Original scientific paper
UDC 577.122.3:543.544.14

PRELIMINARY STEPS FOR VALIDATION AMINO ACIDS ANALYSIS BY ION CHROMATOGRAPHY WITH PULSED ELECTROCHEMICAL DETECTION

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Abstract

Amino acids analysis has applications in various fields such as; biochemistry, food science, microbiology, clinical studies and diagnostic studies. It is indispensable in the quantification of peptides and proteins as it is the only method to determine absolute protein quantities in solution. The aim of this study is represented by validation of a new, versatile technique, for separation and quantification of amino acids in food products, especially cereal based products, by ion chromatography with pulsed electrochemical detection.

Separation and quantification of amino acids were performed using ion chromatography with pulsed electrochemical detection and concentration gradient. NIST SRM 2389a standard solution mixture (17 amino acids) was used for conducting the experiments.

Different parameters such as linearity, repeatability, limit of detection and limit of quantification were preliminary determined in order to perform method validation. Linear regression was used for evaluating correlation coefficients that ranged between 0.8831 and 0.9999. The LODs ranged from 0.041 to 0.283 µmol/L; the LOQs ranged from 0.137 to 0.944 µmol/L. The RSD values for all intra-day repeatability were less than 10% and ranged between 1.48 and 9.71%.

Ion chromatography with pulsed electrochemical detection is a viable technique for separation and quantification of amino acids in particular due to the fact that it does not require pre or post column derivatization. Preliminary steps performed in order to validate the method are promising: good linearity and correlation coefficients and low LOD and LOQ.

***Key words**: Amino acids, Method validation, Pulsed electrochemical detection, Ion chromatography.*

1. Introduction

Amino acids are organic compounds, biologically important, containing two functional groups: amine (-NH_2) and carboxyl (-COOH) and a side chain, different and specific to each amino acid [1].

About 500 amino acids are known (though only 20 appear in the genetic code) and can be classified in many ways [2]. Amino acids are involved in many essential physiological processes and one of the most important of these processes is represented by the construction of peptides and proteins [3].

The key elements of an amino acid are carbon (C), hydrogen (H), oxygen (O), and nitrogen (N), although other elements are found in the side chains of certain amino acids (Figure 1). In the construction process of peptides and proteins only 20 amino acids participate, each with different chemical properties [4].

According to SR EN ISO/CEI 17025:2005, validation represents the confirmation by examination and provision of objective evidence that the requirements for intentional use are met.

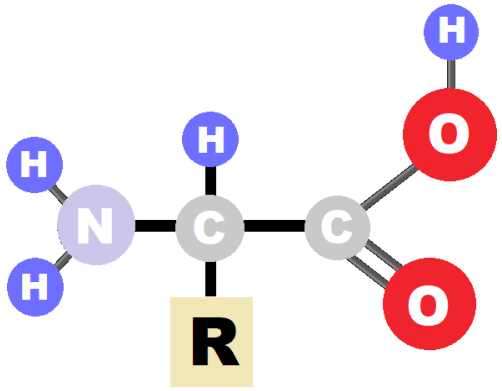

Figure 1. Amino acid structure

The information used to validate a method can be obtained by laboratory testing or by other sources (information provided by reagent/equipment manufacturer, literature, method standards, etc.).

Depending on the purpose of validation, the following parameters can be evaluated: selectivity and specificity, linearity, accuracy, repeatability, intermediate precision, reproducibility, detection limit, quantification limit, recovery, robustness (Figure 2).

