Journal of Chromatography B, 904 (2012) 99–106

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Comparison of amino acid derivatization reagents for LC–ESI-MS analysis. Introducing a novel phosphazene-based derivatization reagent

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ARTICLE INFO

Article history:
Received 8 June 2012
Accepted 25 July 2012
Available online 31 July 2012

Keywords:
Amino acids
Derivatization reagents
LC–ESI-MS

ABSTRACT

Amino acid analysis with high performance liquid chromatography with electrospray ionization mass spectrometry (LC–ESI-MS) is an emerging method. For more sensitive analysis, derivatization is used and next to commercially available derivatization reagents such as dansyl chloride (DNS), 9-fluorenylmethyl chloroformate (FMOC-Cl) and diethyl ethoxymethylenemalonate (DEEMM), new derivatization reagents are designed specially for LC–ESI-MS, like p-N,N,N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide (TAHS) which provides very low limits of detection. In this work, a novel phosphazene based derivatization reagent (FOSF) that provides comparable limits of quantitation (LoQ) to TAHS is introduced. Moreover, a thorough comparison between FOSF, TAHS, DNS, FMOC-Cl and DEEMM is carried out for 7 different amino acids – Arg, Asp, Gly, β-Ala, Pro, Trp and Phe. This is a first time that thorough comparison is carried out on the same instrument for amino acid derivatization reagents. Results on the same instrument for five amino acid derivatization reagents show that novel reagents are sensitive with LoQ values around 80 fmol but have disadvantages such as problematic chromatographic separation. Next to novel reagents, DEEMM offers very good LoQ-s (average of 150 fmol) and wide dynamic linear range.

1. Introduction

Amino acids are building blocks of proteins and play an important role in biochemistry [1]. Amino acid content is measured in all types of biological samples ranging from human bodily fluids and tissues to various foods. Many different techniques have been applied over the years for analysis. However, the pursuit for more sensitive methods is ongoing [2,3] and therefore high performance liquid chromatography with electrospray ionization mass spectrometry (LC–ESI-MS) has become widely used analytical technique. Inherently LC–MS provides low limits of detections, but is strongly dependent on the efficiency of ionization in ESI. One of the ways to achieve low detection limits is to concentrate the analyte in the sample, but often times it is not possible since matrix components may interfere or sample sizes are too small [4]. Another solution, which has been emphasized and has recently gained popularity, is the use of derivatization for signal enhancement [4–7]. Moreover, in some cases, pre-column derivatization of amino acids also allows better chromatographic separation [4].

One of the approaches for choosing a derivatization reagent is to use commercially available ultraviolet (UV) absorbance or fluorescence (FL) detection tags such as dansyl chloride (DNS), 9-fluorenylmethyl chloroformate (FMOC-Cl) and diethyl ethoxymethylenemalonate (DEEMM). These have been used for amino acid analysis with LC–ESI-MS [3,8,9]. In recent years, there has been a rapid growth in design and development of amino acid derivatization reagents that are specially meant for LC–ESI-MS applications: (5-N-succinimidoxy-5-oxopentyl)triphenylphosphonium bromide (SPTPP) [10], 3-aminopyridyl-N-hydroxysuccinimidyl carbamate (APDS) [11], N-hydroxysuccinimide ester of N-alkylnicotinic acid (C_n-NA-NHS) [12] and p-N,N,N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide (TAHS) [2]. Out of these various reagents, APDS is targeted towards better chromatographic separation [11], but SPTPP [10] and TAHS [2] for sensitive analysis in positive ion mode. Moreover, TAHS is claimed to be the most sensitive out of these novel derivatization reagents [2].

However, when characterizing these novel reagents and analyzing their properties as amino acid derivatization reagents, the comparisons are often made to the analysis of underivatized amino acids [2,10]. As for comparing derivatization reagents to each other, there have been various publications for UV and FL detection [13,14]. For ESI-MS analysis, comparison of limits of detections (LoD) for underivatized amino acids and derivatized amino acids (FMOC-Cl, propyl chloroformate and butanol) have been made and results showed that the best results were obtained

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http://dx.doi.org/10.1016/j.jchromb.2012.07.029

by propyl chloroformate [8]. To the best of our knowledge, for LC–ESI-MS analysis, no other comparisons of amino acid derivatization reagents have been published.

In order to choose a derivatization method for a particular analytical task, it is necessary to compare properties of various amino acid derivatization reagents. Comparison of analytical performance of derivatization methods using results published by different workgroups may be inconclusive – aims, instruments and presentation of performance criteria are different. For example, different calculation and presentation methods for LoD/LoQ values are used. Another aspect to consider is that the mass spectrometry systems can vary a lot by their sensitivity. In some cases modifications are made in order to further enhance the signal such as using a sheath solution after the column outlet like in the case of TAHS analysis [2].

The aim of this work was to make a comparison on common grounds for the performance of five amino acid derivatization reagents, out of which 3 are commercially available: DNS, FMOC-Cl and DEEMM, and two are novel reagents designed for LC–ESI-MS analysis: TAHS – previously published by Shimbo et al. [2] and an in-house synthesized reagent 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF).

The choice of derivatization reagents was based on the different structure of the reagents since the focus of the article is on the suitability of reagents for LC–ESI-MS analysis, including chromatographic separation and ionization. FMOC-Cl and DNS represent commercially available and widely used derivatization reagents. DEEMM was chosen since methods have been previously developed in our workgroup and it is known that it provides good chromatographic separation and LoQ-s [15]. TAHS is known to provide low LoQ-s [2] and FOSF is a novel reagent and its properties have not been previously described.

