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Volatile ion-pairing agents for liquid chromatographic–thermospray mass spectrometric determination of amino acids and amino acid amides

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ABSTRACT

The behaviour of volatile ion-pairing agents in terms of performance of the combined liquid chromatography–mass spectrometry is described. The applicability of heptafluorobutanoic acid, tridecafluoroheptanoic acid and nonadecafluorodecanoic acid as volatile ion-pairing agents is demonstrated by the analysis of mixtures of amino acids and the corresponding amino acid amides. Translation of a non-volatile to a volatile ion-pair system that is suitable for thermospray mass spectrometry is described. Using nonadecafluorodecanoic acid, the retention times of the compounds studied were comparable to those obtained with *n*-dodecylsulphonic acid. Further, with nonadecafluorodecanoic acid, the repeatability of the tests and the calibration graphs of the compounds investigated were good. The sensitivity of the mass spectrometer towards the compounds of interest was greatly improved by postcolumn addition of trifluoroacetic acid and ionization with gaseous ammonia. Using nonadecafluorodecanoic acid, the method was applied to the analysis of samples from bio-organic synthesis.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) is one of the most commonly used techniques for the separation of both polar and ionic compounds, with or without using non-volatile ion-pairing agents. In liquid chromatography–thermospray mass spectrometry (LC–TSP-MS), volatile buffers and ion-pairing agents are required for the mass spectrometer. Hence, either replacement of non-volatile substances by volatile equivalents or post-column suppressor techniques [1–3] are necessary.

The use and selectivity of volatile buffers for LC–TSP-MS have been evaluated by several workers [4–6]. Applications of volatile ion-pairing agents with LC–UV and amperometric detection have been reported [6–10]. In these studies short-chain perfluorinated carboxylic acids are used instead of strong non-volatile ion-pairing agents. Compared with alkylsulphonates, perfluorinated carboxylic acids have lower boiling points and are therefore suitable for preparative chromatography [8,9]. So far, only a short-chain perfluorinated carboxylic acid has been reported as a volatile ion-pairing agent for LC–TSP-MS analysis [6].

The aim of this study was to replace the non-volatile additives used in a LC reaction detection system for the determination of amino compounds [11] by volatile additives which are suitable for LC–TSP-MS. To this end, the behaviour of a series of perfluorinated carboxylic acids as volatile ion-pairing agents for LC–TSP-MS was investigated. In the mobile phase, sodium phosphate was replaced with triethylammonium (TEA) formate. The selection of this type of buffer and ion-pairing agent still allowed the use of the reaction detection system [11] for routine analysis, while the LC–TSP-MS system

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could be applied for identification and/or confirmation purposes.

The influence of heptafluorobutanoic acid (HFBA), tridecafluoroheptanoic acid (TDFA) and nonadecafluorodecanoic acid (NDFA) on the retention of several amino acids and amino acid amides was investigated and compared with *n*-dodecylsulphonic acid.

In order to increase the mass spectrometric sensitivity, both postcolumn addition of trifluoroacetic acid (TFA) and chemical ionization with gaseous ammonia [12] were applied. After optimization of the mass spectrometric parameters, spectra of the amino acids and amides were recorded in the full-scan mode. Determination of the compounds was performed in the multiple-ion detection (MID) mode.

The sensitivity, linearity and repeatability of the LC–TSP-MS system were investigated.

EXPERIMENTAL

Chemicals

α-Methylalaninamide (α-Me-Ala-NH_2) was obtained from DSM Research. Other amino acid amides and amino acids were obtained from Sigma (St. Louis, MO, USA). The pairing ions *n*-dodecylsulphonic acid, TDFA and NDFA were supplied by Aldrich (Milwaukee, WI, USA), TFA and HFBA by Janssen Chimica (Beerse, Belgium) and 2-propanol (IPA), ethanol, 2-mercaptoethanol (MCE) and *o*-phthalaldehyde (OPA) by Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade. Water was purified with a Milli-Q system (Millipore). Samples from bio-organic synthesis were supplied by DSM Research.

