CHIMIA 1999, 53, No. 10

Chimia 53 (1999) 506–510

ISSN 0009–4293

# ESI/TOF-MS Detection for Microseparation Techniques

Ulrike Selditz, Stefan Nilsson, David Barnidge, and Karin E. Markides*

*Abstract.* The choice of electrospray ionisation/time-of-flight mass spectrometry (ESI/TOF-MS) as mass analyser for combination with microseparation techniques, such as μLC, CE and CEC, may be recommended due to the features that present-day TOF-MS instruments have to offer. Advanced digital electronics, which ensure a fast and accurate data handling over a more or less 'unlimited' mass range, make these mass spectrometers the instruments of choice to sensitively cope with low flow rates, high-speed analyses and often very narrow bandwidths without risking the loss of valuable information. Recent achievements in interfacing microseparation techniques to ESI/TOF-MS will be presented and discussed.

## 1. Introduction

In the course of miniaturising analytical tools, microseparation techniques (MST) such as capillary electrophoresis (CE), capillary (or micro) liquid chromatography (μLC) and capillary electrochromatography (CEC) have developed rapidly in recent years. Mostly, this can be attributed to their capability to produce very efficient and fast high-resolution separations of complex mixtures. Also, next to a considerable reduction of solvent consumption, the reduced amount of sample required for such analyses is of great importance, especially in the bioanalytical field. Low sample concentrations/volumes and relatively low sample loading capacities require, however, very sensitive detection methods. For this purpose, electrochemical array detection or mass spectrometry (MS) should be favoured over the conventional analyte monitoring methods such as UV/VIS or fluorescence spectroscopy, since high detection sensitivities can be obtained and active chromophores are not required. Furthermore, peak identification by MS is considered to be more reliable.

In particular, the time-of-flight mass spectrometer (TOF-MS) should be recommended for microcolumn separations since its digital electronic features are able to cope with the large amount of data points usually generated during such fast high-resolution separations. Besides the wide mass range, the high-speed data acquisition rate makes the TOF-MS especially useful and attractive for MST detection. While conventional scanning mass analysers (*i.e.*, quadrupole and ion trap) need several seconds to produce a full mass spectrum, the same can be accomplished by a TOF-MS in 50–200 microseconds [1].

Among the ionisation methods for MS, electrospray has been shown to be well compatible with liquid separation techniques. There are several options on how to assist the analyte transfer from the liquid phase separation into the MS. As described in [2], the main techniques include the coaxial sheath-flow interface, the liquid junction interface, and the sheathless interface. We prefer to operate MST-TOF-MS with the sheathless interface because it is simple and there is no risk of sample dilution, allowing us to fully benefit from the advantages of TOF-MS detection.

An important factor to be considered in ESI is that sensitivity can be improved simply by decreasing the inner diameter of the emitter tip. The smaller the droplet, the higher the electric field and, thus, the surface tension to promote an enhanced release of ions, which in turn will enable more ions to be transferred through the sampling orifice into the MS (nano-ESI) [3]. When ESI is combined with TOF-MS, the sampling of ions is performed continuously with an off-axis pulsation of ions into the analysing flight tube.

In this paper, we will present the latest results from our laboratories in the field of sheathless MST-ESI/TOF-MS.

### 1.1. *Short Review of the MST-TOF-MS*

#### 1.1.1. *MicroLC*

In μLC, the smaller column inner diameter (i.d.) is often combined with reduced particle size of the packed bed, which causes a higher flow resistance than in conventional LC. A high backpressure, however, can cause frictional heating of the mobile phase, eventually leading to an increase in analyte retention [4]. Therefore, low flow rates (μl/min) are applied in μLC. This circumstance, and in particular the much lower sample loading capacity as compared to conventional LC, make mass-spectrometric detection favorable in combination with μLC. There are a number of reports dealing with μLC-ESI/MS(MS) employing different types of interfaces [5]. To date, only little can be found on μLC-TOF-MS separations [6].