All the comparisons are carried out with the same instrument and optimized according to each analyte. This way the results are directly comparable. If lower LoD-s are aimed at, several modifications can be used in order to enhance the signal: post-column infusions, splitters, etc. In present work, standard instrument setup was used.

For comparison, 7 amino acids were chosen (Arg, Asp, Gly, β-Ala, Pro, Trp, Phe) so that their properties would represent the variability of amino acid structures. Choice of amino acids was mainly based on their chromatographic behavior (retention): Arg and Asp are polar/ionic and elute fast, Phe and Trp are hydrophobic and elute late, the rest covering the intermediate range. Comparisons are done in positive ion ESI mode and MS/MS is used. Various aspects are compared in order to get widespread information about five amino acid derivatization reagents.

2. Materials and methods

2.1. Materials

HPLC-grade methanol and acetonitrile were obtained from J.T. Baker. Amino acid standards (L-proline, L-phenylalanine, L-aspartic acid, L-arginine, glycine, L-histidine, L-tryptophan) were purchased from Sigma; β-alanine was from Fluka. Derivatization reagents diethyl ethoxymethylenmalonate (DEEMM) and dansyl chloride (DNS) were purchased from Fluka and 9-fluorenylmethyl chloroformate (FMOC-Cl) was from Aldrich. Other chemicals: sodium hydroxide (Chemapol); acetic acid (Lach-Ner); sodium dihydrogensulfate (Merck); hydrochloric acid, boric acid, dichloromethane, iodomethane and ammonium hydroxide were from Reakhim, formic acid, N,N-dimethylamino-p-phenylenediamine (DPD), N,N′-dihydroxysuccinimidyl carbonate (DSC) and ammoniumbicarbonate from Aldrich and ammonium acetate from Fluka. All reagents were of analytical grade unless otherwise stated.

All aqueous solutions were prepared with ultrapure water purified by Millipore Milli-Q Advantage A10 (Millipore).

2.2. Synthesis of TAHS and FOSF

TAHS was synthesized as by Shimbo et al. [2] with minor modifications. DSC (600 mg) was dissolved in 25 mL of acetonitrile at room temperature. DPD (300 mg), dissolved in 25 mL of acetonitrile, was added dropwise to the DSC solution over a period of approximately 2 h. Then the reaction mixture was concentrated by rotary evaporation. The residue was resuspended in 5 mL of acetonitrile and then filtered, to obtain p-N,N-dimethylaminoanilyl N′-hydroxysuccinimidyl carbamate which was dissolved in 10 mL of acetonitrile/dichloromethane (4:1) at room temperature. Iodomethane (0.4 mL, 8 equiv.) was added to the solution, which was then stirred for 23 h at room temperature. After the reaction mixture was filtered, TAHS was obtained.

For synthesis of FOSF (Fig. 1a), DSC (80 mg) was dissolved in 4 mL of acetonitrile at room temperature. Hydrazinotripyrrolidinophosphonium hexafluorophosphate (synthesized as in [16]) (80 mg) dissolved in 4 mL of acetonitrile was added dropwise to the DSC solution over a period of approximately 2 h. Then the reaction mixture was concentrated by rotary evaporation. The residue was resuspended in 2 mL of water. Formed white crystals were filtered from the water. Synthesis had a quantitative yield.

Both for TAHS and FOSF, solutions of approximately 20 mg mL^{-1} in acetonitrile were prepared immediately and stored at −20 °C.

2.3. Instrumentation

HPLC system Agilent Series 1100 LC/MSD Trap XCT (Agilent Technologies, Santa-Clara, USA) was equipped with an in line degasser, a binary pump, an autosampler and a column thermostat. For detection photodiode array detector (PDA) with 6 mm path length flow cell and electrospray interface mass spectrometer (ESI-MS) were used. The system was controlled with Chemstation (Rev.A.10.02) and LCMSD Trap Control (Version 5.2) software. Chemstation (Rev.A.10.02) and DataAnalysis (Version 3.2) were used for UV and MS chromatograms analysis and peak integration.

Chromatographic analysis of DEEMM and TAHS derivatives was performed using an analytical column Synergi Hydro-RP 80A (4.60 mm × 250 mm, 4 μm) (Phenomenex, USA) with guard cartridge 4.0 mm × 2.0 mm, polar endcapped C18 (Phenomenex). For FMOC-Cl and DNS derivatives Eclipse XDB-C18 4.6 mm × 250 mm, 5 μm analytical column with guard column (4.6 mm × 12.5 mm, 5 μm; Agilent) was used. And for FOSF derivatives, Eclipse XDB-C18 4.6 mm × 150 mm, 5 μm analytical column with guard column (4.6 mm × 12.5 mm, 5 μm; Agilent) was used.

2.4. Preparation of standard solutions

Stock solutions of individual amino acids (1–20 mg g^{-1}) were prepared by dissolving respective substances in 0.1 M hydrochloric acid with 30% methanol. Stock solutions containing multiple amino acids, were prepared once and stored at −20 °C. All dilutions (0.5–3000 ng g^{-1}) were made with ultrapure MilliQ water. Working standard solutions were prepared daily.

