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Method Optimization and Separation of Aromatic Biogenic Amines and Precursor Amino Acids by High Performance Liquid Chromatography

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Abstract

In this study, the determination of aromatic biogen amines; histamine, β-phenyl ethylamine, tyramine and tryptamine, and their precursor amino acids; histidine, phenylalanine, tyrosine and tyriptophan was performed without using any derivatization reagents. Mobile phase was optimised by establishing relationships between retention parameters and Reichardt's E_T^N scale of solvent polarity. In addition, selectivity (α) and resolution (R_S) values were used in order to establish a general model relating elution behaviour of substances with the composition of mobile phase. In order to find the optimum pH value, pK_a values of amino acids in the mobile phase were determined by potentiometric method in 20% and 30% (v/v) MeCN media. For biogenic amines and amino acids studied, the optimised mobile phase was composed of acetonitrile-water and 6.10^{-3} mol l^{-1} 1-heptanesulphonic acid sodium salt adjusted at pH 2.45 with a percentage of MeCN-water of 28% (v/v). .

Key Words: Biogenic amines, LC, Optimisation of mobile phase, Reichardt's E_T^N scale of solvent polarity

Introduction

Biogenic amines and their precursor amino acids are aliphatic, alicyclic and heterocyclic organic bases of low molecular mass that are formed as a result of metabolic process in animals, plants and micro-organisms. Histamine, tyramine, tryptamine, β-phenylethylamine are considered to be important aromatic biogenic amines occurring in foods. Biogenic amines may also be considered as carcinogens because of their ability to react with nitrites to form potentially carcinogenic nitrosamines [1,2]. The importance of determination of biogenic amines in foods have received crucial attention in recent years due to the possibility of using amine concentration as an index of food quality [3-5], as the original concentration levels of biogenic amines can be changed during food processing and storage and are influenced by the hygienic conditions employed.

In order to establish separation methodologies and determine biogenic amines and precursor amino acids, liquid chromatography (LC) is the method of choice because of its versatility, precision and sensitivity, and results are obtained in a reasonable time. Due to their important roles in human health and ubiquity in foodstuffs, various reversed phase high-performance liquid chromatographic (RP-HPLC) methods have been developed for determining biogenic amines in fish products, cheese varieties, meat and meat products, and fermented foods [6-8]. Although the methods are usually based on pre- or post column derivatization of amines using several derivatization reagents [9-11], the chromatographic separation of aromatic biogenic amines and their precursor amino acids can be performed using UV detection without using any derivatization agent. The detection methodology that does not require derivatization is preferred for convenience and simplicity.

The two most useful optimization parameters for ionogenic solutes in LC are the polarity and the pH of the mobile phase. The approach for optimizing the organic modifier concentration in the mobile phase during chromatographic separation of aromatic biogenic amines and precursor amino acids has been made by establishing a relationship between the retention parameters and Reichardt's E_T^N scale of solvent polarity [12]. Moreover, pH of the mobile phase is a major factor that affects the chromatographic behavior of biogenic amines and precursor amino acids. Because they contain ionogenic functions such as carboxylic, amino and hydroxylic groups.

The retention of the solutes in LC depends on the degree of ionized and non-ionized species of each compound. Thus, knowledge of the acid-base dissociation constants of studied compounds in acetonitrile (MeCN)-water binary mixtures, which are used as mobile phase, can help to improve the analytical method and would lead to a better understanding of the behavior of studied compounds.

In this work, the proportion of organic modifier of the hydro-organic mobile phase was optimized in order to separate eight compounds which were four biogenic amines and their precursor amino acids, namely tyramine-tyrosine, histamine-histidine, β-phenylethylamine-phenylalanine and tryptamine-tryptophan. The components were detected by using their natural absorbances in the UV region. The relationships between the retention parameters (k) of the compounds and Re-

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ichard's E_T^N scale of solvent polarity were used to optimize the proportion of organic modifier in the mobile phase. In order to find the optimum pH value, the pK_a values of amino acids in 20 and 30 %(v/v) MeCN-water mixtures were determined with using potentiometric method according to the rules and procedures endorsed by IUPAC [13,14]. The dissociation constants determined correspond to the dissociation of the terminal carboxylic and protonated amino groups of the compounds studied.

