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ORIGINAL PAPER

Isocratic micellar liquid chromatography using mixed anionic and non-ionic surfactants as mobile phase additives for separation of 17 free amino acids

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Abstract
In this work, an isocratic micellar chromatographic method for separation of 17 free amino acids was first established. Mixed anionic and non-ionic surfactants were used as mobile phase additives. Besides, a chromatographic environment was simulated and the possible mechanism was discussed. Pre-column derivatization with 9-fluorenylmethyl chloroformate was used before chromatographic analysis. The optimized chromatographic conditions were the mobile phase A (0.075 M sodium dodecyl sulfate solutions and 0.010 M polyoxyethylene lauryl ether containing 20 mM ammonium acetate at pH 3.5) and B (100% acetonitrile) (85:15, v/v) running at 1.2 mL/min by a Venusil XBP C18 column (5 μm, 250×4.6 mm) at 35.0 °C. Compared with the conventional reversed phase liquid chromatography, the consumption of organic solvent in the method is lower which reduces the analysis cost and being environment-friendly. In addition, complex gradient elution is not required. This provides an alternative way for the separation of amino acids.

Keywords Amino acids · Micellar liquid chromatography · Mixed surfactants · Polyoxyethylene lauryl ether · Sodium dodecyl sulfate

Introduction

Micellar liquid chromatography (MLC) is a kind of reversed phase liquid chromatography that uses aqueous solutions of surfactants above critical micellar concentration (CMC) and low organic solvent content (Ruiz-Ángel 2011; Dong et al. 2015; Ramezani et al. 2018). The addition of surfactants makes it distinguish from the traditional organic solvent–water system, with the advantages of low toxicity, low cost, high efficiency, direct injection of biological samples without deproteination and gradient elution (means surfactant concentration gradient) without the column re-equilibration (Nakao et al. 2012). Because of these factors, MLC is known as "green chromatography" (El-Shaheny et al. 2015). The surfactant monomers are adsorbed on the surface of the nonpolar stationary phase, producing a charged or uncharged layer (depends on the types of surfactant) that modifies its properties; meanwhile, the monomers in the mobile phase integrate to micellar clusters, changing the elution strength of mobile phase (El-Shaheny et al. 2015). The variety of interactions that occurs in the column complicates the retention mechanism and provides a high versatility to MLC, allowing the resolution of mixtures of solutes with different charges and hydrophobicity values using an isocratic elution (Albiolchiva et al. 2018). Some surfactants including anionic, cationic and non-ionic surfactants can be used in MLC to develop analytical methods, being the anionic sodium dodecyl sulfate (SDS) the most common (Ruiz-Ángel et al. 2014). SDS monomers are adsorbed on the surface of the nonpolar stationary phase, producing a negatively charged layer that modifies its properties. Besides, there are few reports in which polyoxyethylene lauryl ether (Brij-35, non-ionic surfactant) is used for

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the separation of compounds (Memon et al. 2005; Dhote et al. 2013; Dong et al. 2015; Peris-García et al. 2016). The combination ways between Brij-35 and the stationary phase is similar to that of SDS. Moreover, Brij-35 can increase the polarity of stationary phase without bringing any extra charge. Effective separation of those compounds can be obtained in the system.

Mixed surfactants are widely used in the field of physical chemistry at the beginning (Angarska et al. 2004; Rangel-Yagui et al. 2005). In recent years, the system has also been applied to develop MLC and micellar electrokinetic chromatography (MEKC) analysis methods. One of the most widely used combinations is SDS and Brij-35 (Ebrahimi and Hadjmohammadi 2006; Ruiz-Ángel et al. 2015; Nakao and Halldin 2013). When the two kinds of surfactants are added to the mobile phase, hydrophobic interactions will exist between their hydrophobic tails, ion–dipole interactions will exist between head groups and hydrophilic interactions between the hydrophilic groups (Ruiz-Ángel et al. 2015). This forces the formation of mixed micelles which is different from simple summation of the two single systems. The hybrid system may improve the separation effectively due to the combination of advantages possessed by both of the single ones. It has also been reported that SDS micelles were used and the amino acids were fluorescently derivatized prior to injection. This method is more sensitive, but they detect fewer amino acids and the measurement process is susceptible to environmental interference (Chiu and Chang 2007; Sueyoshi et al. 2011; Chiu and Tai-Chia 2013).

Amino acids (AAs) are the main constituent of proteins and the organic molecules in the body that play an important role in various biological functions (Moughan et al. 2014). The analysis of AAs has been a hot topic in the field of analytical chemistry. However, AAs (except for three with benzene ring structures) have no UV absorption because of the absence of chromophores (Ziegler and Abel 2014). To make amino acids be detected effectively and improve the sensitivity of high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) method, derivatization is routinely used (Jambor and Molnar-Perl 2009; Khuhawar and Majidano 2011; Tuberoso et al. 2015; Tian et al. 2014). Some no derivatization methods are also employed, such as indirect UV detection, other detection methods such as evaporative light scattering detection and online coordination interactions between AAs and Cu (II) ions for direct analysis (Wang et al. 2011; Pérez-Palacios et al. 2014; Luo et al. 2017). Amino acid analyzer is known as an automatic instrument for AAs analysis based on the principle of post-column derivatization (Zeng et al. 2015). The specificity of instrument causes its comparatively low popularity in common laboratories. On the contrary, the pre-column derivatization has low requirement for instrument, low cost and high sensitivity, which is employed with 9-fluorenylmethyl chloroformate (FMOC-Cl) in this work. Traditional organic solvent–water reversed phase liquid chromatography (RPLC) is generally utilized after pre-column derivatization of AAs. Nevertheless, the method requires complex gradient elution program, high content of organic solvent (being harmful to the environment and increasing analysis cost) and long analysis time. Consequently, an effective method needs to be developed to solve the above problems.

Some reports used MLC for separation of AAs. However, the numbers of AAs measured in most of these reports were only 4–9 (Safa and Hadjmohammadi 2005; Gil-Agustí et al. 2007), leading to the limitations in the real sample determination. Loginova et al. studied the effect of aliphatic carboxylic acid modifiers on the separation of 2,4-dinitrophenyl AAs in MLC, and made it possible to separate 12 AAs using isocratic elution (Boichenko et al. 2007). On this basis, high concentration of organic solvents was found to be required if appropriate analysis time is wanted when we attempted to separate the AAs with a single micellar system and gradient MLC method (Ke et al. 2017). Therefore, we adopted mixed surfactants as mobile phase additives and got satisfactory results, achieving the separation of 17 AAs under isocratic condition.