2.5. Derivatization procedure

DEEMM derivatization: to 1 mL of sample 30 μL of DEEMM in 1.5 mL methanol, and 3.5 mL of 0.75 M sodium borate buffer (pH 9.0) were added [15,17]. The derivatized mixture was kept at room temperature protected from direct light for 24 h. LC–MS analysis has to be carried out at least 24 h but not more than 48 h after the derivatization [15]. Prior to LC–MS analysis, the sample

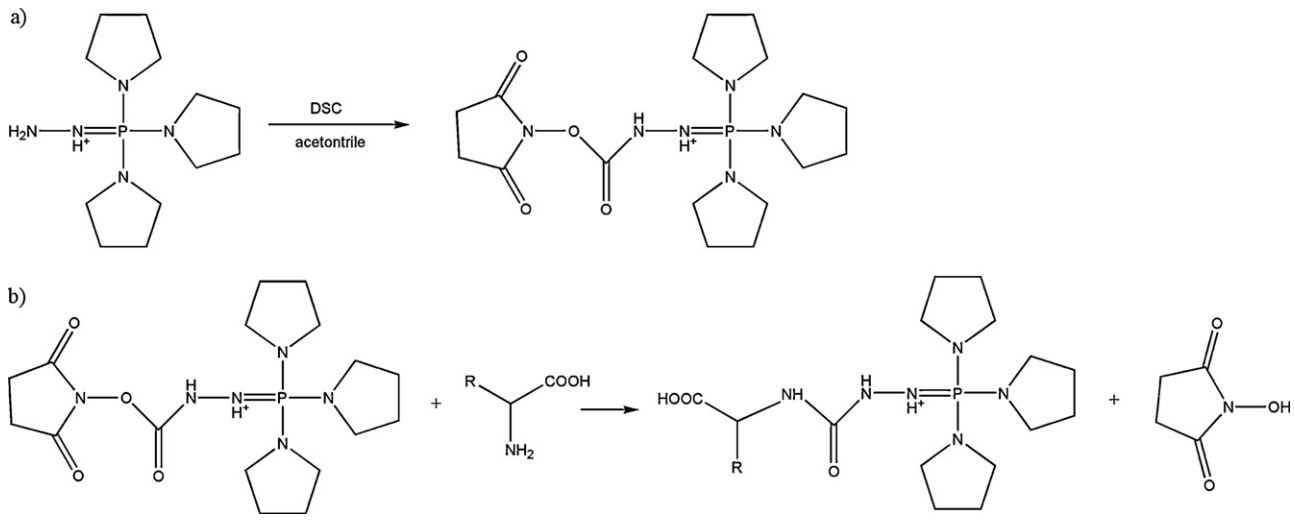

Fig. 1. (a) Synthesis of FOSF and (b) FOSF derivatization reaction with amino acids.

solutions were filtered through 0.45 μm cellulose acetate syringe filter (Whatman). The molar ratio of DEEMM to amino acids was over 500.

FMOC-Cl derivatization: to 300 μL of amino acid solution 300 μL of 0.75 M sodium borate buffer (pH 9.0) and 300 μL of FMOC-Cl (5 mM in acetonitrile) were added and vigorously mixed. The mixture was kept at room temperature for 5 min and then 300 μL of histidine solution (8 mg g^{-1} in MilliQ) was added and vigorously mixed again [18]. Prior to HPLC analysis, the sample solutions were filtered through 0.45 μm regenerated cellulose syringe filter (Agilent). The molar ratio of FMOC-Cl to amino acids was over 200.

DNS derivatization: to 100 μL of amino acid solution, 20 μL of 2 M NaOH and 30 μL of concentrated $NaHCO_3$ and 500 μL of DNS solution (10 mg mL^{-1} in acetone) were added. Reaction mixture was kept in refrigerator (approximately at 6 °C) in the dark for 45 min. Reaction was stopped with 10 μL of 25% NH_4OH [19]. Prior to LC–MS analysis, the sample solutions were filtered through 0.45 μm regenerated cellulose syringe filter (Agilent). The molar ratio of DNS to amino acids was over 25,000.

TAHS and FOSF (Fig. 1b) derivatization: with little modifications from the Ref. [2]. To 10 μL of amino acid solution 30 μL of 0.2 M sodium borate buffer (pH 9.0) and 20 μL of TAHS or FOSF solution (approximately 20 mg mL^{-1} in acetonitrile) were added. Reaction was carried out at room temperature and stopped after 10 min with 200 μL of 0.2% acetic acid in MilliQ. Heating was not necessary since tyrosine is not analyzed. The molar ratio of TAHS and FOSF to amino acids was over 23,000.

2.6. LC–UV–MS analysis

HPLC conditions for DEEMM derivatives were as follows: mobile phase A: buffer solution (pH = 3.2; 1 mM ammonium acetate in 0.1% formic acid); mobile phase B: acetonitrile. Gradient program was as follows: 0–12 min, 20–25%; 12–20 min, 25%; 20–50 min, 25–60% B. The eluent flow rate was 0.9 mL min^{-1} and the column was maintained at 40 °C and 5 μL of the sample was injected [15].

HPLC conditions for FMOC-Cl derivatives were as follows: mobile phase A: 0.1% formic acid; mobile phase B: acetonitrile. Gradient program was as follows: 0–45 min, 30–100% B. The eluent flow rate was 0.8 mL min^{-1} and the column was maintained at 30 °C and 10 μL of the sample was injected.

HPLC conditions for DNS derivatives were as follows: mobile phase A: 0.1% formic acid; mobile phase B: acetonitrile. Gradient program was as follows: 0–45 min, 10–100% B. The eluent flow rate was 0.8 mL min^{-1} and the column was maintained at 30 °C and 5 μL of the sample was injected.