Materials and Methods

Chemicals and Reagents

Analytical reagent grade chemicals were used, unless otherwise indicated. Histidine, phenylalanine, tyrosine, tyrptophan, histamine, tyramine, β-phenyletilamine and tryptamine (Figure 1a, b) purchased from Sigma (MO, USA) and used without further purification. Water, with conductivity lower than 0.05 μS cm^{-1}, was obtained with a Milli-Q water purification system (Millipore Corp. Billerica, USA). MeCN (HPLC grade), sodium hydroxide, potassium hydrogen phthalate (dried at 110 °C before use), hydrochloric acid were supplied by Merck (NJ, USA). 1-heptansulphonic acid sodium salt as ion pair reagent was purchased from Fluka (MO, USA). Potassium hydroxide, potassium chloride were also purchased from Merck (NJ, USA) and were used in potentiometric studies. Potassium hydroxide solutions (0.025 mol l^{-1}) were prepared in each MeCN–water mixture from 1.000 mol l^{-1} potassium hydroxide solution (Titrisol, Merck) by dilution. Hydrochloric acid solutions were prepared by dilution from 1.000 mol l^{-1} hydrochloric acid (Titrisol, Merck) in each MeCN-water mixture. The ionic strength was adjusted at 0.1 mol l^{-1} with potassium chloride.

Apparatus

Chromatographic equipment consisted of a Shimadzu Model LC 10 ADVP pump with an auto injector (SIL 10 ADVP) and a diode-array detector system (SPDM 10 A DAD) was used for chromatographic separation. This equipment has a column oven (CT 10 AVP) and a degasser system (DGU 14 A). A YMC-Pack ODS-AM column (250 × 4.6 mm I.D., 5 μm) was used at 25 °C.

The e.m.f measurements to evaluate the pH of the mobile phase were performed with a model Metleer-Toledo MA 235 pH/ion analyzer with Hanna HI 1332 Ag/AgCl combined pH electrode system (±0.1 mV). The measurements were performed in triplicate to ensure stability and reproducibility of the potentiometric system. While adjusting the pH, solutions were thermostated externally at 25±0.1 °C. Potassium hydrogen phthalate solutions (0.05 mol kg^{-1}) prepared in appropriate MeCN–water binary mixtures were used as primary standard buffer references [15,16].

In order to obtain the pK_a values using potentiometric technique, potential values of the potentiometric cell were measured with the same pH meter. Triplicate titrations of each system were performed. The glass electrode was stored in water when not in use and soaked for 20-25 min in MeCN-water mixture before potentiometric measurements. The stabilization criterion for the potential readings was 0.2 mV within 120 s. In all instances, the electrode system gave stable and reproducible potentials within 5 min. The cell was thermostated externally at 25 ^{0}C ± 0.1 with a cooler system water bath (Heto CBN 8-30) and temperature control unit (Heto HMT 200) and the test solution was stirred magnetically under a continuous stream of purified nitrogen. All titrations were carried in double-walled and thermostated vessel.

Chromatographic procedure and measurements

Stock standard solutions of biogenic amines and their precursor amino acids were prepared in water at concentrations of approximately 1000 mg l^{-1}. Working solutions were diluted with the corresponding mobile phase before injections. Stock standard solutions were stored in deep freezer and diluted working solutions were stored at 4 °C.

In this study, the mobile phases assayed were MeCN–water (26-30 (v/v)) with 6.10^{-3} mol l^{-1} 1-heptansulphonic acid sodium salt as ion pair reagent. The pH of the mobile phase was adjusted to 2.45 with hydrochloric acid. The flow-rate was maintained at 0.8 ml/min. For each compound and for every mobile phase composition, the retention time values, t_R , were determined from three different injections. The retention factors were established for each mobile phase composition and pH studied. A wavelength of 215 nm was selected as being optimal for the simultaneous determination of all eight compounds.

