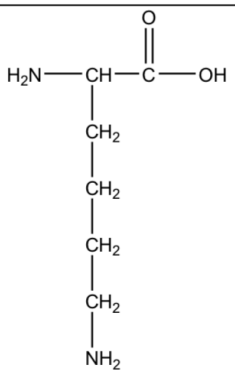
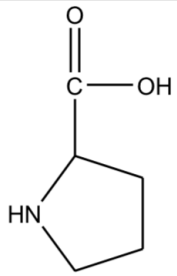
HPLC has become a very popular method offering reproducible analytical techniques in food analysis. HPLC becomes the method of choice for the determination of AAs in food samples due to its capability to offer a high-throughput feature, provide good separations of AAs in a relatively short time, and quantify low levels of AAs with good sensitivity. However, this technique has some problems regarding the need for extensive sample preparations to remove the interferences that may be present in food samples (Csapó *et al*., 2008). Analysis of AAs using HPLC consisted of three main steps namely (1) hydrolysis of individual AAs from the protein backbones, (2) separation of individual AAs using HPLC procedure and (3) detection and quantification of the separated AAs (Otter, 2012).

2.1 Sample preparation

Sample preparation is often a critical step in the analysis of AAs. The main objective of sample preparation in the analysis of AAs using HPLC is to release AAs completely from the sample matrix with minimum interferences resulting in the acceptable recoveries of analyte(s). During sample preparation, the optimization should be undertaken to get ideal conditions such as minimum use of chemical solvents and reagents to support the green analytical technique. The optimum condition of sample preparation should reduce analysis time, and sources of error, enhance sensitivity and enable unequivocal identification and quantification of the AAs (Gałęzowska *et al*., 2021).

AAs can be either in the form of free AAs or in the form of protein backbones. The release of AAs from protein backbones in the food samples is a challenging task because the different hydrolysis conditions are required for the optimal release of AAs in different food matrices. The hydrolysis of protein into AAs is a critical step for analysis contributing to the main source of

Table 1. Common amino acids along with chemical structure, isoelectric point (pI) and symbols using three letters and one letter (Csapó *et al*., 2008).

Acidic amino acids
 Aspartic acid (**Asp, D**), pI = 2.98
 Glutamic acid (**Glu, E**), pI = 3.08
Neutral amino acids
 Alanine (**Ala, A**), pI = 6.11
 Asparagine, (**Asn, N**) pH = 5.43
 Cysteine (**Cys, C**), pI = 5.15
 Glutamine, (**Gln, Q**), pI = 5.65
Basic amino acids
 Arginine (**Arg, R**) pI =10.76
 Histidine (**His, H**) pI = 7.64
 Lysine (**Lys, K**) pI = 9.47
Imino acid
 Proline (**Pro, P**), pI = 6.3

analytical errors (Rutherfurd and Gilani, 2009). Conceptually, the hydrolysis procedure of AA is simple in which the use of acids (HCl) or bases of 4.2 M NaOH (105°C for 20 hrs) at a certain time and temperatures was capable of hydrolyzing the peptide bond between adjoining AAs to release free AAs (Hirs *et al*., 1954; Dai *et al*., 2014). This procedure can efficiently hydrolyze most of the peptide bonds. These methods are effective and convenient because HCl can be either easily evaporated or readily neutralized with NaOH after the hydrolysis process is completed. But, at the same time, the levels of certain amino acids are reduced or even destroyed completely. In addition, some AAs obtained during hydrolysis of proteins using HCl or NaOH applying heat are unstable (Mæhre *et al*., 2018). The sample matrix also affected the hydrolysis of AAs as a consequence the hydrolysis condition must be optimized for every AAs and type of food sample. The hydrolysis of protein using acids could result in the destruction of Trp, the conversion of Glutamine (Gln), Asparagine

(Asn), and Cys into Glutamic Acid (Glu), Asp, and cystine, respectively, while hydrolysis using bases [NaOH, KOH, LiOH, and $Ba(OH)_2$] at 105–110°C for 20 –24 hrs resulted in little loss of Trp and decomposing Arg, Asn, Cys, cysteine, Gln, Met, Ser and Thr. In order to overcome some problems related to the loss of AA residues, enzymes are typically applied for the hydrolysis of proteins from animal tissues and foods (Wu *et al*., 2011). Some efforts were made by protecting the amide groups of Gln and Asn using *bis*(1,1-trifluoroacetoxy) iodobenzene and by oxidizing Cys with performic acid to form cysteic acid before protein hydrolysis. Due to Trp destroyed during conventional hydrolysis, some protective agents such as phenol, 2-mercaptoethanol (ME), thioglycolic acid, indole and tryptamine have been employed to recover tryptophan (Ingle and Lill, 2007).