In this study, we first applied isocratic MLC containing SDS and Brij-35 to the separation of 17 FMOC-Cl derived AAs (the structures and basic properties of these compounds were shown in Table S1). The optimization process for the derivatization reaction conditions was based on our previous study work (Ke et al. 2017). Meanwhile, we compared the retention performance and selectivity between the hybrid micelles system and each single micelle. Finally, the method was successfully applied to the analysis of Compound Amino Acid Injection. In addition, we studied the possible separation mechanism. In spite of the long analysis time for about 76 min, the consumption of organic solvent in the method is lower which may reduce the analysis cost and being environment-friendly, besides, complex gradient elution is not required.

Experimental

Materials

Chemicals

Chromatographic-grade methanol (MeOH), acetonitrile (ACN) and 1-propanol were purchased from Yuwang Reagent Factory (Yucheng, China). Ammonium acetate (NH_4OAc), acetic acid (AcOH), and 1-propanol were all of analytical-grade and were bought from Tianjin Chemical Industrial Company (Tianjin, China). Sodium dodecyl sulphate (SDS, content ≥ 99.0%) and cetyltrimethylammonium

bromide (CTAB, content ≥ 99.0%) were from Shuangshuang Chemical Company (Yantai, China), and polyoxyethylene(23) lauryl ether (Brij-35, content ≥ 99.0%) were purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China). 1-propanol was distilled before use. Sodium hydroxide (NaOH) and boric acid were analytical-grade and purchased from Tianjin Chemical Industrial Company (Tianjin, China). Derivatization reagents 9-fluorenylmethyl chloroformate (FMOC-Cl) were purchased from Bailingwei (Beijing, China). Distilled water was from the GLP lab of Lanzhou University (Lanzhou, China) and was used to prepare all aqueous solutions.

Reference standards of AAs: L-proline(Pro), L-phenylalanine(Phe), L-asparagine(Asp), L-serine(Ser), L-valine(Val), L-methionine(Met), L-glutamic acid(Glu), L-threonine(Thr), L-glycine(Gly), L-alanine(Ala), L-cysteine(Cys), L-lysine(Lys), L-tryptophan(Try), L-histidine(His), L-leucine(Leu), L-isoleucine(Ile) and L-arginine(Arg) were purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China). Compound Amino Acid Injection (250 mL) was from Qidu Pharmaceutical Co., Ltd (Shandong, China).

Sodium borate buffer was made from boric acid solution (0.50 M) and adjusted to pH 9.0 with 2 M sodium hydroxide solution. Buffer solution can be stored for 1 week at room temperature. FMOC-Cl (3.0 mM) was prepared daily by dissolving 7.8 mg FMOC-Cl in 10.0 mL ACN.

Equipment

A DionexUltiMate 3000 HPLC was used with a 10 µL loop manual injector, an Ultimate 3000 photodiode array detector, and a column compartment, which was controlled by the chromatographic workstation Chromeleon Client (Dionex Corporation, Sunnyvale, CA). FE20 pH meter (Mettler Toledo Instrument Co., LTD, Shanghai, China) was used to measure the pH of the mobile phase. A KH-300DB ultrasonic cleaner (Kun Shan He Chuang Ultrasonic Instruments Co., LTD, Kunshan, China) was employed for controlling ultrasonic cleaning device. AP-01P vacuum pump (AutosciEnce Instruments Co., LTD, Tianjin, China) was used to filter solution by 0.22 µm nylon membrane. An venusil XBP C18 column (5 µm, 250 mm × 4.6 mm id) was used as stationary phase.

Standards and sample preparation

The AAs stock solutions (1.0 mg/mL) were prepared by dissolving the reference substances in distilled water. All standard stock solutions were stored under refrigeration at − 20 °C and were placed at room temperature before use. Subsequently the dilution in water was made to yield concentrations of 50 µg/mL. Compound Amino Acid Injection was diluted in water for 100 times and then for the derivatization procedure.

Derivatization procedure

The optimization of the derivatization procedure of AAs has been investigated in our previous study work (Ke et al. 2017) and we applied the optimized derivatization reaction conditions in this work. The concentration of derivatization reagents (FMOC-Cl) was 3 mM; the pH value of the sodium borate buffer was 8.5 and the derivatization time was 10 min. Briefly, the procedure of derivatization of AAs was as follows: a 250 µL aliquot of amino acid standard solution (or sample) was mixed with 250 µL of sodium borate buffer; then, 500 µL of FMOC-Cl (3.0 mM) was added, mixed on a vortex-mixer and allowed to proceed at ambient temperature for 10 min; the reaction was then stopped by the addition of 2.5 mL *n*-pentane. The mixture was used for HPLC analysis after filtration through a 0.22 µm nylon membrane filter.

Optimized chromatography conditions

Venusil XBP C18 column (5 µm, 250 × 4.6 mm,); detection wavelength was set at 266 nm; mobile phase A was 0.075 M SDS and 0.010 M brij-35 containing 20 mM NH_4OAc and adjusted to pH 3.5 by addition of glacial acetic acid and mobile phase B was 100%ACN (85:15, v/v); the flow rate was 1.2 mL/min at 35 °C and the injection volume was 10 µL.

Results and discussion

Optimization of the separation conditions

Influence of surfactant type

SDS (anionic surfactant), CTAB (cationic surfactant) as well as Brij-35 (non-ionic surfactant) are considered as the most commonly used surfactants in MLC. These three popular surfactants were investigated in this work to optimize separation conditions. We chose pH3.5, 15%ACN (v:v) and 35 °C for column temperature as the chromatographic conditions by simple preliminary inquiry. The concentrations of SDS, Brij-35 and CTAB solutions were 0.075 M, 0.010 M and 0.050 M, respectively.