HPLC conditions for TAHS derivatives were as follows: mobile phase A: pH = 3.2; 1 mM ammonium acetate in 0.1% formic acid; mobile phase B: acetonitrile. Gradient program was as follows: 0–30 min, 5–70% B. The eluent flow rate was 0.8 mL min^{-1} and the column was maintained at 30 °C and 5 μL of the sample was injected [2].

HPLC conditions for FOSF derivatives were as follows: mobile phase A: 1 mM ammoniumbicarbonate pH = 7; mobile phase B: acetonitrile. Gradient program was as follows: 0–20 min, 20–40% B. The eluent flow rate was 0.8 mL min^{-1} and the column was maintained at 30 °C and 5 μL of the sample was injected.

For all measurements, the wavelength for UV detection was 280 nm (full spectra were acquired for additional confirmation). ESI source parameters were same for all derivatization reagents: nebulizer gas (nitrogen) pressure 50 psi (345 kPa), drying gas (nitrogen) flow rate 12 L min^{-1} and drying gas temperature 350 °C. Other MS parameters were optimized for all the reagents and all amino acids. If derivatization is not used, the analyte solution can be directly used for optimization. When derivatization is employed, buffer solution is added, which contaminates the ESI source and interferes optimization. In order to obtain solutions of derivatized analytes free of buffer salts and excess of derivatization reagent (byproducts, hydrolysis products) following procedure was used: in high concentration (at mg g^{-1} levels) amino acid standards were derivatized and injected to the chromatographic system. For optimization, at the corresponding retention time of an amino acid derivative, effluent was collected and used for the MS optimization procedures.

Common MS parameter optimization is performed by ramping parameter values while standard solution of the analyte is infused and the procedure used for optimization was: tee-piece was used to mix the chromatographic solvent (0.8 mL min^{-1} or 0.9 mL min^{-1} for DEEMM-derivatives) with the infused amino acid effluent. Optimization with flow rate was applied since it has provided more optimal parameters [20]. The composition of the chromatographic solvent corresponds to the solvent composition at the time each amino acid derivative elutes. For the optimization, the starting point for all amino acid derivatives was the default parameters that the software provided for the target mass m/z 300.

In the early steps of method development, MS was used but once the chromatographic separation and derivatization procedures were fixed, MS/MS analysis was targeted. MS/MS parameters were optimized for all analytes.

3. Results and discussion

DEEMM, FMOC-Cl and DNS are amino acid derivatization reagents, which have been widely used for various applications over the years and are commercially available through various chemical suppliers. However, TAHS and FOSF are new reagents, specially designed for LC–ESI-MS analysis and not commercially available. Synthesis and derivatization procedure of TAHS has been published in literature [2]. FOSF was synthesized in-house.

3.1. Synthesis of new derivatization reagent

One of the reagents used for the comparison was a reagent called FOSF that has been designed for better LC–ESI-MS sensitivity. FOSF is based on phosphazenes and designed keeping in mind the electrospray ionization efficiency scale, which brings out that phosphazenes are among the compounds of highest ionization efficiency [21]. Phosphazenes have been previously synthesized in our workgroup [16]. One of them was used as a starting material for the synthesis of FOSF. In order to make the phosphazene into a derivatization reagent, the reaction scheme used was similar to the previously published TAHS synthesis. High ionization efficiency of TAHS is achieved incorporating permanently charged quaternary ammonium group in its structure by alkylation of tertiary ammonium [2]. However, since FOSF is a strong base, there is no need for the additional step of creating permanently charged group. Therefore, the use of harmful iodomethane is not necessary in FOSF synthesis. Synthesis of phosphazene based derivatization reagent was successful and a novel derivatization reagent was created, called FOSF. Derivatization procedure was chosen the same as for TAHS.

3.2. General comparison

When comparing derivatization properties of DEEMM, FMOC-Cl, DNS, TAHS and FOSF, DEEMM is poor for analysis of Pro. DEEMM-Pro derivative is not stable resulting in high LoQ of the analysis [17,22]. In this work, no LC–MS signal could be registered for DNS-Asp even at elevated Asp concentrations. However, previously DNS has been used for Asp analysis [23–25]. FMOC-Cl proved to be suitable for derivatization and analysis of all the tested amino acids. TAHS provided very good signal for all amino acids, similar to FMOC-Cl. Elevated concentration of Arg solution was needed for FOSF derivatization – FOSF-Arg signal could not be obtained at normal concentration. This is most probably related to high basicity of Arg side chain.

3.3. Derivatization procedures

One aspect of the derivatization procedure is the preparation of a derivatization reagent solution. For FMOC-Cl and DNS, it is a general practice that a fresh solution is made before each measurement due to the instability of the solutions. For FOSF and TAHS, no such information is available. However, experiments showed that there were no problems with FOSF and TAHS derivatization reagent solutions (20 mg mL^{-1}) for about a 9-month period (12 for TAHS). After that time the ability to derivatize amino acids decreased significantly and it was not possible to use the same solution again. Therefore, even though the solution is stable for a relatively long time, the preferred storage of FOSF would be a solid instead of a solution form. Even though the article for TAHS [2] does not suggest it, the same would be preferable for TAHS. In conclusion, for all amino acid reagents, except DEEMM, it is preferable that a freshly prepared derivatization reagent solution is made before each measurement. For DEEMM, this practice is not necessary since DEEMM is used for derivatization without any dissolution and over the course of approximately 8 years of usage, no problems were observed with derivatization.