Potentiometric procedure

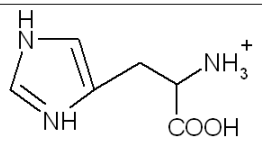
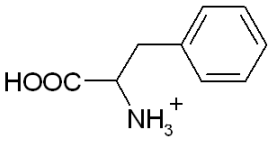
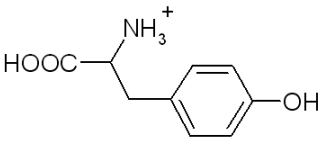
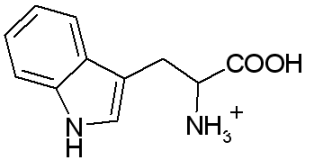
The pK_a values were determined by titration of the appropriate solution of amino acids (3 mM) in 20 and 30 %(v/v) MeCN-water mixtures, using KOH solution as titrant, in 0.1 mol l^{-1} ionic strength (KCl) according to the criteria endorsed by IUPAC [13,14].

The standardisation of the electrode system was carried out, each time in MeCN-water binary mixtures studied by Gran's method [17,18]. The potential was allowed to stabilize after each addition of acid and the potential values obtained were used to calculate the standard potential of the cell, E^0. Usually, about 10 or 12 additions to background solution were enough for verify E^o to be accurately determined. The calculation of pK values requires an iterative cycle for each point of the potentiometric titration at which e.m.f. was measured and were carried out by making use of the program written in PASCAL, PKPOT [19].

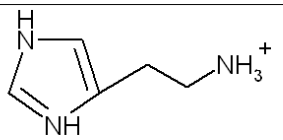
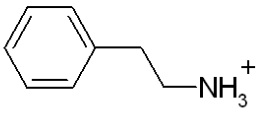
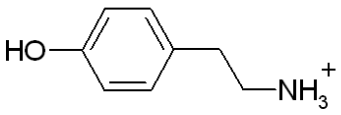
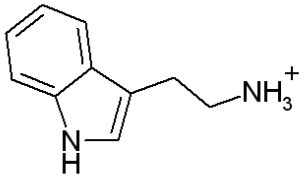
Results and Discussion

In this study, the dissociation constants of amino acids (Figure 1) in MeCN-water binary mixtures, which are used as mobile phase, were determined to establish the appropriate pH of the mobile phase. As an example for potentiometric studies, series of measurements for titra-

tion of histidine and phenylalanine in 30% and 20% (v/v) MeCN using KOH solution in the same solvent as titrant respectively are shown in Figure 2a and 2b. The symbols indicate experimental values and solid line indicates the theoretical titration curve in the figures.

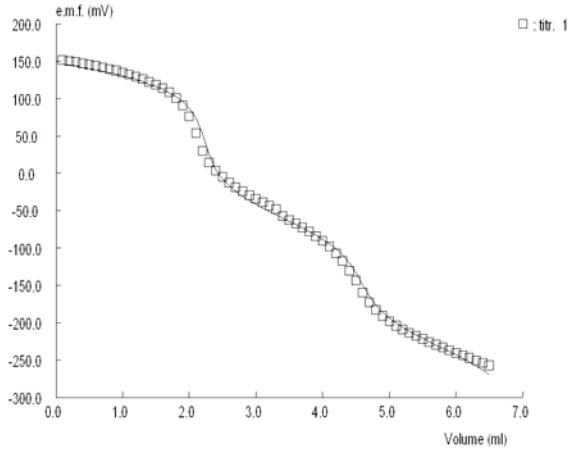
Compounds name	Structure
Histidine	
Phenylalanine	
Tyrosine	
Tryptophan	

(a)