The results showed that CTAB-mediated method suffers the longest analysis time (300 min), and poorest peak shape and selectivity. The adsorption way of CTAB on the stationary phase relies on the cationic head groups that strongly adsorbed on the surface of stationary phase and alkyl chains. Therefore, the hydrophobic tails point to the polar solution, which is opposed to that of SDS and Brij-35 (Ruiz-Ángel

et al. 2013). This, undoubtedly, increases the hydrophobicity of stationary phase surface. Accordingly, the retention of analytes will also increase due to their weak polarity, thereby increasing the retention. The compounds can be eluted within 180 min, obtaining the shortest analysis time and the best peak shape among these three situations when SDS is used as additives. The time of Brij-35 condition (210 min) was slightly longer than SDS. As mentioned above, the binding ways of SDS and Brij-35 on the stationary phase are different from CTAB, so their coating increases the polarity of the stationary phase, thereby accelerates the compounds elution. In addition, adsorption of SDS monomers produces a layer of negative charge, leading to the existence of repulsive electrostatic interactions between the analytes and stationary phase; accordingly, SDS was chosen as the mobile phase additive for the separation of AAs.

Concentrations of the mixed surfactants mobile phase

It can be seen from the above that although single SDS micellar system obtained the shortest separation time and the best selectivity, it is still unable for the single one to baseline separate the 17 AAs simultaneously within appropriate time. Therefore, we combined SDS and Brij-35 together in mobile phase to form mixed micelle system, and studied its separation ability. We found the separation time was significantly shortened and the selectivity greatly enhanced when proper amount of Brij-35 was added. Brij-35 competed for the binding site on the stationary phase with SDS in a same combined way. Consequently, the density of negative charge on stationary phase and mobile phase is reduced; Fig. 1 shows the chromatograms of the single SDS micellar system and mixture of SDS and Brij-35 micellar conditions.

To get the best separation, we optimized the two surfactant concentrations by changing one of them while fixing another. When the SDS concentration was kept at 0.075 M, the retention time decreased as the Brij-35 concentration was changed in the range of 0.005–0.03 M (Fig. S1a). This can be explained by the addition of Brij-35 that cuts down the negative charge density in both of the mobile phase and the stationary phase and increases the polarity of mobile phase, which is responsible to the reduced retention. Similarly, when the concentration of Brij-35 was set at 0.01 M, the retention time was also reduced with the increasing SDS concentrations in the range of 0.025–0.125 M (Fig. S1b). This can be explained by an increase in the SDS content resulting in an increase in the negative charge density on the stationary phase, so the electrostatic repulsion causes a decrease in the retention time of the analytes. The above two cases illustrate that hydrophobic interaction is the main factor that determines the retention of AAs in chromatographic system. Thus, 0.075 M SDS and 0.01 M Brij-35 were chosen as micellar mobile phase additives.

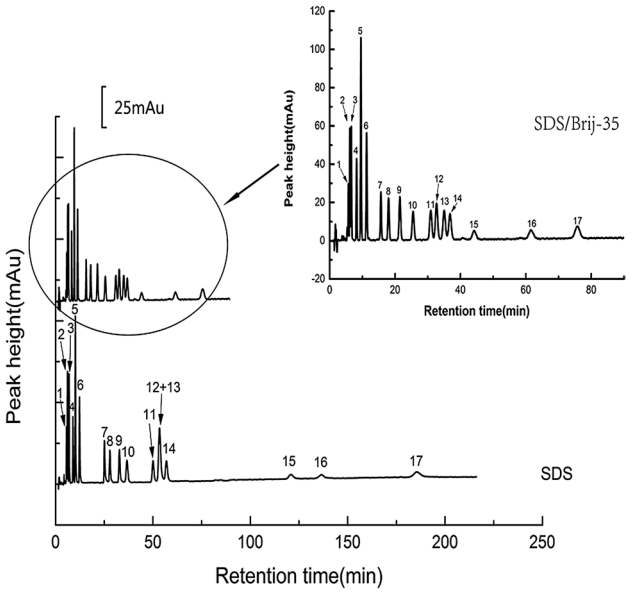

Fig. 1 Chromatograms of mixing standard solution of AAs in single SDS system (0.075M SDS, pH = 3.5) and mixed SDS/Brij-35 system (0.075M SDS and 0.01 M Brij-35, pH = 3.5)1. Asp; 2. Glu + Pro; 3. Ser; 4. Thr; 5. Gly; 6. Ala; 7. FMOC-OH; 8. Met; 9. Val; 10. Try; 11. Phe; 12. His; 13. Ile; 14. Leu; 15. Arg; 16. Lys; 17. Cys. Conditions: the working concentration of the standard solutions was 50 μg/mL; Venusil XBP C18 column (5 μm, 250 × 4.6 mm); column temperature was 35 °C; flow rate was 1.2 mL/min; injection volume was 10 μL

Influence of pH values

The pH value of the mobile phase is another important condition in MLC. It determines the charged state of the analytes, which directly affects the retention behaviors in chromatographic systems. As shown in the Table S1, the structures of the amino acids after the derivatization reaction were changed to weak acids. The effects of pH values were examined in the range of pH 3.5–6.5 to obtain the best conditions. As shown in Fig. 2, the retention of the compounds decreases with the increasing pH. The pKa values of the compounds analyzed are within the range of 3.26–3.95, that is to say, the amount of negative charge carried by them will increase when pH value is growing. When pH is greater than 5.95 (pKa values of all the compounds were less than the solution pH for two units), AAs are almost all in their anionic forms. Electrostatic repulsion exists between the compounds and the stationary phase modified by SDS, which will increase as the negative charge carried by analytes grows. Therefore, the retention reduced. Although the relatively high pH condition can shorten the analysis time, it will reduce the resolution of the analytes. Taking both of the time and resolution into consideration, we chose pH 3.5 for subsequent experiments.

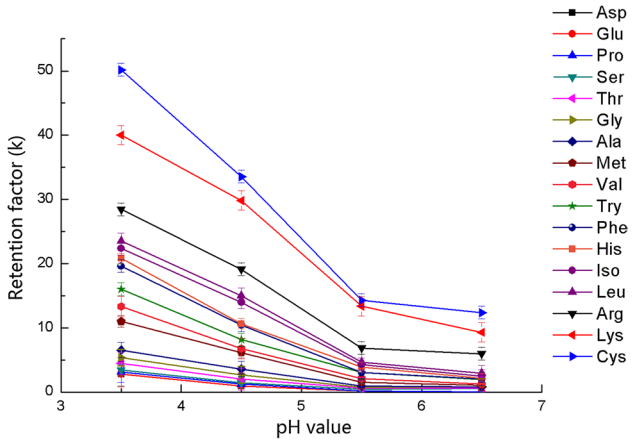

Fig. 2 The influence of different pH values of mobile phase on retention factor (k) of AAs. Conditions: the working concentration of the standard solutions was 50 μg/mL; Venusil XBP C18 column (5 μm, 250×4.6 mm); column temperature 35 °C; flow rate 1.2 mL/min; injection volume was 10 μL; the mobile phase consisted of aqueous solution (0.075 M SDS and 0.010 M Brij-35 containing 20 mM NH_4OAc at pH 3.5) with 15% (v/v) of ACN