For most amino acid derivatization reagents, the derivatization procedures are quite similar: derivatization reaction is carried out at high pH for some relatively short time and then the reaction is ended with a compound that would react with the excess reagent or the pH of the medium is changed in order to stop the reaction.

Out of these 5 derivatization procedures, for FMOC-Cl and DNS, the reaction was stopped by using up the excess reagent: solution of histidine for FMOC-Cl and ammonia for DNS. For TAHS and FOSF, the pH of the derivatization mixture was changed with acetic acid and excess reagent is not removed since it does not interfere with the chromatographic analysis and MS detection. The simplest derivatization reaction is for DEEMM – excess DEEMM does not need to be removed or the pH changed. However, the downside is that, before analysis, the reaction mixture should stand for about 24 h in order to proceed with the LC–ESI-MS analysis [15].

It is also important to discuss the possibilities for method automatization, which has been addressed in Ref. [26]. As for derivatization reagents under comparison, DEEMM, TAHS and FOSF can in principle be used with an automated system. Moreover, FMOC-Cl has been used with automated derivatizations [27,28] as well as DNS [29].

3.4. Repeatability

With MS, the signal stability can be very different depending on the analyte, solvents and also the cleanliness of the ESI-source. Stability of the signal was evaluated at higher (amino acid concentrations around 3000 ng g^{-1}) and lower concentrations (amino acid concentrations around 50 ng g^{-1}). Relative standard deviations of peak areas of six consecutive injections were calculated. Relative standard deviations at higher concentrations were all under 10%, which is acceptable in MS analysis and comparable to other LC–ESI-MS methods for derivatization reagents [8,10]. However, differences between the reagents emerged when signal stability at lower concentrations was assessed. Results showed that for most reagents, even at low analyte concentration, relative standard deviation is below 10% and therefore very good for measurements at LoQ levels. However, for DNS, signals for different amino acids at low concentrations provided poor stability (9–25%). Even though the absolute signals at low concentration levels were high compared to DEEMM and FMOC-Cl, the repeatability was poor. Reasons for that are unknown but might be related to the composition of derivatization mixture, which might produce signal modifiers, which are influential at low concentrations.

3.5. Chromatographic separation

Before the comparison, chromatographic methods for all the reagents were evaluated. Methods for DEEMM and FMOC-Cl derivatives have been previously developed with acetonitrile [30]. Moreover, for DNS, TAHS and FOSF, chromatographic separation with acetonitrile proved to be better than with methanol.

For DNS, the same column used for FMOC-Cl was applied (Eclipse XDB-C18 4.6 mm × 250 mm, 5 μm). For TAHS, with Eclipse, it was more difficult to obtain separation between the derivatives and therefore, the same column used for DEEMM derivatives was chosen, since it has shown better separation properties (Synergi Hydro-RP 80A 4.60 mm × 250 mm, 4 μm) for polar analytes. For FOSF, the choice of column is described below.

For the buffer component of the chromatographic eluent, 1 mM ammonium acetate in 0.1% formic acid and 0.1% formic acid were tested. Choices were based on the better ionization properties: 1 mM ammonium acetate in 0.1% formic acid for TAHS and DEEMM and 0.1% formic acid for FMOC-Cl and DNS.

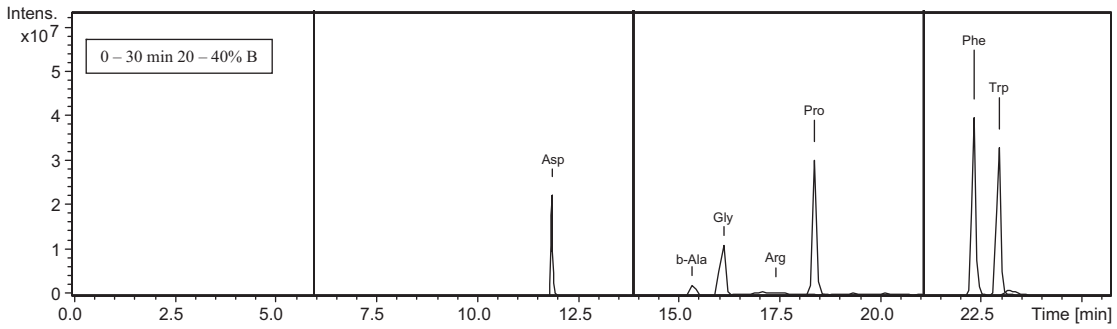

Fig. 2. Representative chromatogram of FOSF-derivatives.

For FOSF, several eluent compositions and components (methanol, acetonitrile, 1 mM ammonium acetate in 0.1% formic acid and 0.1% formic acid), pH values in range of 2–7 and gradient programs were tested (including isocratic). The best chromatographic separation of FOSF derivatives was achieved with 100 mm shorter column and ammoniumbicarbonate buffer (pH = 7). Moreover, peak shapes were better with these conditions compared to longer columns and more acidic eluents (Fig. 2).

However, getting all amino acids separated would be a challenge for the charged derivatization reagents – FOSF and TAHS [2].

Various reagents are very different by their chromatographic separation properties. Differently from traditional derivatization reagents, novel reagents are charged at chromatographic conditions, which is unfavorable for their reversed phase separation. Separation is more easily obtained for DNS, FMOC-Cl and DEEMM. For all amino acid derivatives, the retention times obtained are presented in Table 1. Comparison shows that for commercial derivatization reagents, the elution order of amino acid derivatives is the same, but for TAHS and FOSF, the order of elution is slightly different.