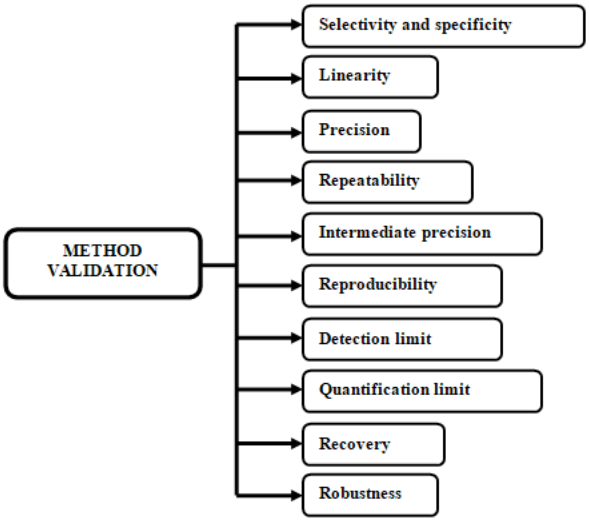

Figure 2. Parameters evaluated in method validation

Analytical methods for detecting and analyzing amino acids are numerous: LC/HPLC-MS [5 - 8], GC-MS [9 - 10], NMR [11 - 12] and others. Most of these mentioned techniques involve pre or post-column derivatization.

This study aims to validate other analytical technique for amino acids analysis, namely ion-chromatography with pulsed electrochemical detection, which does not need derivatization. Preliminary steps for validation of the method were performed and the most important parameters were analyzed.

2. Materials and Methods

2.1 Reagents

The standard solution mixture SRM 2389a, containing: alanine (Ala), arginine (Arg), aspartic acid (Asp), cystine (Cys), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), valine (Val) in 0.1 mol/L hydrochloric acid, was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, USA). Norleucine (N1398) and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Deionized water (18 MΩ·cm), sodium hydroxide (50% w/w) and sodium acetate were purchased from Fisher Scientific (Hampton, USA).

2.2 Standard solutions

4 mM stock solution of norleucine (524.8 mg/L) was prepared in 0.1 M HCl then diluted 1 : 500 with a deionized water solution containing ca. 20 mg/L of sodium azide (NaN_3). This diluent solution is stable for month if stored in a refrigerator and was used to dilute aliquots of SRM 2389a in order to obtain: 1 µM, 2 µM, 3 µM, 4 µM and 5 µM standard solutions for calibration. The prepared standard solutions will remain stable for weeks, if stored in a refrigerator. The trace of NaN_3 introduced with the diluents solution stabilizes standards for up to 48 hours at room temperature.

2.3 Equipment

An ICS-3000 System (Dionex) including dual pump system, electro-chemical detector, autosampler, eluent organizer, dionex chromeleon work station and Chromeleon software (version 6.80) loaded on computer was used.

The analytical column was an AminoPAC PA10 column, packed with a hydrophobic, polymeric, anion exchange resin stable over the range of pH 0 - 14.

Resin characteristics: particle size - 8.5 µm; pore size - microporous (< 10 Å); ion exchange capacity - 60 µ equivalents/column (2 x 250 mm).

Latex characteristics: functional group - alkyl quaternary ammonium ions; latex diameter: 80 nm. Typical operating parameters: pH range = 0 - 14; temperature limit: 40 ^{0}C; pressure limit: 4000 psi; organic solvent limit: 100% acetonitrile, methanol, (acetone if required for cleaning).

3. Results and Discussions

To determine the linearity of the 17 types of amino acids, five different concentrations of standards were used: 1, 2, 3, 4 and 5 µmol/L (except Cys). Each standard solution was injected 10 times. Linear regression was used for evaluating correlation coefficients that ranged between 0.8831 (for Glu) and 0.9999, as presented in Table 1.

The limit of detection (LOD) was estimated using the following formula: SD/b·3.3, where SD and b represent the standard deviation of the intercept and slope of the regression line, respectively. For the limit of quantification (LOQ) was used the following formula: SD/b·10, where SD and b are the same as above. For estimating LOD and LOQ each solution was injected 10 times. The results are presented in Table 2. LODs ranged from 0.041 µmol/L to 0.283 µmol/L and LOQs ranged from 0.137 µmol/L to 0.944 µmol/L.