Buffer, eluent, reagent and sample preparation

Sodium phosphate and triethylammonium (TEA) formate buffers were prepared by dissolving the corresponding bases (0.1 *M*) in water and, after adding the required amount of ion-pairing agent, adjusted the pH to 3.0 with phosphoric acid and formic acid, respectively. The eluents were mixtures of buffer containing different amounts (0.5–8 m*M*) of ion-pairing agent and 20% (v/v) 2-propanol. Potassium borate buffer (0.4 *M*, pH 10.0) was prepared by dissolving boric acid in water and adjusting the pH to 10.0 with potassium hydroxide solution. OPA–MCE reagent was a mixture of potassium borate buffer (0.4 *M*, pH 10.0), OPA (6 m*M*), MCE (0.1%) and ethanol (1.0%). Standard solutions of the amino acids and the amino acid amides were dissolved in the appropriate eluent. Samples were diluted with 0.1 *M* formic acid to pH 3.0.

Instrumentation

A Gilson (Villiers-le-Bel, France) Model 305 pump was used for solvent delivery; injection was performed with a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 20-μl loop. The column used was a Nucleosil-120-C_{18} (250 × 4.0 mm I.D., 5 μm) from Macherey–Nagel (Düren, Germany). The flow-rate was 1.0 ml/min. The separations were carried out at ambient temperature.

For reaction detection, the OPA–MCE reagent was added to the column effluent by a mixing T-piece. This was effected with a Gilson Model 305 pump at a flow-rate of 1.0 ml/min. The reaction was carried out in a coiled capillary stainless-steel tube (12 m × 0.35 mm I.D., coil diameter 12 mm) at ambient temperature. The fluorescence detector was a Waters Model 420, equipped with a 338-nm band-pass filter for excitation and a 415-nm long-pass filter for emission. Peak integration and quantitative analysis were performed with a Hewlett-Packard Model 3350 laboratory data system.

For MS detection, a Finnigan MAT TSQ-70 triple quadrupole mass spectrometer equipped with a thermospray interface was used. To maintain optimum ionization conditions, postcolumn addition of 2% (v/v) aqueous TFA solution at a flow-rate of 0.1 ml/min was effected with a Gilson Model 302 pump. The solution was added to the column effluent using a Lee (Frankfurt, Germany) visco-jet micromixer. Gaseous ammonia, obtained from Hoek Loos (Amsterdam, Netherlands), was added at an optimum source pressure of 120 Pa (0.9 Torr). After optimization, the vaporizer temperature, the repeller voltage and the source temperature were kept at 85°C, 20 V and 190°C, respectively. The electron multiplier was operated at 2 kV. Scanning was performed from mass 80 to 310 with a scan time

of 2 s. MID with a scan time of 0.24 s per m/z (*ca.* 2 s) was used for quantitative analysis on the $[M + TEA + H]^+$ ions at m/z 191 and 190 (Ala and Ala-NH_2), m/z 205 and 204 (α-Me-Ala and α-Me-Ala-NH_2), m/z 219 and 218 (Val and Val-NH_2) and m/z 233 and 232 (Leu and Leu-NH_2).

RESULTS AND DISCUSSION

Influence of the pairing ion on retention and separation of amino compounds

For the separation of a mixture of four amino acids and the corresponding acid amides, the influence of four types of perfluorinated ion-pairing agents was investigated. Fig. 1 shows the separation of the mixture using HFBA, TDFA and NDFA. The concentration of the pairing agent was 4 m*M*. For comparison, the chromatogram obtained using *n*-dodecylsulphonate (0.5 m*M*) is also given in Fig. 1. With increasing chain length of the perfluorinated pairing agent, an increase in retention was noted for all amino acids and amino acid amides. The best separation of the compounds was obtained using NDFA as ion-pairing agent (Fig. 1d). Seven out of eight amino compounds could be separated using this type of pairing agent. With *n*-dodecylsulphonate (0.5 m*M*), six out of eight compounds were separated (Fig. 1e). Regarding the chromatographic performance of NDFA, it can be stated that this ion-pairing agent is a good substitute for *n*-dodecylsulphonate in the separation of amino acids and acid amides.