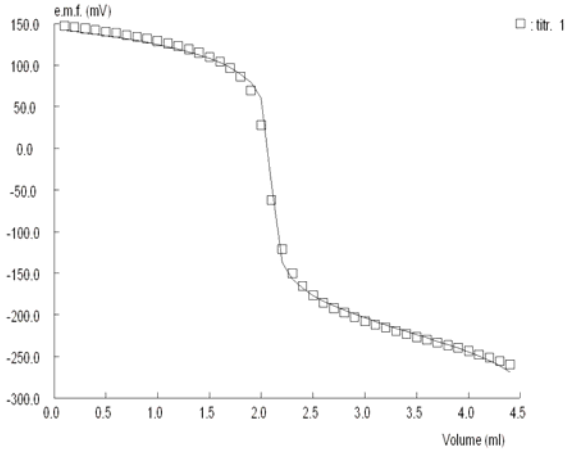
Compounds name	Structure
Histamine	
β-Phenylethylamine	
Tyramine	
Tyriptamine	

(b)

Figure 1. a) Structural formulae of studied amino acids; b) Biogenic amines



(a)



(b)

Figure 2. a) Plot of potentiometric measurement for histidine in 30% (v/v) MeCN. b) Plot of potentiometric measurement for phenylalanine in 20% (v/v) MeCN. Solid lines indicate the theoretical values calculated by PKPOT.

Table 1. The pKa values of amino acids obtained by potentiometric method in MeCN-water media.

Compounds	pK_a	
	20 % (v/v) MeCN	30 % (v/v) MeCN
Histidine	2.85 (0.03) 5.97 (0.07) 9.14 (0.05)	3.00 (0.09) 6.07 (0.02) 9.22 (0.08)
Tyrosine	3.04 (0.02) 8.88 (0.09) 9.50 (0.03)	3.15 (0.03) 9.15 (0.10) 10.91 (0.13)
Phenylalanine	3.03 (0.03) 8.74 (0.09)	3.14 (0.03) 8.96 (0.06)
Tryptophan	3.16 (0.01) 8.89 (0.13)	3.24 (0.01) 9.11 (0.02)

Table 1 shows the dissociation constants of amino acids studied obtained by the potentiometric method using the PKPOT program [19] in 20% and 30% (v/v) of MeCN and the values in parenthesis are standard deviations. The first pK_a values correspond to carboxylic acid group dissociation while the second corresponds to protonated amino groups dissociation.

Potentiometric results showed that when pH<3 all these compounds were in protonated forms hence pH of the mobile phase was adjusted to 2.45 taking care of limiting pH values of the columns.

Nowadays to optimize the chromatographic separation by using a few experimental data, criteria that have to be evaluated to achieve the good separation and minimum analysis time are retention factor, k, resolution, R_S, and selectivity, α. The normalized scale of solvent polarity was shown a significantly better linearity than similar plots against the volume fraction of organic modifier and proposed by Reichardt [12]:

$$\log k = C + eE_T^N \quad (1)$$

Retention factor values for biogenic amines and their precursor amino acids studied were obtained experimentally in MeCN-water binary mixtures which values are in the range of 0.880-0.867 (26-30% (v/v) MeCN).

The correlation between the experimental log k values of the solutes studied over the whole experimental range of MeCN contents and the E_T^N values of the mobile phases are shown in Table 2.

Table 2. Relationships between log k for studied compounds and E_T^N parameters, with correlation coefficients (r).

Compounds	Equation	r
Histidine	$\log k = 22.609E_T^N - 20.085$	0.991
Tyrozine	$\log k = 17.937E_T^N - 15.871$	0.988
Tyramine	$\log k = 20.802E_T^N - 18.199$	0.978
Phenylalanine	$\log k = 21.955E_T^N - 19.087$	0.984
Tryptophan	$\log k = 26.699E_T^N - 23.031$	0.982
β-Phenylethyl amine	$\log k = 27.515E_T^N - 23.689$	0.999
Tyriptamine	$\log k = 32.034E_T^N - 27.493$	0.999
Histamine	$\log k = -48.241E_T^N + 42.493$	0.905

According to Eq. (1) logarithms of the retention factor correlate linearly (r > 0.98) with the polarity of the mobile phase for the studied compounds except histamine (Figure 3).