#### 1.1.2. *CE*

Since its introduction in the mid 1980s, CE, particularly capillary zone electrophoresis (CZE), has enjoyed a tremendous growth, mostly due to its ability to pro-

*Correspondence: Prof. K.E. Markides
Department of Analytical Chemistry
Institute of Chemistry
Uppsala University
Box 531
S–751 21 Uppsala
Tel.: +46 18 471 36 90
Fax: +46 18 471 36 92
E-Mail: Karin.Markides@kemi.uu.se

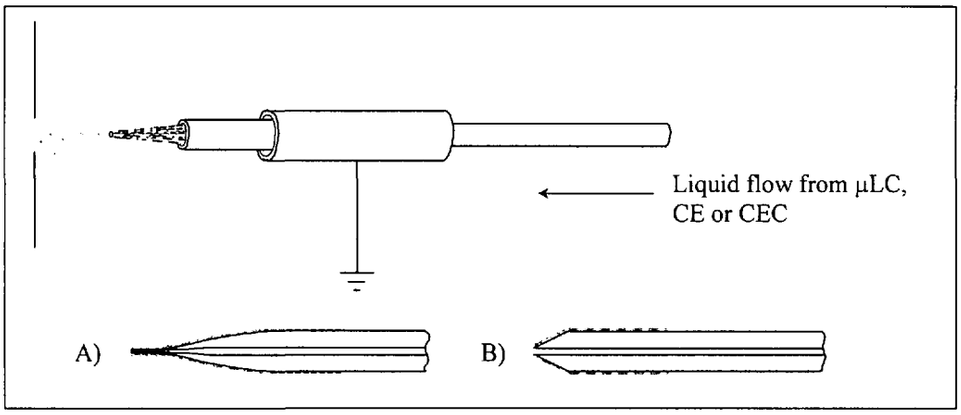


Fig. 1. *Schematic drawing of the interface applied for sheathless electrospray ionisation. A)* Pulled tip with 'fairy dust' coating. *B)* Mechanically shaped tip with 'fairy dust' coating.

duce highly efficient separations of charged analyte mixtures within a short time period and usually also with high resolution. Since only nanoliter fractions of a sample are introduced into the CE capillary, this technique appears to be very suitable for bioanalytical problems where only small sample volumes are available. The drawbacks of CE are mostly related to disturbances in EOF generation due to analyte adsorption to the inner wall surface, heating effects at higher voltages, as well as poor selectivity and sample loading capacity. However, proper surface deactivation and a well chosen experimental set-up can often help to overcome these problems. In recent years, the number of reports on CE-MS separations has grown steadily, and the evaluation of interfaces is often the driving force behind these investigations [7]. With respect to CE-TOF-MS, several groups have published their results on high-speed high-resolution separations of peptides and proteins [8], but also on small organic molecules [9]. Furthermore, *Lazar et al.* reported on their efforts to optimize CE-*o*TOF-MS, whereas *Wu et al.* reviewed their work in the field of microcolumn separations with IT/reTOF-MS detection [10].

#### 1.1.3. *CEC*

Capillary electrochromatography is often considered as a hybrid technique of CE and μLC, combining major characteristics from both separation techniques, *i.e.*, the high efficiency of the former with the enhanced selectivity of the latter. Commonly, fused-silica tubing is packed with small particles having combined surface characteristics of retentive interaction with analytes and charges to give electroosmotic flow when the particles are wetted with an electrolyte solution and brought under the influence of an electrical field. Due to the self-generating electroosmotic flow along the packed bed, CEC is not, like μLC, limited by backpressure, and thus, much smaller particles can be used.

In recent years, a number of CEC-MS reports have appeared, mostly employing sheath-flow,assisted ESI with scanning MS [11]. CEC Separations combined with TOF-MS as detection tool are still in the pioneering stage [10b][12].

## 2. Experimental

### 2.1. *Chemicals and Materials*

*LiChrosolv* methanol (MeOH) and acetonitrile (ACN), acetone, hydrochloric acid, sodium chloride, and ammonium acetate, *p.a.*, were all purchased from *E. Merck* (Darmstadt, Germany). Trimethoxyaminopropylsilane from *Aldrich*, trimethylchlorosilane (TMCS) from *Fluka* (*Sigma-Aldrich Sweden AB*, Sweden), and triethylamine (TEA), HPLC grade, from *Pierce* (Rockford, IL) were used in deactivation procedures of the capillary inner surface. De-ionized water was taken from a *Milli-Q*$^+$ water purification system (*Millipore Corp.*, Marlborough, MA). The peptide standards were purchased from *Sigma* (*Sigma-Aldrich Sweden AB*, Sweden) and used without further purification.