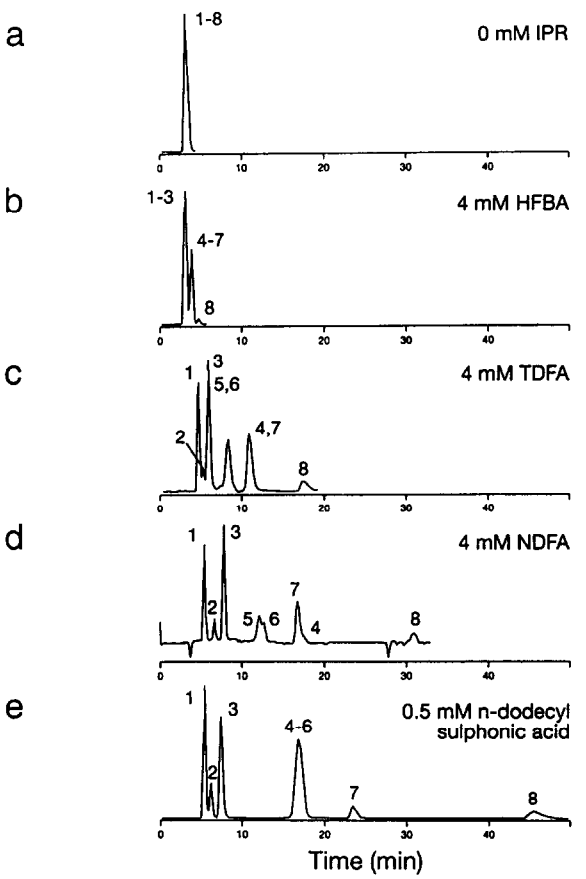

Fig. 1. Chromatograms of amino acids and amino acid amides. Buffer: (a–d) 0.1 *M* triethylammonium formate; (e) 0.1 *M* sodium phosphate. Peaks: 1 = Ala; 2 = α-Me-Ala; 3 = Val; 4 = Leu; 5 = Ala-NH_2; 6 = α-Me-Ala-NH_2; 7 = Val-NH_2; 8 = Leu-NH_2. Detection, fluorescence. For other conditions, see Experimental.

The dependence of the retention times of the amino compounds on the concentration of TDFA and NDFA is shown in Fig. 2. An increase in the retention times of all compounds was noted with an increase in the concentration of TDFA.

For NDFA, a substantial increase in retention was seen up to 1 m*M*. At higher concentrations of NDFA, only a slight effect of the ion-pairing agent concentration on the retention could be observed.

Mass spectrometric data

Postcolumn addition of TFA to the column effluent resulted in an effluent pH of less than 2, which facilitated the ionization of the amino acids, resulting in an increase in sensitivity. Addition of gaseous ammonia also increased the sensitivity to both amino acids and amino acid amides. The sensitivity to the amino acids and amino acid amides was enhanced overall by a factor 3 and 5, respectively.

The base peak in all the mass spectra was the $[M + TEA + H]^+$ ion (M + 102). The $[M + H]^+$ ion (M + 1) and the $[M + IPA + H]^+$ ion (M + 61) were also present as abundant ions in the mass spectra of the compounds investigated. As an example, the mass spectra of Val and Val-NH_2 are shown in Fig. 3.