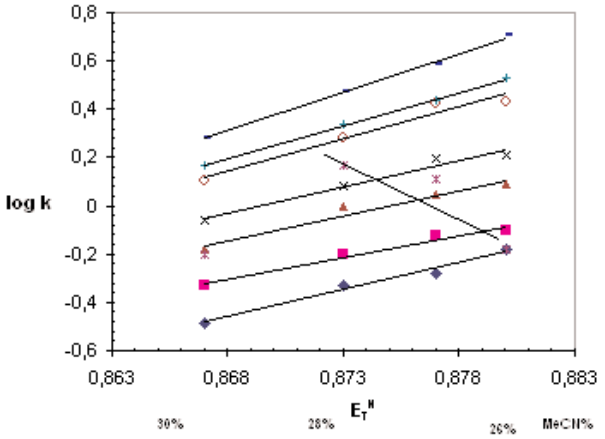


Figure 3. Plot of normalized scale of solvent polarity vs. percentage of MeCN. Symbols: •: Histidine, ■ : Tyrosine, ▲: Tyramine, x : Phenylalanine, * : Histamine, o: Tryptophan, + :β-Phenylethylamine, -:Tryptamine. Experimental conditions: mixtures containing acetonitrile-water at different percentages, containing 6.10^{-3} mol l^{-1} 1-heptansulphonic acid sodium salt as ion pair reagent, and pH adjusted to 2.45.

The structural features of MeCN-water mixtures, explored by Marcus and Migron [21], show three regions which are water-rich, microheterogeneity and MeCN-rich medium. The limit of MeCN molar fraction, X_{MeCN}, for water-rich medium is ≤ 0.1 that corresponds to approximately 28% (v/v). In this work, the linear correlation between log k versus were obtained up 30% (v/v) MeCN for the studied seven compounds. Histamine is the most sensitive compound by the polarity of the mobile phase and the behavior of the compound is different than the others. The slope of the straight line (up to 28%) is greater than the others but correlation coefficient is smaller. Changing the proportion of MeCN (even 1%) caused the change of the elution order of the compounds and gave twin peaks. Retention times of histamine were dramatically influenced by the polarity of the mobile phase.

The selectivity for adjacent solute pairs of the studied compounds was calculated in the usual way, $\alpha = k_i / k_j$. The selectivity between adjacent pairs of compounds versus percentage of MeCN in the mobile phase is shown in Figure 3. As can be observed from Figure 3, there is a good concordance of both selectivity values at 28% (v/v) MeCN over the whole MeCN range for all compounds studied.

Capacity factors (log k) and selectivity factors (α) obtained for biogenic amines and precursor amino acids studied with the mobile phase of 28% (v/v) MeCN are given in Table 3.

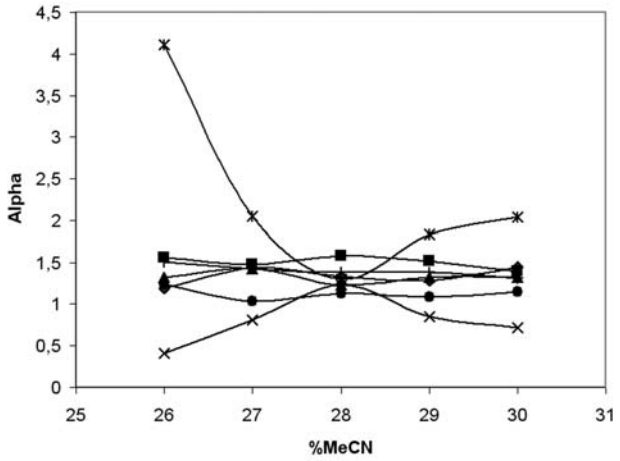


Figure 4. Plot of selectivity, α, vs. percentage of MeCN for pairs of studied compounds. Symbols: •:Tyrosine/Histidine, ■:Tyramine/ Tyrosine, ●: Phenylalanine / Tyramine, X:Histamine / Phenylalanine, +: Tryptophan/Histamine, ▲: β-Phenylethylamine/Tryptophan, *:Tryptamine/β-Phenyl ethylamine. Experimental conditions as in Figure 3.

Table 3. Chromatographic capacity factors, selectivity and resolutions of studied compounds at 28% (v/v) MeCN.