For DEEMM, in previous works, complete separation was obtained for 23 amino acids [15]. Moreover, selenomethylselenocysteine and selenomethionine can be separated from 23 amino acids with DEEMM also, providing chromatographic separation for 25 amino acids in total [3]. This is quite significant since for many derivatization reagents, separation of that many amino acids could be a challenge. For the seven amino acids under interest, FMOC-Cl and DNS provide relatively good separation also. However, in case of FMOC-derivatives, separation of Gly and β-Ala derivatives was problematic.

Retention times of the derivatives of different amino acids vary since the content of acetonitrile needed for elution depends on the amino acid and reagent used. FMOC-Cl and DNS need significantly higher content of acetonitrile for elution. In case of DEEMM, the difference in the content of acetonitrile in the eluent between the fastest eluting peaks and the latest eluting peak is the largest. This results in a longer run and better chromatographic separation. In the case of TAHS and FOSF, peaks start to elute at significantly lower acetonitrile content and peaks are eluting at quite similar eluent compositions resulting in a poor chromatographic separation and also shorter run times.

3.6. Linearity

Linearity of amino acid derivative calibration graphs was assessed. It was observed that for different derivatization reagents, the linear dynamic ranges differed significantly (Table 2). Therefore, it was briefly investigated by visual inspection of residual plots and squared correlation coefficients.

By its nature, LC–ESI-MS/MS does not have a very wide linear dynamic range and for certain analytes, linear range can be even narrower [31]. Therefore, a comparison of linear range between the reagents for LC–ESI-MS/MS analysis was included. For DEEMM, FMOC-Cl and DNS linear dynamic ranges are quite wide and this is their advantage over novel reagents, which seem to have quite limited linear dynamic range. This could be due to their very sensitive nature in ESI-source and moreover, due to the fact that they carry a charge. For DEEMM, the linear dynamic range is very large and this means that it is easy to apply for samples with wide range of amino acid concentrations.

In the case of novel reagents, these could be used for especially sensitive analysis and also in the very narrow amino acid concentration ranges. This is not desirable for applications where the analyte concentration in the sample can vary to a large extent.

3.7. LoD and LoQ values

The limits of detection (LoD) were calculated from amino acid standards and expressed as three times the standard deviation ($n = 6$) and the limits of quantitation (LoQ) were calculated from the injections of amino acid standard solutions and are expressed as ten times the standard deviation ($n = 6$). Since all methods were equally optimized, results can be compared. Analysis was done with LC–ESI-MS/MS and LoD and LoQ are expressed in femtomoles on the column (Table 3).

Table 1
Retention times in minutes and percentage of acetonitrile content at the time of the elution for amino acid derivatives.

	DEEMM	FMOC-Cl	DNS	TAHS	FOSF
Arg	14.2 (25%)	13.0 (46%)	15.2 (35%)	14.8 (17%)	14.0 (27%)
Asp	23.0 (29%)	19.8 (57%)	[a]	15.6 (18%)	9.0 (19%)
Gly	27.5 (34%)	22.1 (60%)	22.0 (49%)	15.2 (17.5%)	13.5 (26%)
β-Ala	31.4 (38%)	22.5 (61%)	23.1 (51%)	17.3 (20%)	12.8 (25%)
Pro	35.0 (43%)	25.8 (66%)	27.4 (60%)	19.1 (21%)	15.8 (30%)
Trp	45.6 (55%)	28.7 (70%)	28.7 (62%)	31.6 (34%)	19.7 (35.5%)
Phe	47.0 (57%)	30.2 (73%)	30.4 (65%)	29.9 (32%)	20.3 (36%)

[a] The signal of Asp was not obtained for DNS analysis.

Table 2
Dynamic linear range for amino acid derivatization reagents in fmol.

	DEEMM	FMOC-Cl	DNS	TAHS	FOSF
Arg	108–27,871	222–5502	142–14,126	26–230	[c]
Asp	156–40,295	488–8025	[b]	37–335	60–333
Gly	266–68,863	1662–14,114	364–36,238	24–589	98–492
β-Ala	212–54,790	1288–10,933	282–28,071	52–456	30–141
Pro	[a]	363–9543	246–24,501	16–398	53–254
Trp	52–13,541	108–2618	67–6722	13–109	4–47
Phe	50–13,004	103–2737	70–7026	12–114	9–239

[a] Pro was unstable for DEEMM analysis and not added to the comparison.
[b] The signal of Asp was not obtained for DNS analysis.
[c] The signal of Arg for FOSF was not stable and not considered for linear range.

Table 3
LoQ values for the studied amino acid derivatives obtained at $n = 6$ and expressed in fmol on column.

	DEEMM	FMOC-Cl	DNS	TAHS	FOSF
Arg	84	259	365	81	[c]
Asp	154	943	[b]	117	96
Gly	384	3615	3887	61	168
β-Ala	227	1687	377	101	54
Pro	[a]	174	1381	31	130
Trp	53	164	55	92	7
Phe	26	193	252	22	41

[a] Pro was unstable for DEEMM analysis and not added to the comparison.
[b] The signal of Asp was not obtained for DNS analysis.
[c] The signal of Arg for FOSF was not stable and LoD/LoQ values obtained not reliable.