The research to find the optimum conditions capable of reducing the hydrolysis time, having high throughput and offering good stability of some AAs continue (Otter, 2012). This is caused by fact that the completeness of protein hydrolysis is affected by many factors, including sample matrix, the temperature, time, reagents, and additives of hydrolysis (Dai *et al*., 2014). Marino *et al*. (2010) have compared six combinations (hydrolysis agent and temperature-time conditions) with the AOAC method (hydrolysis with boiling 6 N HCl under vacuum at 110°C for 24 hrs) for hydrolysis of AAs in milk samples. The result showed that the use of 6 N HCl at 160°C for 1 hr resulted in comparable results with the standard method of AOAC with the advantage of a shorter time of hydrolysis. Using this optimum condition, the average recovery value of the single amino acids was 98.38%.

The hydrolysis procedure of proteins is the rate-limiting step for the analysis of AAs. In order to speed up the hydrolysis process, some innovations were made. Microwave-assisted acid hydrolysis has been explored as one method for hydrolysis of AAs in monofloral be pollens. Hydrolysis was carried out using HCl 6 M with the addition of phenol (0.5%, w/v) in a microwave at 150°C for 60 mins at a pressure of 70 psi (Themelis *et al*., 2019). The use of microwaves during hydrolysis is capable of reducing the hydrolysis time significantly in which the complete hydrolysis can be achieved using microwave irradiation at 175°C for 10 mins (Ingle and Lill, 2007). Other methods for hydrolysis of protein are the use of enzymes capable of cleavage the peptide bonds using a range of broad-spectrum proteases, but some enzymatic methods only yield the hydrolysis of proteins partially (Asano, 2019). Indeed, the selection of mixed enzymes (proteases) having broad-spectrum specificity for cleavage of the peptide bonds in protein can result in the recovery of Glu, Gln, Asp and Asn satisfactorily (Tavano, 2013). The method will be suitable for testing due to its simple derivatization procedures, stable derivatives, and ability to react with primary and secondary (Rebane and Herodes, 2012).

2.2 HPLC separation of amino acids

Over the last decades, HPLC methods using specific detectors of UV-Vis and fluorescence have been applied for the analysis of AAs in protein hydrolysates coming from food samples. Because most AAs are not chromophoric and not fluorescent, the derivatization of AAs with appropriate reagents to produce chromophoric and or fluorescent compounds is necessary for the sensitive detection of AAs.

Currently, HPLC methods for AAs involve either pre -column or post-column derivatization of AAs (Callejón *et al*., 2010). The pre-column derivatization approach is typically in reversed-phase separation, while post-column derivatization is useful for separation of AAs using cation-exchange resin, and has the advantage of removing interfering substances before derivatization reaction occurs. Compared to ion-exchange chromatography, the resolution of AAs on reversed-phase columns is faster and superior but their life spans are shorter due to the possible development of backpressure. Because of its versatility, reversed-phase HPLC is now preferred for the analysis of AAs in protein hydrolysates (Ferré *et al*., 2019).