Compound	k	log k	α	R_S
Histidine	0.469	-0.329	-	-
Tyrosine	0.625	-0.204	1.333	1.029
Tyramine	0.988	-0.005	1.581	3.285
Phenylalanine	1.210	0.083	1.225	1.446
Histamin	1.482	0.171	1.225	1.420
Tryptophan	1.924	0.284	1.298	4.214
β-Phenylethylamine	2.165	0.335	1.125	2.574
Tryptamine	2.986	0.475	1.379	4.762

Achieving a good resolution between all of the analytes of interest is the main goal of a chromatographic separation. In terms of fundamental chromatographic parameters, the resolution between two adjacent peaks, Rs , is given by:

$$R_S = (1/4)(N)^{1/2} \quad [(\alpha - 1)/\alpha] \quad [k/(1+k)] \qquad (2)$$

Efficiency Selectivity Retention

where N is the number of theoretical plates. Although the selectivity term is generally regarded as the most important in LC, full attention must be given to all of the terms in Equation (2). Also, Table 3 shows the variation of Rs for adjacent solute pairs for 28% (v/v) MeCN.

The separation of ionizable compounds (such as amines and amino acids) by reversed – phase HPLC can be achieved by means of ion-pair chromatography. Ion pair reagents have long been known and used for their ability to change selectivity and increase retention of polar compounds on reversed-phase (RP) analytical columns. In this study alkanesulfonate salt (RSO_3^-) was chosen as ion pair agent and the pH of the mobile phase should be adjusted to lower than 3 to protonate amine groups of the amino acids.

Figure 5 shows a chromatogram of the studied compounds in a mobile phase of MeCN – water with 28% (v/v) organic modifier at pH 2.45. Under the chosen conditions a good separation was obtained with an analysis time of 12 min. The best peak shape and resolution is obtained when 6.10^{-3} mol l^{-1} 1-heptansulphonic acid sodium salt was used. The elution order was histidine, tyrozine, tyramine, phenylalanine, histamine, tryptophan, β-phenylethylamine and tyriptamine.

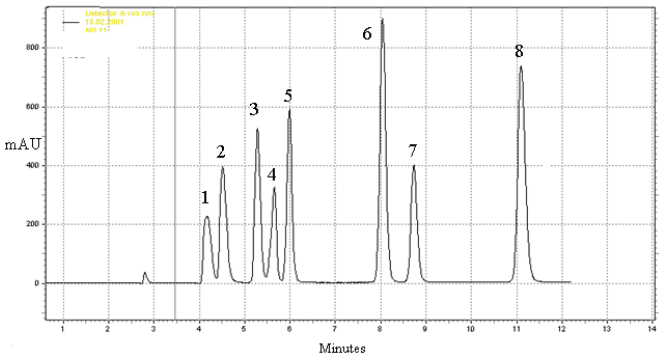


Figure 5. Chromatogram of a standard mixture of aromatic biogenic amines and their precursor amino acids, with a mobile phase of MeCN-water (28:72, v/v) containing 6.10^{-3} mol l^{-1} 1-heptansulphonic acid as ion pair reagent, with the pH adjusted to 2,45 with HCl. Histidine (1), tyrozine (2), tyramine (3), phenylalanine (4), histamine (5), tyriptophan (6), β-phenylethylamine (7), tryptamine (8).

Conclusion

The separation of eight compounds of aromatic biogenic amines and their precursor amino acids has been performed by RP-HPLC in isocratic mode without using any derivatization reagent. The mobile phase consists of MeCN-water (28:72, v/v) containing 6.10^{-3} mol l^{-1} 1-heptansulphonic acid sodium salt as ion pair reagent, and the pH adjusted to 2.45 with HCl. This optimum condition can be used for adequate separation for quantitative analysis of biogenic amines and their precursor amino acids in fer-

mented foods and beverages applying the appropriate extraction methodologies.