Table 1. Linearity and regression analysis

Amino acid	Retention time (min.)	Linearity range (µmol/L)	Regression equation	Correlation coefficient (r^2)
Arg	1.86	1 – 5	y = 10.4087 + 1.3072·x	0.9999
Lys	3.08	1 – 5	y = 7.0541 + 1.8145·x	0.9937
Ala	5.36	1 – 5	y = 3.9984 + 0.2329·x	0.9995
Thr	5.66	1 – 5	y = 12.5733 - 0.4013·x	0.9998
Gly	6.22	1 – 5	y = 4.7050 + 0.1036·x	0.9998
Val	6.78	1 – 5	y = 3.0677 - 0.0174·x	0.9999
Ser	8.29	1 – 5	y = 22.3058 - 0.0620·x	0.9993
Pro	9.97	1 – 5	y = 3.2030 - 0.0708·x	0.9962
Ile	10.65	1 – 5	y = 2.9137 - 0.0853·x	0.9993
Leu	11.29	1 – 5	y = 10.4087 + 1.3072·x	0.9975
Met	12.33	1 – 5	y = 2.8796 + 7.7565·x	0.9961
His	20.57	1 – 5	y = 10.5347 + 11.5441·x	0.9795
Phe	21.35	1 – 5	y = 8.8559 + 1.3480·x	0.9957
Glu	21.95	1 – 5	y = 0.4546 + 0.9967·x	0.8831
Asp	23.08	1 – 5	y = 2.5156 - 0.2570·x	0.9969
Cys	24.13	0.5 – 2.5	y = 21.7580 + 0.6249·x	0.9996
Tyr	26.86	1 – 5	y = 9.4142 + 1.9788·x	0.9995

Table 2. LOD and LOQ

Amino acid	LOD (µmol/L)	LOQ (µmol/L)
Arg	0.077	0.258
Lys	0.087	0.291
Ala	0.171	0.570
Thr	0.283	0.944
Gly	0.074	0.248
Val	0.121	0.403
Ser	0.050	0.167
Pro	0.158	0.528
Ile	0.169	0.564
Leu	0.118	0.393
Met	0.201	0.670
His	0.222	0.740
Phe	0.041	0.137
Glu	0.106	0.353
Asp	0.195	0.650
Cys	0.054	0.180
Tyr	0.048	0.161

Table 3. Intra-day repeatability and trueness

Amino acid	Concentration (µmol/L) (*)	SD (µmol/L)	RSD (%)
Arg	4.33	0.13	2.38
Lys	4.97	0.15	2.93
Ala	4.67	0.29	1.42
Thr	4.86	0.47	9.71
Gly	4.90	0.12	2.53
Val	4.74	0.20	4.24
Ser	4.64	0.08	1.79
Pro	4.70	0.26	5.62
Ile	4.63	0.28	6.10
Leu	4.96	0.20	3.97
Met	5.39	0.33	6.21
His	4.01	0.37	9.24
Phe	4.62	0.07	1.48
Glu	4.31	0.18	4.10
Asp	4.42	0.32	7.75
Cys	2.41	0.05	1.87
Tyr	4.12	0.08	1.95

(*) Average values obtained from 10 injection of a 5 µmol/L. SRM 2389a solution (2.5 µmol/L for Cys).

The repeatability (intra-day) was also calculated by injecting 10 times a 5 µmol/L solution (SRM 2389a). Average obtained concentrations as well as SDs and RSDs are shown in Table 3. RSD values for all intra-day repeatability were less than 10% and ranged between 1.42% and 9.71%.

4. Conclusions

- Ion chromatography with pulsed electrochemical detection is a viable technique for separation and quantification of amino acids in particular due to the fact that it does not require pre or post column derivatization.

- Preliminary steps performed in order to validate the method are promising: good linearity and correlation coefficients and low LOD and LOQ. The studies will be further conducted to analyze other important parameters in method validation such as reproducibility, recovery and robustness.

Acknowledgements

This work was performed through the NUCLEU Program, carried out with the support of the Ministry of Research and Innovation, the project no PN 16 46 01 06.

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