Choice of organic modifiers and concentration

Aliphatic alcohols are often used in MLC as the organic modifiers, such as alcohol, 1-propanol besides, methanol and ACN are also used in some reports. To investigate the effects of different organic solvents on the separation, we studied MeOH, ACN and 1-propanol in the experiment. The results showed that ACN is superior to the others in both resolution and peak shape. Furthermore, we studied the effects of the organic solvent content. It is known to us that the content of organic solvent can be used in MLC is limited, otherwise, the micelles in mobile phase will disintegrated, which brings on another chromatographic mode named high submicellar chromatography. It is accepted that the content of ACN in MLC should not be exceed 30% (v:v). We found the retention of compounds decreased with the increasing organic solvent content, which is similar to that of in conventional RPLC. Despite the shortened retention, the resolution is also reduced. Therefore, we may reduce the solvent consumption as much as possible under the precondition that reasonable analysis time could be obtained. This will save the analysis cost and make the method be more ecological. Therefore, 15% ACN (v:v) was selected.

Peak profiles in the mixed micellar systems

The linear correlations between the two peak half-widths of chromatographic peak and the retention time can effectively evaluate the changes of peak width and symmetry under certain conditions (Ruiz-Ángel et al. 2010). Suppose that A and B represent the left and right half-widths, respectively (where A and B are determined at 10% peak height to avoid the interference of baseline noise in the measurement (Ruiz-Ángel et al. 2015), then

$$A = m_A t_R + A_0, \tag{1}$$

$$B = m_B t_R + B_0, \tag{2}$$

where m_A (left) and m_B (right) are the slopes of the linear correlations; and A_0 and B_0 are the corresponding intercepts; t_R means the retention time.

Equations (1) and (2) can be used to predict the peak half-widths of the compounds corresponding to the different retention time. Furthermore, the summation of m_A and m_B ($m_A + m_B$) represents the peak broadening ratio in the column, and their ratio (m_A/m_B) illustrates the symmetry of chromatographic peaks. These parameters are effective in the optimization of chromatographic conditions.

In this work, the chromatographic efficiency of 17 AAs in 0.075 M SDS (Fig. 3a) and 0.075 M SDS/0.010 M Brij-35 mixed micellar (Fig. 3b) systems was discussed based on the principles above. Linear relationship in Fig. 3a is good while m_B is much greater than m_A, indicating the tailing peaks; and in Fig. 3b, symmetry of chromatographic peaks of good as m_B tended to be close to m_A. The results shown that good peak symmetry of weak acidic compounds could be obtained in mixed micellar system, which may be related to the protection of Brij-35 on stationary phase by reducing the interaction of analytes and silicon hydroxyl groups owing to the longer alkyl chains in Brij-35. It should be noted that the sum of the two slopes ($m_A + m_B$) in mixed SDS/Brij-35 mobile phase is greater than that in single SDS system, that is to say, the extent of peak broadening is greater. This may be explained by that the carbon content in alkyl chains of mixed surfactant modified on the stationary phase is more than that in the single ones, resulting in the slower mass transfer in stationary phase and thus increasing peak broadening.

Discussion of the possible mechanism

Based on the above results, we simulated a simple chromatography environment of SDS/Brij-35 mixed mode for AAs separation which is shown in Fig. 4 Since the AAs are derived with bring the large group in derivatization reagents, their structures and chemical properties are relatively similar (Table S1). Thus, only one symbol was used to present the analytes in the diagram. There are organic phase, water phase, SDS and Brij-35 monomers and SDS/Brij-35 micellar groups in mixed micellar system. These two surfactants monomers are sharing a single core to form micelles and the nonpolar hydrocarbon chains point to the center. The stationary phase is modified by SDS and Brij-35 monomers with a

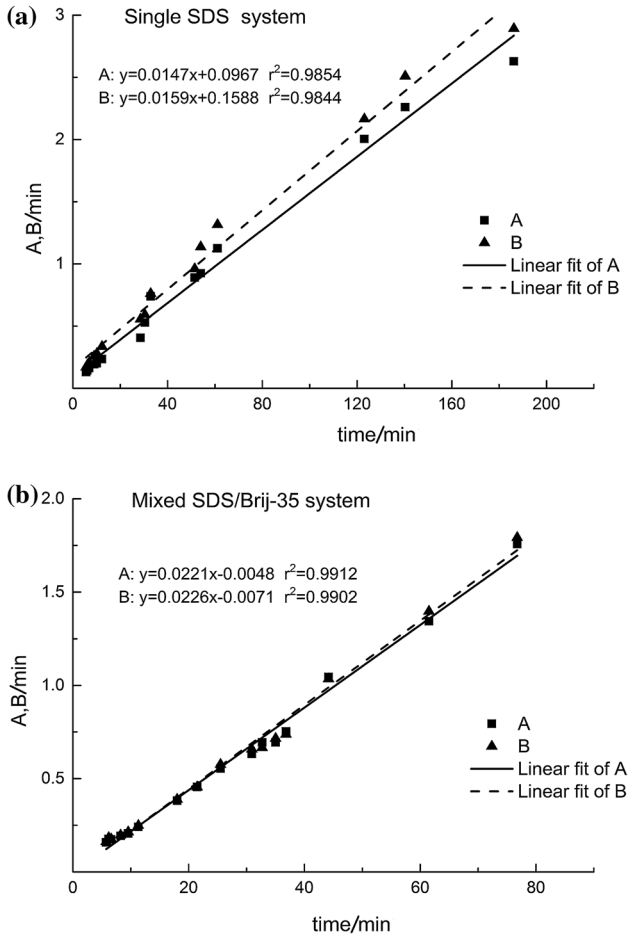

Fig. 3 Evaluation of half-width of AAs in mobile phase. Mobile phase with different composition of surfactants. **a** Single SDS system; **b** mixed SDS/Brij-35 system. Conditions: the working concentration of the standard solutions was 50 μg/mL; Venusil XBP C18 column (5 μm, 250×4.6 mm); column temperature was 35 °C; flow rate was 1.2 mL/min; injection volume was 10 μL; the mobile phase: **a** aqueous solution (0.075 M SDS containing 20 mM NH_4OAc at pH 3.5) with 15% (v/v) of ACN; **b** aqueous solution (0.075 M SDS/0.010 M Brij-35 system containing 20 mM NH_4OAc at pH 3.5) with 15% (v/v) of ACN

layer of negative charge on the surface, and the exposed silicon hydroxyl groups still exist (Fig. 4). The retention of the solutes is determined by the equilibrium among the aqueous phase, the micellar phase and the modified stationary phase.