Acknowledgments

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References

1. Shalaby, A.R., Significance of biogenic amines to food safety and human health, Food Res. Int., 29, 675-690, 1996.
2. Armağan, Ö., A review: Current analytical methods for the determination of biogenic amines in foods, Food Chemistry, 103, 1475–1486, 2007.
3. Edwards, R.A., Dainty, R.H., Hibbard, C.M., Ramantanis, S.V., Amines in fresh beef of normal pH and the role of bacteria in changes in concentration observed during storage in vacuum packs at chill temperatures. J. Appl. Bacteriol., 63, 427-434, 1987.
4. Lange, J., Thomas, K., & Wittmann, C., Comparison of a capillary electrophoresis method with high-performance liquid chromatography for the determination of biogenic amines in various food samples. J. Chromatogr. B., 779, 229–239, 2002.
5. Innocente, N., Biasutti, M., Padovese, M., Moret, S., Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract, Food Chemistry, 101, 1285–1289, 2007.
6. Loukou, Z., and Zotou, A., A Comparative Survey of the Simultaneous Ultraviolet and Fluorescence Detection in the RP-HPLC Determination of Dansylated Biogenic Amines in Alcoholic Beverages, Chromatographia, 58, 579-585, 2003.
7. Vidal-Carou, C., Lahoz-Portolés, F., Bover-Cid S., and Mariné-Font A., Ion-pair high-performance liquid chromatographic determination of biogenic amines and polyamines in wine and other alcoholic beverages, J. Chromatogr. A. 998, 235-241, 2003.
8. Lozanov, V., Petrov, S., and Mitev, V., Simultaneous analysis of amino acid and biogenic polyamines by high-performance liquid chromatography after precolumn derivatization with N-(9-fluorenylmethoxycarbonyloxy) succinimid, J.Chromatogr. A. 1025, 201-208, 2004.
9. Levin, S., and Grushka, E., Reversed-phase liquid chromatographic separation of amino acids with aqueous mobile phases containing copper ions and alkylsulfonates, Anal. Chem., 57, 1830-1835, 1985.
10. Walker, T.A., Pietrzyk, D.J., Ion-interaction chromatographic separation of free amino acids, J. Liquid Chromatogr.10 (1), 161-174, 1987.
11. Hajos, G., Sass-Kiss, A., Szerdahelyı, E., Bardocz, S., Changes in biogenic amine content of Tokaj Grapes, wines and Aszu wines, J. Food Sci., 65 (7), 1142-1144, 2000.
12. Reichardt, C., Solvent and Solvent Effects in Organic Chemistry, VCH, Weinheim. 1988.
13. Rondinini, S., Mussini, P.R., Mussini, T., References value standard and primary standard for pH measurements in organic solvents and water-organic solvents mixtures of moderate to high permittivities, Pure Appl. Chem., 59, 1549-1560, 1987.
14. Mussini, P.R., Mussini, T., Rondinini, S., Reference value standards for pH measurements in D2O and aqueous–organic solvent mixtures: New accessions and assessments, Pure Appl. Chem.69, 1007-1014, 1997.
15. Barbosa, J., Sanz-Nebot, V., Preferential solvation in acetonitrile-water mixtures. Relationship between solvatochromic parameters and standard pH values. J. Chem. Soc. Faraday Trans., 90, 3287-3292, 1994.
16. Barbosa, J., Marqués, I., Barrón, D., Sanz-Nebot, V., The application of factor analysis to solvatochromic parameters and pHs values for the standardization of potentiometric sensors in mobile phases used in liquid chromatography, TRAC Trends Anal. Chem., 18, 543-549, 1999.
17. Gran G., Determination of the equivalence point in potentiometric titration. Part II. Analyst, 77, 661-665, 1952.
18. Gameiro, P., Reis, S., Lima, J.L.F.C., De, Castro B., Calibration of pH glass electrodes by direct strong acid/strong base titrations under dilute conditions, Anal. Chim. Acta, 405, 167-172, 2000.
19. Barbosa, J., Barron, D., Beltran, J.L., Sanz-Nebot, V., PKPOT A program for the potentiometric study of ionic equilibria in aqueous and non-aqueous media, Anal. Chim. Acta.317, 75-81, 1995.
20. Marcus, Y., and Migron, Y., Polarity, hydrogen bonding, and structure of mixtures of water and cyanomethane. J. Phys. Chem., 95, 400-406, 1991.