In Table I, the intensities of the most abundant ions in the mass spectra of the compounds studied are shown. For the amino acids, the intensity of the $[M + 61]^+$ ion was higher than

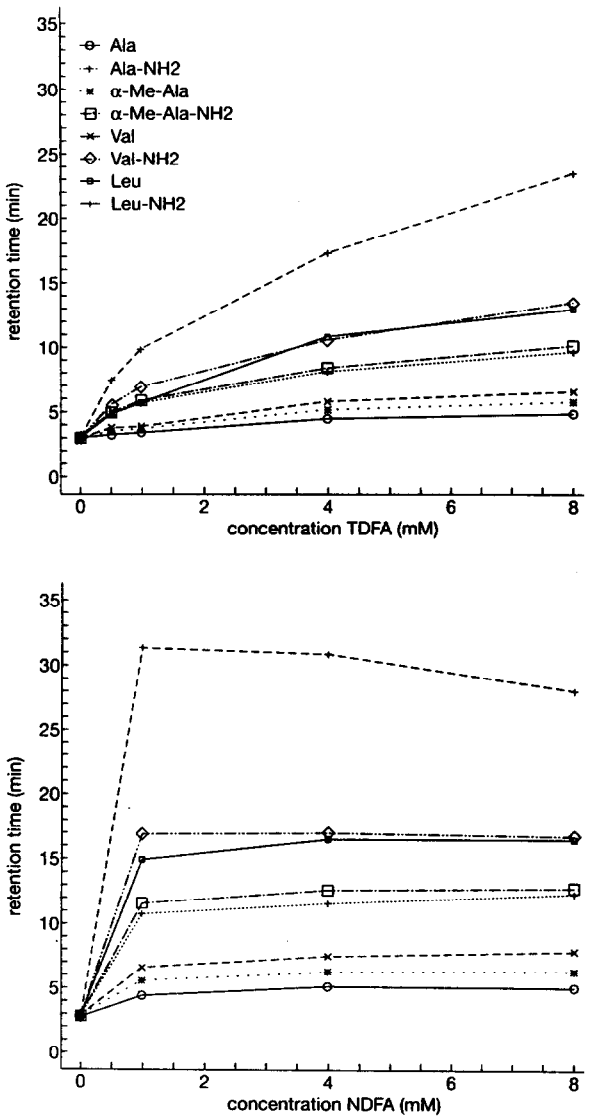

Fig. 2. Dependence of retention times on the concentration of TDFA and NDFA. Mobile phase, 0.1 *M* triethylammonium formate; detection, fluorescence. For other conditions, see Experimental.

that of the $[M + 1]^+$ ion, whereas for the amino acid amides the opposite was observed. This difference may be explained by the difference in proton affinity of the α-amino group of the acid and the acid amide. As the $[M + TEA + H]^+$ ion was the most intense ion in the mass spectra (Table I), this ion was selected for MS detection.

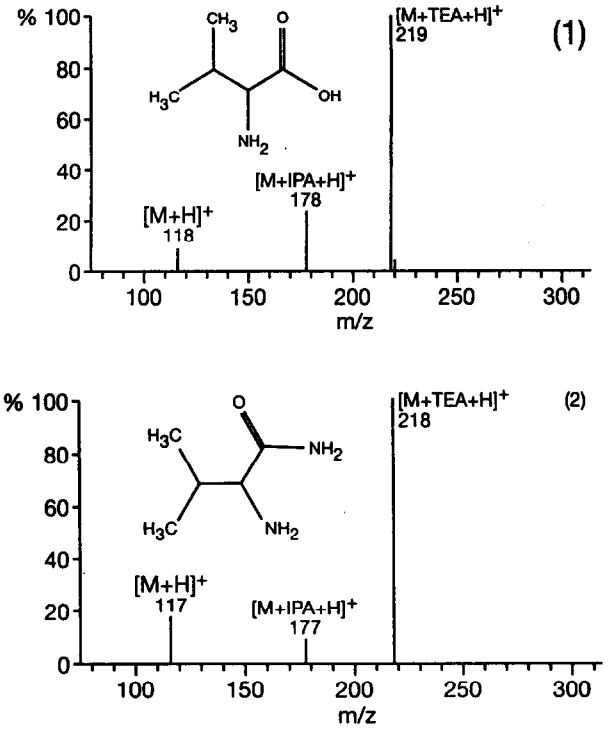

Fig. 3. Mass spectra of (1) Val and (2) Val-NH_2.