Fused-silica tubing of the stated dimensions was purchased from *Polymicro Technologies Inc.* (Phoenix, AZ). The RP stationary phase particles *YMC ODS-AQ*, 3 μm, 120-Å pore size were kindly provided by *YMC Europe GmbH* (Schermbeck/Weselerwald, Germany). For frit purposes, glass microfibre filters *GF/A* from *Whatman International Ltd.* (Maidstone, Kent, UK) were used. The epoxy glue *'Rapid'* (*Bostik AB*, Sweden) was purchased in a nearby shop. Gold powder with a mean particle size of 2 μm was obtained from *Goodfellow* (Cambridge, UK).

Generally, for packing capillary columns and during μLC-MS experiments, a *Jasco PU-980 Intelligent* HPLC pump (*Jasco Corp.*, Tokyo, Japan) was utilized.

### 2.2. *Time-of-Flight Mass Spectrometer*

A *Jaguar* orthogonal time-of-flight MS (*Sensar Corp.*, Provo, UT) was used throughout the studies. A detailed description of this apparatus can be found in [13].

Sheathless electrospray ionisation/mass spectrometry was conducted. The position of the microcolumns with integrated emitter tip was adjusted relative to the orifice of the TOF (1–1.5 cm) by means of a home-built xyz-positioning stage. On applying the electrospray (ES) voltage to a stainless steel capillary (see *Fig. 1*), electrical contact with the eluting liquid phase was made through a novel emitter tip design, which we refer to as 'fairy dust' (gold powder dusted onto a supporting coating), which is described in detail elsewhere [14].

Two types of emitter tips were used: a pulled and a mechanically shaped tip. More information about the fabrication of such tips can be found elsewhere [16][17]. The *SprayTof* software, version 1.61 for *Windows NT 4.0* developed by *Sensar* (Provo, UT) was used for data processing and instrument control.

### 2.3. *MicroLC*

#### 2.3.1. *Column Preparation*

Fused-silica tubing of 150 μm i.d. × 365 μm o.d. was used for preparing a reversed-phase column. Since problems were experienced earlier when coupling a packed column to an electrospray emitter tip *via* a teflon sleeve connection, the 'integrated system' approach by *Robins* and *Guido* [5g] was adapted. Shortly, the procedure included pulling a tip on one end of the capillary, rinsing with 6M hydrochloric acid, drying at 120° under $N_2$-flush, silanisation with a solution of TEA/TMCS 1:2 and washing with ACN before slurry-packing the capillary with 3 μm *YMC ODS-AQ* particles against a glass microfibre frit. The final length of the obtained column was 22 cm.