There are hydrogen bonding, hydrophobic and electrostatic interactions in the chromatographic system. The retention mechanism relies on the relative magnitude of the three forces. First, it is easy to form intermolecular hydrogen bonds between the exposed silicon hydroxyl groups and the analytes due to the presence of carboxyl and (or) amino groups. Different numbers of hydrogen bond receptors in the compounds determine the strength of the hydrogen bonds interactions. As can be seen from their structures (Table S1), the receptor numbers are from 1 to 6 for all the analytes. AAs, such as Pro, which have less numbers, obtain relatively weak retention on the stationary phase; while the ones such as Arg, Try and Lys that have more receptors were strongly retained and thereby eluted later.

Second, hydrophobic interaction existed among the compounds and the stationary and mobile phase. The oil/water partition coefficient (Log Po/w) values of the solutes were in the range of 2.31–4.79, which indicated the weak hydrophobic properties of these compounds. The polarity of the stationary phase increased due to the surfactants absorption. The solubilization capability of micelles made analytes interact with silicon hydroxyl groups, uncovered alkyl-bonded layer and hydrophobic chain of SDS and Brij-35 more easily, which improved the retention of them. MLC still belongs to RPLC conditions in spite of the modification, therefore, the larger the Log Po/w values of the compounds were meant the stronger the retentions of the compounds were. When increasing the concentration of organic phase, the elution strength also increased, which is responsible for the decreased retention.

Last, with their pKa values (3.26–3.95) close to the pH (3.5) of the mobile phase, the compounds existed in the form of molecules and anions together in solution, and the larger the pKa values are, the more negative charges are. The carboxylic acid ionization of target compounds produces negatively charged carboxylate ions which participate in electrostatic repulsion with modified stationary phase. The stationary phase has a negative charge due to the adsorption of SDS, hence there is an electrostatic repulsive interaction between the analytes and the stationary phase, and the compounds with larger pKa values would flow out from the column more quickly. However, the negative charge of the compound itself is not much and meanwhile, gap of pKa values among these AAs is not large enough (≤ 0.7), making electrostatic repulsive interaction be far from the decisive force to change the retention behavior.

Method validation

To examine the proposed MLC method for the determination of AAs, the accuracy and precision, linearity and range, limit of detection and limit of quantification were calculated under the optimal chromatographic conditions described previously.

The linearity and range were performed with the diluted standard mixtures of AAs at five different concentrations under the optimized conditions, and plotting peak areas as a function of analytes concentration expressed in μg/mL. The linear relationships among the concentrations of these AAs and the corresponding peak areas were summarized in Table 1 and the results indicated good linearity for each analyte. In addition, because Glu and Pro could not be separated in isocratic mode of this method, there were

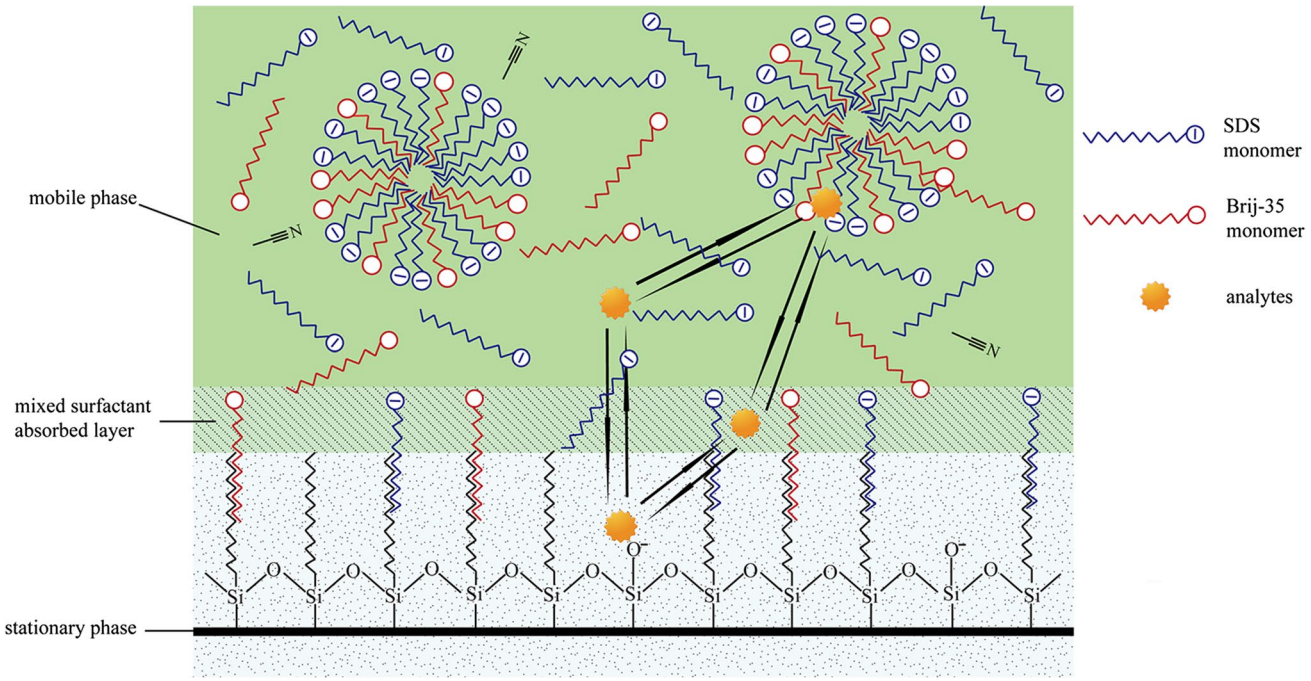

Fig. 4 The retention mechanism diagram in mixed MLC mode

Table 1 Linearity, correlation coefficient, limit of detection (LOD), limit of quantification (LOQ) and precision of the method