Linearity, precision and sensitivity of the LC–TSP-MS method

The linearity of the amount *versus* MID response relationship was established over the range 200 ng–20 μg for each of the compounds studied. Linear regression analysis from calibration graphs indicated that the correlation co-

TABLE I

INTENSITIES (%) OF THE MOST ABUNDANT IONS IN THE MASS SPECTRA OF THE AMINO COMPOUNDS STUDIED

Compound	M + 102	M + 61	M + 1
Ala (M_r 89)	100	80.3	16.6
Ala-NH_2 (M_r 88)	100	<1	12.9
α-Me-Ala (M_r 103)	100	3.0	<1
α-Me-Ala-NH_2 (M_r 102)	100	<1	<1
Val (M_r 117)	100	24.4	9.3
Val-NH_2 (M_r 116)	100	4.8	13.9
Leu (M_r 131)	100	19.6	12.3
Leu-NH_2 (M_r 130)	100	<1	9.5

TABLE II

MINIMUM DETECTABLE AMOUNTS (MDA) WITH MID DETECTION

Compound	MDA (μg)	Compound	MDA (μg)
Ala	0.5	Val	0.3
Ala-NH_2	0.2	Val-NH_2	0.2
α-Me-Ala	0.1	Leu	0.9
α-Me-Ala-NH_2	0.2	Leu-NH_2	0.1

efficients were >0.9995, except for Ala (0.994), Ala-NH_2 (0.998) and Val (0.998). The within-run precision of the assay gave a relative standard derivation (R.S.D.) of <5% ($n = 3$; 1–3 μg level) for Val-NH_2, α-Me-Ala, α-Me-Ala-NH_2, Leu and Leu-NH_2. For Ala, Ala-NH_2 and Val, an R.S.D. of >5% ($n = 3$, 1–3 μg level) was found.

The detection limits for the compounds studied using MID, based on a signal-to-noise ratio of 3, are given in Table II.

As an application, a representative chromatogram of a Val-NH_2 sample from a chemo-enzymatic reaction performed on the laboratory scale is shown in Fig. 4. Using MID at m/z 218 and 219, selective detection of Val-NH_2 and Val, respectively, could be achieved. At m/z 219, the isotope peak of Val-NH_2 is clearly visible.

In this manner, LC–TSP-MS analysis was used for confirmation of the results obtained by means of the LC reaction detection system.

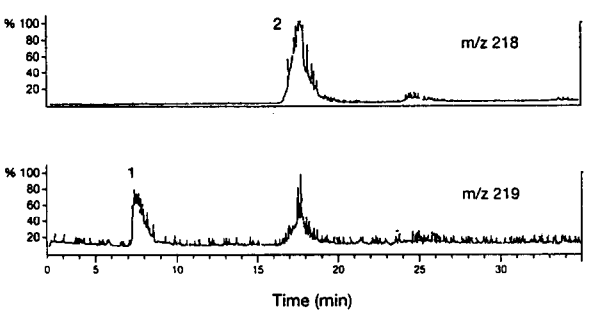

Fig. 4. Mass chromatograms of a Val-NH_2 sample from an enzymatic hydrolysis experiment. Peaks: 1 = Val; 2 = Val-NH_2. Mobile phase, 0.1 *M* triethylammonium formate and 8 m*M* NDFA. For other conditions, see Experimental.

CONCLUSIONS

For the determination of amino acids and amino acid amides, a non-volatile mobile phase consisting of sodium phosphate buffer and *n*-dodecylsulphonic acid has been successfully translated to a volatile system containing triethylammonium formate and nonadecafluorodecanoic acid, which is suitable for LC–TSP-MS analysis. The predominant ions in the mass spectra of the amino compounds are $[M + TEA + H]^+$, $[M + IPA + H]^+$ and $[M + H]^+$. Target LC–TSP-MS determination of amino acids and amino acid amides is possible with these ions, even when LC separation is not optimum. Increased MS sensitivity was achieved by postcolumn addition of TFA and chemical ionization with gaseous ammonia. Calibration graphs with good linearity were obtained and the repeatability was satisfactory. The method was suitable for the analysis of samples from bio-organic synthesis.

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