Some derivatizing agents have been introduced and used for the derivatization of amino acids as reviewed by Callejón *et al*., 2010 and Dai *et al*., 2014) including ortho-phtalaldehyde (OPA), 7-phenanthroline-5,6-dione, phenylisothiocyanate (PITC), known as Edman's reagent, 9-fluorenylmethyl chloroformate, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, butylisothiocyanate, dimethylaminoazobenzene-4-sulfonyl chloride, 1-dimethylaminonaphthalene-5-sulfonyl chloride, diethyl 2 (ethoxymethylidene) propanedioate, 2,4-dinitrofluorobenzene, fluorescamine, 4-fluoro-7-nitro-2,1,3-benzoxadiazole, fluorescein-5-isothiocyanate, 1-fluoro-2,4- dinitrobenzene, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, naphthalene-2,3-dialdehyde, and ninhydrin. Some scientists continuously developed some derivatizing agents offering simple steps and sensitive derivatives to be detected using HPLC detectors of UV-Vis and fluorescence (Cardinael *et al*., 2015). Recently, a novel derivatizing agent of 2-(9-acridone)-ethyl chloroformate has been synthesized and applied for the quantitative analysis of free AAs by HPLC using a fluorescence detector at excitation and emission wavelengths of 268 nm and 438 nm, respectively (Gao *et*

al., 2020). Jin *et al*. (2021) also developed a new derivatization reagent of DBD-trans-2-methyl-L-proline for pre-column derivatization of AAs to be detected by fluorescence detector at excitation and emission wavelengths of 450 nm and 560 nm.

The derivatization procedure of AAS can be done either using pre-column derivatization (before injection) or post-column derivatization (after analyte(s) out from column) and more rarely, using the column derivatization. The most commonly used derivatizing agent is orto-phtalaldehyde (OPA) in the presence of 2-mercaptoethanol or 3-mercaptopropionic acid which react with primary AA to form a highly fluorescent adduct. OPA does not react with secondary AAs, therefore, in the analytical procedure OPA was combined with 6-aminoquinolyl-N-hydroxysuccinymidyl carbamate (AQC) (Gwatidzo *et al*., 2013). The fluorescence of OPA derivatives is monitored at excitation and emission wavelengths of 340 and 455 nm, respectively. Detection limits are 50 mol for AAs. OPA method provides a useful tool for monitoring AA composition in proteins of animal tissues and foods (Dai *et al*., 2014). Some drawbacks were also met including long derivatization times (PITC, dansyl chloride), need for removal of excess reagent after derivatization (PITC, FMOC-Cl), unstable derivatives, failure to react with secondary amines (OPA) and decreased derivatization efficiency in the presence of buffers and detergents (FMOC, PITC).

3. Application of reversed HPLC for analysis of AAs in food samples

For food matrix application, AAs analysis needs to continue to minimizing hydrolysis times, increasing throughput, decreasing AAs degradation and rapid method for example the use of derivatization-free LC with triple quadrupole tandem MS detection (Thiele *et al.*, 2008; Qiu *et al.*, 2020). But the importance of the methods is must be sufficiently robust to handle the diverse range of food products presently being produced (Otter, 2012). Table 2 compiles some successful applications of HPLC methods using reversed-phase columns which appear in scientific publications year 2019 to 2021. Special emphasis was given to the use of derivatizing agents applied during the analysis of AAs composition to be detected with detectors of UV-Vis or fluorescence. Nowadays, the HPLC trends are often dispensed with automated techniques for reversed-phase or ion-pair chromatographic systems (Held *et al*., 2011). The development of AAs analysis is associated with equipment miniaturization and the use of multidimensional chromatography for the complete separation of analytes ranging from non-polar to polar compounds.

4. Conclusion

HPLC using UV-Vis or fluorescence detectors is considered a standard method for the determination of AAs previously derivatized with certain reagents in food samples offering acceptable specificity and sensitivity. In the future, the HPLC method using newly developed derivatizing agents offering simple and environmentally friendly must be developed and validated to support green analytical techniques.

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Table 2. Example of Application of HPLC for analysis of derivatized amino acids in food samples using certain derivatizing agents.