Analytes	Linear equation	Correlation coefficient	Ranges (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)	Retentiontime (RSD, %)		Peak area (RSD, %)	
						Intra-day ($n=5$)	Inter-day ($n=5$)	Intra-day ($n=5$)	Inter-day ($n=5$)
Asp	$y=0.1160x-0.8284$	0.9936	2.00–100	0.93	2.62	0.5	2.7	2.6	6.0
Ser	$y=0.2372x+0.6306$	0.9981	1.50–50	0.56	1.65	0.6	1.9	2.0	7.4
Thr	$y=0.1897x+1.2154$	0.9995	2.00–100	1.05	3.23	1.0	1.6	3.9	6.3
Gly	$y=1.1203x+3.8637$	0.9966	1.00–200	0.48	1.39	1.2	2.8	1.7	5.3
Ala	$y=0.3295x+0.7806$	0.9991	2.00–100	0.82	2.29	0.8	2.8	2.7	4.9
Met	$y=0.3290x-1.8901$	0.9984	3.00–100	2.16	6.61	0.6	2.3	3.8	4.9
Val	$y=0.3537x-3.4785$	0.9950	5.00–100	2.39	7.20	1.3	1.6	3.8	5.1
Try	$y=0.1257x+0.9823$	0.9927	5.00–50	3.18	10.55	1.2	2.7	3.0	6.9
Phe	$y=1.1465x+2.5095$	0.9912	10.00–100	4.51	13.64	0.9	2.6	4.1	7.8
His	$y=0.3499x-1.0245$	0.9971	10.00–100	4.32	13.62	0.8	3.0	3.6	6.9
Ile	$y=0.2318x+0.4587$	0.9993	10.00–100	5.25	16.78	0.7	2.0	2.7	5.2
Leu	$y=0.2081x-0.8858$	0.9981	10.00–100	5.88	15.92	1.7	3.1	4.8	8.2
Arg	$y=0.1392x-4.0214$	0.9879	30.00–200	13.78	42.98	0.9	2.0	3.2	5.8
Lys	$y=0.8775x-0.5641$	0.9981	30.00–200	12.55	38.67	1.4	3.1	3.5	7.3
Cys	$y=1.4366x-1.0922$	0.9952	30.00–200	11.97	35.65	2.1	4.0	4.4	7.9

no relevant data in Table 1. The LOD and LOQ values were estimated at signal-to-noise ratios of 3:1 and 10:1, respectively and the results were summarised in Table 1. It is worth noting that the LODs and LOQs in the method are not satisfying enough for the last three compounds. The reason may be that lysine (Lys), tryptophan (Try) and arginine (Arg) contain a relatively large number of hydrogen bond receptor groups (4–6), so they have a strong interaction with the exposed silicon–hydroxyl group in the stationary phase. Due to the long retention time, the peak widens resulting in a low sensitivity of the three amino acids.

The precision of the method for each amino acid in the experiment was performed by injecting five standard samples in a single day for the intra-day precision and across five different days for the inter-day precision. The results are shown in Table 1. The precision expressed by relative standard deviations (RSDs) of retention time and peak area for intra-day precision were ≤ 2.19% and ≤ 4.89%, and for inter-day precision were ≤ 3.47% and ≤ 8.23%, respectively.

The accuracy was determined with recovery studies using the standard addition method. Known amounts of standard AAs with three different concentration levels were added to Compound Amino Acid Injection. Recoveries were calculated by comparing the obtained amounts with those added. The results are as in Table 2 that showed and indicated good accuracy. In addition, because Glu and Pro could not be separated in isocratic mode of this method, there were no relevant data in Table 2.

Application

After validation, free AAs were determined in an injection preparation named Compound Amino Acid Injection by employing the established and validated method. Figure 5a, b shows the HPLC chromatograms of the standard solution and injection, respectively. There is still difficulty in the separation of Glu and Pro in the isocratic mode. We will continue to try to improve this issue in subsequent research. The concentration of amino acids in the sample was calculated according to the calibration curves in Table 1. The results in Table 2 showed that the content of amino acids in the sample of Compound Amino Acid Injection ranged from 10.36 to 75.63 μg/mL. The Compound Amino Acid Injection was spiked with the analyses at three levels. Three levels of analytes were added to the amino acid injection. The results are shown in Table 2 that the recoveries for amino acids ranged from 86.2 to 111.3% with the RSDs between 0.1 and 5.0%. These results indicated that the method was accurate and reliable for analysis of amino acids.

Conclusions

In this work, we established an effective isocratic micellar chromatographic method using mixed anionic and nonionic surfactants as mobile phase additives for separation of 17 free AAs. Besides, the possible separation mechanism was discussed. In spite of the long analysis time for 76 min, the consumption of organic solvent in the method is lower which may reduce the analysis cost and being environment-friendly. In addition, complex gradient elution is not required. Although the method has been further modified, the LODs and LOQs in the method are not satisfying enough

Table 2 Results of the recovery experiments in Compound Amino Acid Injection

Analytes	Sample (μg/mL)	Added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD (%)
Asp	23.83	20	41.02	93.5	2.8
		25	42.13	86.2	1.9
		30	48.82	90.6	3.5
Ser	10.36	5	13.27	86.3	2.7
		10	17.93	88.0	0.7
		15	24.08	94.9	2.9
Thr	25.17	20	45.20	100.0	4.3
		25	47.78	95.2	2.0
		30	51.15	92.7	4.1
Gly	75.63	70	152.1	104.4	5.0
		80	168.6	108.3	3.1
		90	176.9	106.8	2.5
Ala	19.38	15	38.29	111.3	2.4
		20	43.09	109.4	0.8
		25	43.78	98.6	1.4
Met	29.15	20	50.32	102.3	3.2
		30	56.78	115.5	3.9
		40	65.33	94.4	0.7
Val	30.66	20	46.77	92.3	2.0
		30	59.31	97.7	0.8
		40	68.89	97.5	1.3
Try	11.26	5	15.24	93.7	3.8
		10	23.20	109.1	3.2
		15	24.92	94.9	1.5
Phe	47.88	40	82.93	94.3	3.0
		50	95.33	97.3	0.1
		60	108.1	100.2	3.6
His	20.97	15	36.37	101.1	4.6
		20	43.25	105.5	2.3
		25	44.63	97.0	1.5
Ile	30.64	20	45.99	90.8	1.9
		30	63.33	104.4	2.8
		40	62.68	88.7	1.2
Leu	47.65	40	85.53	97.5	4.4
		50	95.02	97.3	1.2
		60	113.3	105.2	0.8
Arg	40.35	30	74.42	105.7	3.2
		40	85.60	106.5	1.2
		50	91.06	100.7	1.0
Lys	33.16	20	45.82	86.1	2.5
		30	57.05	90.3	0.6
		40	69.12	94.4	0.8
Cys	35.33	20	48.25	87.2	3.0
		30	60.17	92.1	1.2
		40	70.36	93.4	2.4