To the best of our knowledge, comparison of LoD and LoQ values for five different amino acid derivatization reagents with the same LC–ESI-MS/MS system has not been reported previously. When comparing overall results, it is clear that different reagents do provide different results. It can be concluded from the results that the difference in the LoD/LoQ values is mostly due to the differences in the molecular structure of the reagents. It has been proposed that with higher organic content in the ionization source, the ionization efficiency is better [8]. However, in the current case, the LoD/LoQ values are lower for reagents that elute with lower acetonitrile content (TAHS and FOSF) and LoD/LoQ values are higher for those derivatives that elute at the higher acetonitrile content. This is also related to the fact that TAHS and FOSF are carrying a permanent charge.

Moreover, amino acids differ from each other also, meaning that with the same reagent, LoD/LoQ values for different amino acids vary significantly. For example, for FMOC-Cl, the LoD/LoQ values for Trp and Phe are significantly lower than for Gly and β-Ala (up to 18 times). For amino acids that elute in the end of the chromatogram, with higher organic percentage in the eluent, LoD/LoQ values are much lower than for amino acids that elute in the beginning of the chromatogram. It can be attributed to the fact that ionization is better with higher organic percentage in the eluent [5]. In addition, for all reagents, Gly derivatives provide much higher LoD/LoQ values compared to other amino acids (except for TAHS). It could be related to the fact that Gly is also the simplest amino acid by its structure and therefore, the ionization efficiency is determined mostly by the reagent side. Moreover, their retention on the reversed phase chromatography is poor and they elute at low organic solvent composition meaning that the ionization is not as good as for the later eluting amino acids. In order for a molecule to carry a charge and at the same time have a good chromatographic retention, the charge should be sterically shielded. This is taken into account with a design of FOSF.

Comparison of the reagents to each other shows that novel reagents, TAHS and FOSF, do differ from the commercially available derivatization reagents providing lower LoD and LoQ values. From commercially available reagents, DEEMM shows comparable results to new reagents. Moreover, DEEMM has been used for very sensitive selenoamino acid detection [3] and proves that DEEMM is very sensitive for LC–ESI-MS/MS analysis. Besides, it is commercially available and provides much better chromatographic separation than novel reagents because DEEMM does not carry a permanent charge. This is a useful concept for novel LC–MS derivatization reagents – derivative part should be uncharged for better chromatographic separation, but easily charged in ESI. According to ionization efficiency scale in [21], diesters like DEEMM, are therefore good candidates for derivatization reagents.

As for FMOC-Cl and DNS, these provide higher LoD/LoQ values. It is important to note that DNS provides signal at very low concentrations but due to poor signal stability, the values for LoD/LoQ indicate that DNS is not as good as DEEMM. If the stability problem with DNS is addressed, its LoD/LoQ values could be lowered.

LoD/LoQ values of novel derivatization reagents are quite similar to each other. One could not be preferred to the other by these results.

Since the results are calculated in fmol on column, LoD/LoQ could be improved by using bigger injection volume or decreasing volumes of derivatization solvents, in order to increase the amount of analyte entering the column.

In conclusion, choosing a derivatization reagent carefully can significantly influence the method sensitivity.

3.8. MS/MS analysis

In most cases, MS/MS analysis is targeted since MS/MS provides more sensitive analysis. Therefore, a discussion of the MS/MS analysis with different reagents is included. Supplementary Table S1 shows the parent and fragment ions chosen for the MS/MS analysis. In most cases, the $[M+H]^+$ is used as parent ion (DNS, TAHS, FOSF). Upon fragmentation DNS-, TAHS- and FOSF-derivatives, loss of neutral amino acid part occurs and fragments with constant m/z for each reagent are observed (m/z 252 for DNS, m/z 177 for TAHS and m/z 298 for FOSF) (Fig. 3). However, for FMOC-Cl and DEEMM derivatives, Na-adduct is fragmented (except Arg) since Na-adducts were more abundant than $[M+H]^+$. Moreover, signal from the protonated form was missing from the MS spectra of FMOC-derivates. For FMOC-Cl, all derivatives (except Arg) gave the same fragment, 263. However, Arg is different than other 6 amino acids by not giving a Na-adduct and fragmenting differently, giving a fragment with m/z 336. This can be explained by FMOC-Cl reacting with the more basic side-chain amino group rather than α-amino group. The same applies to DEEMM-Arg that does not have a Na-adduct either. Fragmentation of DEEMM derivatives differs from FMOC-Cl derivatives, since for each amino acid, different fragment is observed and neutral 46 amu fragment is lost. This makes DEEMM fragmentation different from all other derivatization reagents discussed in this work. Regarding MS analysis modes, single reaction monitoring (SRM) can be used for both types of derivatives. Additionally, neutral loss scan can be used for DEEMM derivatives and parent ion scan for other derivatives. Both modes can be utilized for analysis of complex mixtures.

It is discussed that TAHS has been designed keeping MS/MS fragmentation in mind [2]. Other derivatives included in the present study proved to be also suitable for MS/MS analysis. In all cases, LoQ values were lower for MS/MS than for MS.

3.9. Practical considerations

The choice of amino acid derivatization reagents is wide and the comparison of five reagents for the LC–ESI-MS/MS analysis brings out the differences between the commercially available reagents and the novel reagents.

From practical point of view, it is better to use reagents that have been in use longer and are also suitable for LC–ESI-MS/MS analysis. If very sensitive analysis is targeted, the use of novel reagents could be considered. However, since little method optimization information is available, much care must be taken in order to optimize the derivatization procedure and chromatographic separation.