Samples	Derivatizing agents	HPLC condition	Detector	Results	References
Tea products (green, black, oolong, and white).	OPA + 3MPA	Agilent Zorbax Exclipe XDB- C_{18} column (5 µm, 250 mm×4.6 mm i.d., Agilent Technologies, U.S.A.). Mobile phase: two gradient phases, acetate buffer (pH 7.6) containing 3% THF [A] and a mixture of acetate buffer (pH 7.6): acetonitrile: methanol (1:2:2, v/v/v) [B]. The linear ranges of 0.5–50 nmol/L	Fluorescence detection at λ_{Ex} of 340 nm and λ_{Em} at 450 nm	HPLC method using this condition was valid as indicated by acceptable validation parameters and is successfully applied in tea products. The levels of AAs in tea are highly dependent on the tea type, grade, and quality	Kazan *et al*. (2019)
Fishes of mahi-mahi (*Coryphaena hippurus*) and yellowfin tuna (*Thunnus albacares*)	Dansyl chloride	Column: Kinetex® 1.3 µm C_{18} Column, 50 × 2.1 mm set at 30°C. Mobile phase: 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Mobile phase was delivered in gradient manner with flow rate of 0.5 mL/ min; 0 min 5% B; 1 min 5% B; 5 min 41% B; 11 min 68% B; 14 min 95% B; 14.5 min 95% B; 14.6 min 5% B; 17.5 min 5% B.	UV detector at a wavelength of 254 nm.	The method is valid for the quantification of amino acids in fishes as indicated by acceptable performance characteristics.	Bai *et al*. (2019)
Honey samples	2-(9-acridone)-ethyl chloroformate	Hypersil ODS C_{18} column (250 × 4.6 mm, 5 µm). Mobile phase was delivered in a gradient manner with a flow rate of 1 mL/min using four different eluents	Fluorescence detector at λ_{ex} and λ_{em} of 268 nm and 438 nm.	The method is valid with R2 > 0.99. Detection (LoD) and quantification (LoQ) limits of AAs using this method were in the range of 0.61–2.67 µg/ kg and 2.07–8.35 µg/ kg. This method is successfully applied for the detection of trace levels of AAs in honey samples	Gao *et al*. (2020)
Mead (a fermented alcoholic beverage made from honey)	PITC. The sample was added with water, sodium acetate TEA and PITC.	Column: Zorbax SB-Aq (150 × 3 mm, 3.5 µm particles). Mobile phases of [A] and [B] in gradient manner: 0 min –6 % B, 1 min –10 % B, 4 min –18 % B, 8.5 min –60 % B, 9 min –100 % B. [A] = 70 mM ammonium acetate dissolved in water (pH 6.55) [B] = 60% acetonitrile diluted in water. The flow rate was 0.6 mL/min, temperature 30 °C.	UV 254	The method was valid. The linearity evaluation gave an acceptable R^2 (> 0.99). LODs = 3.2–389.3 µg/L, and LOQs = 10.8-1297.6 µg/L. The percentage of recovery values of 106, 98, and 103% for the addition of standard solutions at concentrations of 5, 50, and 100 mg/L.	Klikarová *et al*. (2021)

OPA = o-phthaldialdehyde, 3MPA = 3-mercaptopropionic, PITC = Phenyl isothiocyanate, FMOC-Cl = 9-fluorenylmethylchloroformate, THF = Tetrahydrofuran, λ_{Ex} = excitation wavelength, λ_{Em} = emission wavelength.

Table 2. Example of Application of HPLC for analysis of derivatized amino acids in food samples using certain derivatizing agents.

Samples	Derivatizing agents	HPLC condition	Detector	Results	References
Natto (a traditional Japanese soybean food fermented with *Bacillus subtilis* var. natto)	DBD-trans-2-methyl-L-proline (DBD - M-Pro) by precolumn derivatization	Column: ACQUITY™ BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm, Waters) set at 40°C and mobile phase A was 0.05% FA in 10 mM ammonium solution; mobile phase B was 0.1% FA in CH_3CN. The whole analysis process was performed by gradient analysis in 20 min. The gradient steps were as follows 0–20 min from 18 to 40% solvent B, and the flow rate was 0.35 mL/min.	Fluorescence detector at λ_{ex} and λ_{em} 450 nm and 560 nm.	HPLC-fluorescence detector is valid and successful for analysis of AAs with a detection limit of 0.25 –2.5 pmol. The average recovery (%) of AAs was in the range of 86.65–118.70%.	Jin *et al.* (2021)

OPA = o-phthaldialdehyde, 3MPA = 3-mercaptopropionic, PITC = Phenyl isothiocyanate, FMOC-Cl = 9-fluorenylmethylchloroformate, THF = Tetrahydrofuran, λ_{Ex} = excitation wavelength, λ_{Em} = emission wavelength.

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