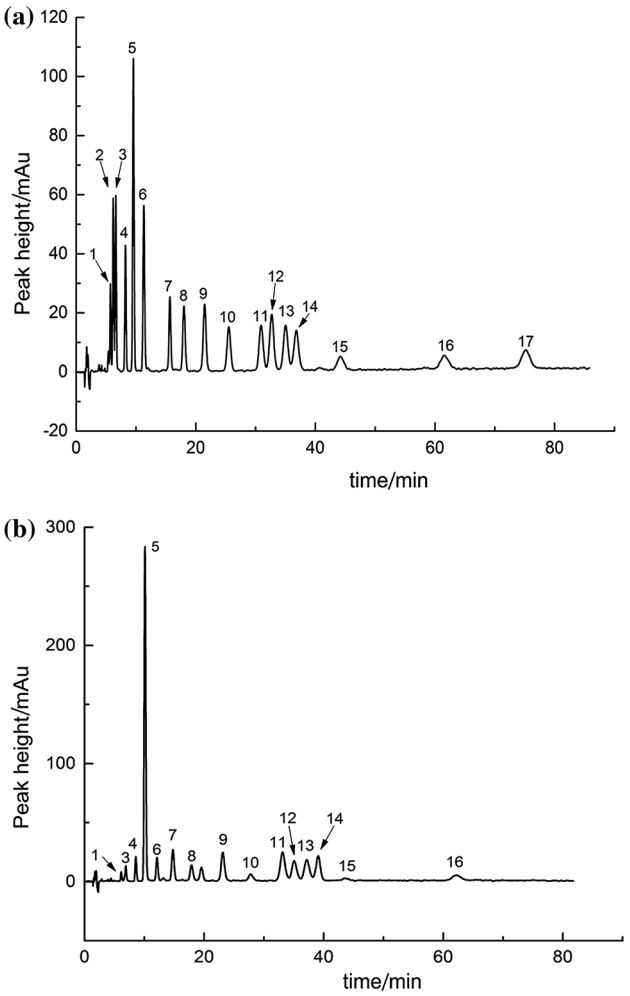

Fig. 5 Chromatograms of: **a** the standard solution and **b** Compound Amino Acid Injection. 1. Asp; 2. Glu + Pro; 3. Ser; 4. Thr; 5. Gly; 6. Ala; 7. FMOC-OH; 8. Met; 9. Val; 10. Try; 11. Phe; 12. His; 13. Ile; 14. Leu; 15. Arg; 16. Lys; 17. Cys. Conditions: Venusil XBP C18 column (5 μm, 250 × 4.6 mm); column temperature 35 °C; flow rate 1.2 mL/min; injection volume was 10 μL; the mobile phase consisted of aqueous solution (0.075 M SDS and 0.010 M Brij-35 containing 20 mM NH_4OAc at pH 3.5) with 15% (v/v) of ACN

especially for the last three compounds. The situation needs to be improved in the follow-up studies by our group.

References

Albiolchiva J, Esteveromero J, Perisvicente J (2018) Development of a method to determine axitinib, lapatinib and afatinib in plasma by micellar liquid chromatography and validation by the european medicines agency guidelines. J Chromatogr B 1074–1075:61–69. https://doi.org/10.1016/j.jchromb.2017.12.034

Angarska JK, Tachev KD, Denkov ND (2004) Composition of mixed adsorption layers and micelles in solutions of sodium dodecyl sulfate and dodecyl acid diethanol amide. Colloid Surf A 233:193–201. https://doi.org/10.1016/j.colsurfa.2003.11.020

Boichenko AP, Kulikov AU, Loginova LP, Iwashchenko AL (2007) Aliphatic carboxylic acids as new modifiers for separation of 2,4-dinitrophenyl amino acids by micellar liquid chromatography. J Chromatogr A 1157:252–259. https://doi.org/10.1016/j.chroma.2007.05.012

Chia TC (2013) Recent advances in on-line concentration and separation of amino acids using capillary electrophoresis. Anal Bioanal Chem 405:7919–7930. https://doi.org/10.1007/s00216-013-6906-1

Chiu TC, Chang HT (2007) Stacking and separation of fluorescent derivatives of amino acids by micellar electrokinetic chromatography in the presence of poly (ethylene oxide). J Chromatogr A 1146:118–124. https://doi.org/10.1016/j.chroma.2007.01.113

Dhote S, Deshmukh L, Paliwal L (2013) Use of nonionic tween-80 surfactant mobile phase in thin layer chromatography of heavy metal cations. J Mater Sci Technol 2:10–23. https://doi.org/10.7726/ajmst.2013.1002

Dong YM, LiN An Q, Lu NW (2015) A novel nonionic micellar liquid chromatographic method for simultaneous determination of pseudoephedrine, paracetamol, and chlorpheniramine in cold compound preparations. J Liq Chromatogr Relat Technol 38:251–258. https://doi.org/10.1080/10826076.2014.903850

Ebrahimi P, Hadjmohammadi MR (2006) Simultaneous optimization of resolution and analysis time in mixed micellar liquid chromatography of coumarins by use of a utility function. Anal Bioanal Chem 384:851–858. https://doi.org/10.1007/s00216-005-0216-1

El-Shaheny RN, El-Maghrabey MH, Belal FF (2015) Micellar liquid chromatography from green analysis perspective. Open Chem 13:877–892. https://doi.org/10.1515/chem-2015-0101

Gil-Agustí M, Carda-Broch S, Monferrer-Pons L, Esteve-Romero J (2007) Simultaneous determination of tyramine and tryptamine and their precursor amino acids by micellar liquid chromatography and pulsed amperometric detection in wines. J Chromatogr A 1156:288–295. https://doi.org/10.1016/j.chroma.2007.02.090