Moreover, pricewise, DEEMM is significantly cheaper than other reagents. DNS and FMOC-Cl are both commercially available but price difference with DEEMM is tens of times. As for TAHS and FOSF, in addition to the necessary synthesis effort, materials for their synthesis are expensive. Therefore, from the availability point of view, DEEMM is one of the best choices for amino acid derivatization.

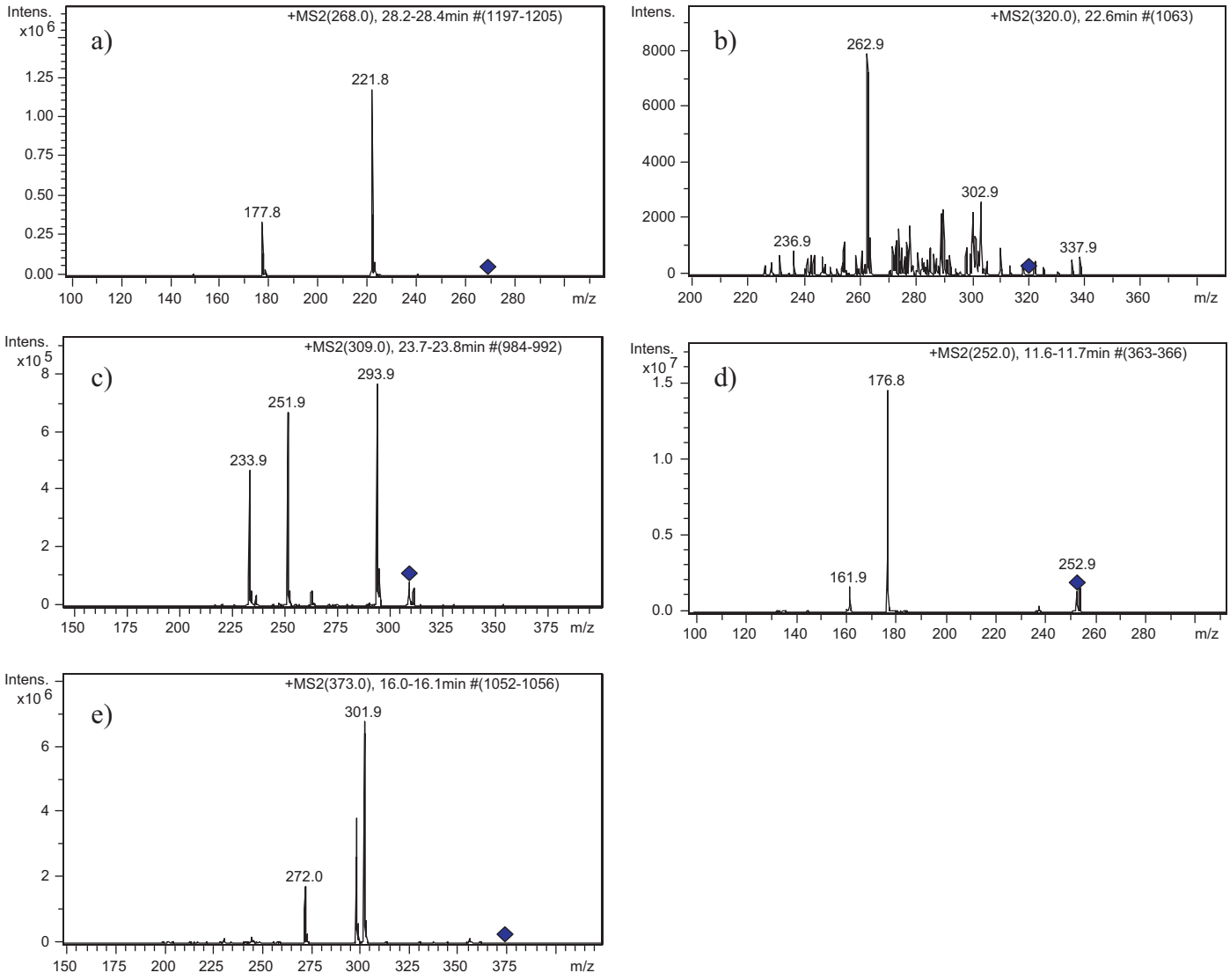

Fig. 3. Fragmentation spectra for Gly derivatives: (a) DEEMM ($m/z = 222$), (b) FMOC-Cl, (c) DNS, (d) TAHS and (e) FOSF.

4. Conclusions

Comparison of five amino acid derivatization reagents for LC–ESI-MS/MS analysis shows that there are big variations in chromatographic separation, LoD/LoQ values and also dynamic linear ranges.

By their properties, novel derivatization reagents (TAHS and FOSF), specifically synthesized for sensitive LC–ESI-MS analysis, and commercially available derivatization reagents (DNS, FMOC-Cl and DEEMM), group together. Novel reagents provide poorer chromatographic separation and smaller dynamic linear range than DEEMM, FMOC-Cl and DNS. The main advantage of novel reagents is that since they have been specifically synthesized for sensitive LC–ESI-MS analysis, they provide lower LoD/LoQ values. However, from the commercial derivatization reagents, DEEMM provides similar LoD/LoQ values to novel reagents in addition to good chromatographic separation and wide dynamic linear range. Only limitation of DEEMM is that with Pro it has higher LoD/LoQ values. All derivatization reagents were suitable for sensitive MS/MS analysis and provided very stable signal.

It could be concluded that even though novel reagents provide lower LoD/LoQ values, commercially available reagents have some advantages from the performance and availability point of view. Therefore, out of compared derivatization reagents, DEEMM is most optimal for amino acid derivatization.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.07.029.

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