Jambor A, Molnar-Perl I (2009) Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride Literature overview and further study. J Chromatogra A 1216:3064–3077. https://doi.org/10.1016/j.chroma.2009.01.068

Ke J, Dong YM, Luo T, Xie YF (2017) Development of a gradient micellar liquid chromatographic method eluting from micellar mode to high submicellar mode for the rapid separation of free amino acids. Anal Method 9:1762–1770. https://doi.org/10.1039/c6ay03453e

Khuhawar MY, Majidano SA (2011) GC analysis of amino acids using trifluoroacetylacetone and ethyl chloroformate as derivatizing reagents in skin samples of psoriatic and arsenicosis patients. Chromatographia 73:701–708. https://doi.org/10.1007/s10337-011-1957-y

Luo T, Ke J, Xie YF, Dong YM (2017) Determination of underivatized amino acids to evaluate quality of beer by capillary electrophoresis with online sweeping technique. J Food Drug Anal 25:789–797. https://doi.org/10.1016/j.jfda.2017.03.003

Memon N, Bhanger MI, Khuhawer MY (2005) Determination of preservatives in cosmetics and food samples by micellar liquid chromatography. J Sep Sci 28:635–638. https://doi.org/10.1002/jssc.200500060

Moughan PJ, Ravindran V, Sorbara JOB (2014) Dietary protein and amino acids—consideration of the undigestible fraction. Poult Sci 93:2400–2410. https://doi.org/10.3382/ps.2013-03861

Nakao R, Halldin C (2013) "Mixed" anionic and non-ionic micellar liquid chromatography for high-speed radiometabolite analysis of positron emission tomography radioligands. J Chromatogr A 1281:54–59. https://doi.org/10.1016/j.chroma.2013.01.071

Nakao R, Schou M, Halldin C (2012) Direct plasma metabolite analysis of positron emission tomography radioligands by micellar liquid

chromatography with radiometric detection. Anal Chem 84:3222–3230. https://doi.org/10.1021/ac2032657

Pérez-Palacios T, Melo A, Cunha S, Ferreira IMPLVO (2014) Determination of free amino acids in coated foods by GC–MS: optimization of the extraction procedure by using statistical design. Food Anal Method 7:172–180. https://doi.org/10.1007/s12161-013-9615-7

Peris-García E, Ubeda-Torres MT, Ruiz-Angel MJ, García-Álvarez-Coque MC (2016) Effect of sodium dodecyl sulphate and brij-35 on the analysis of sulphonamides in physiological samples using direct injection and acetonitrile gradients. Anal Method 8:3941–3952. https://doi.org/10.1039/C6AY00390G

Ramezani AM, Absalan G, Ahmadi R (2018) Green-modified micellar liquid chromatography for isocratic isolation of some cardiovascular drugs with different polarities through experimental design proach. Anal Chim Act 1010:76–85. https://doi.org/10.1016/j.aca.2017.12.021

Rangel-Yagui CO, Hsu HWL, Pessoa-Jr A, Tavares LC (2005) Micellar solubilization of ibuprofen: influence of surfactant head groups on the extent of solubilization solubilizaçãomicelar do ibuprofeno: influência do grupo polar dos tensoativos no grau de solubilização. Braz J Pharm Sci 41:237–246. https://doi.org/10.1590/S1516-93322005000200012

Ruiz-Ángel MJ (2011) Comparison of the performance of non-ionic and anionic surfactants as mobile phase additives in the RPLC analysis of basic drugs. J Sep Sci 34:623–630. https://doi.org/10.1002/jssc.201000840

Ruiz-Ángel MJ, Carda-Broch S, Garcia-Alvarez-Coque MC (2010) Peak half-width plots to study the effect of organic solvents on the peak performance of basic drugs in micellar liquid chromatography. J Chromatogr A 1217:1786–1798. https://doi.org/10.1016/j.chroma.2010.01.041

Ruiz-Ángel MJ, Carda-Broch S, García-Álvarez-Coque MC (2013) Chromatographic efficiency in micellar liquid chromatography: should it be still a topic of concern? Separ Purif Method 42:1–27. https://doi.org/10.1080/15422119.2011.653033

Ruiz-Ángel MJ, Carda-Broch S, García-Álvarez-Coque MC (2014) High submicellar liquid chromatography. Sep Purif Method 43:124–154. https://doi.org/10.1080/15422119.2012.743917

Ruiz-Ángel MJ, Peris-García E, García-Alvarez-Coque MC (2015) Reversed-phase liquid chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: a method for the analysis of basic compounds. Green Chem 17:3561–3570. https://doi.org/10.1039/C5GC00338

Safa F, Hadjmohammadi MR (2005) Simultaneous optimization of the resolution and analysis time in micellar liquid chromatography of phenyl thiohydantoin amino acids using Derringer's desirability function. J Chromatogra A 1078:42–50. https://doi.org/10.1016/j.chroma.2005.04.081

Sueyoshi K, Hashiba K, Kawai T, Kitagawa F, Otsuka K (2011) Hydrophobic labeling of amino acids: transient trapping–capillary/microchip electrophoresis. Electrophoresis 32:1233–1240. https://doi.org/10.1002/elps.201000567

Tian M, Zhang J, Mohamed AC, Han Y, Guo L, Yang L (2014) Efficient capillary electrophoresis separation and determination of free amino acids in beer samples. Electrophoresis 35:577–584. https://doi.org/10.1002/elps.201300416

Tuberoso C, Congiu F, Serreli G, Mameli S (2015) Determination of dansylated amino acids and biogenic amines in Cannonau and Vermentino wines by HPLC-FLD. Food Chem 175:29–35. https://doi.org/10.1016/j.foodchem.2014.11.120

Wang X, Tan X, Gao M, Peng J (2011) Determination of underivatized glycine and proline in vinegar turtle shell by HPLC-ELSD. China J Chin Mater Med 36:2107–2109. https://doi.org/10.1097/mop.0b013e328341d1da

Zeng Y, Cai W, Shao X (2015) Quantitative analysis of 17 amino acids in tobacco leaves using an amino acid analyzer and chemometric resolution. J Sep Sci 38:2053–2058. https://doi.org/10.1002/jssc.201500090

Ziegler J, Abel S (2014) Analysis of amino acids by HPLC/electrospray negative ion tandem mass spectrometry using 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) derivatization. Amino Acids 46:2799–2808. https://doi.org/10.1007/s00726-014-1837-5