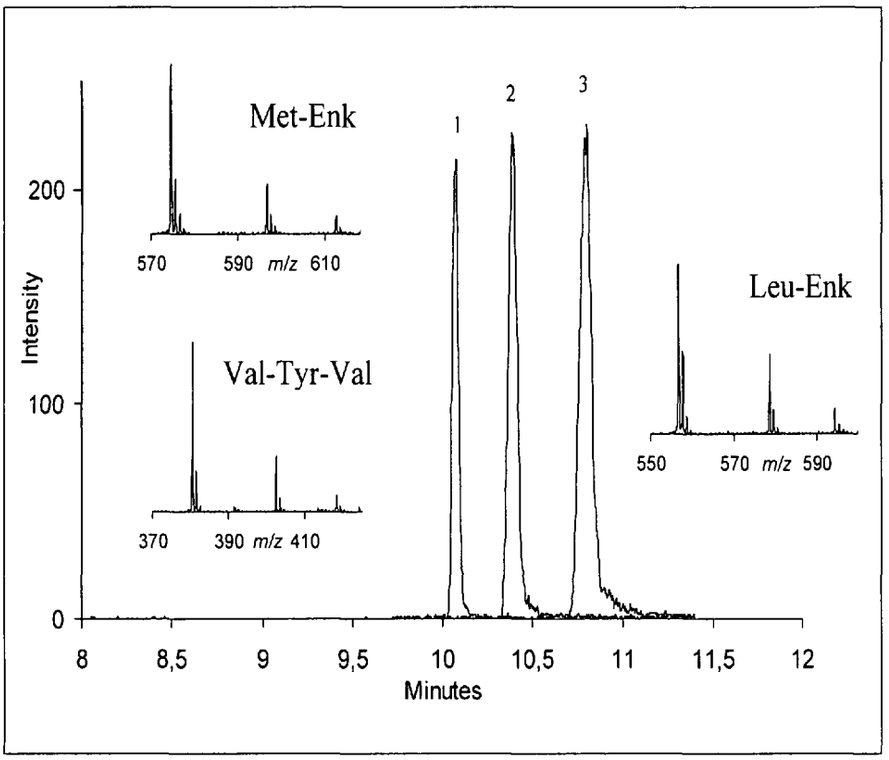


Fig. 2. *Isocratic µLC-ESI/TOF-MS separation of a three-peptide mixture.* Column dimensions: 150 µm i.d. x 365 µm o.d. x 22 cm length, pulled emitter. Stationary phase: 3 µm ODS-AQ, 120 Å; mobile phase: 50% ACN/50% 20 mM ammonium acetate, pH 5. Peak identification: 1 = Val-Tyr-Val, 2 = Met-Enk, 3 = Leu-Enk.

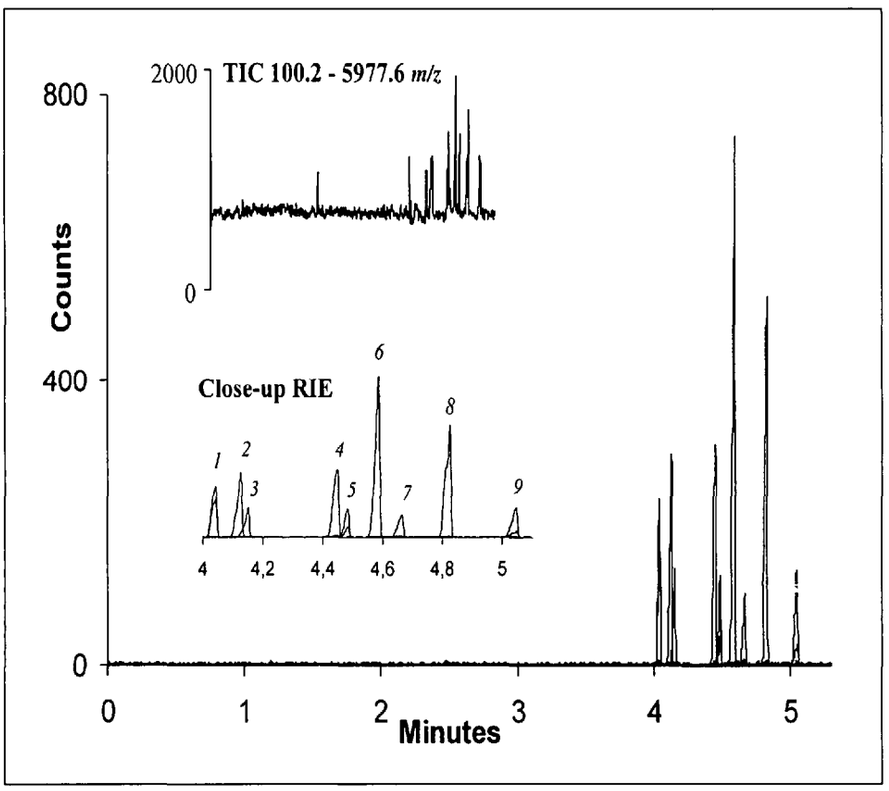


Fig. 3. *Separation of a 9-peptide mixture on a 1-m long APS column with mechanically shaped emitter tip.* For peak identification, see the *Table*. Upper inset: total ion electropherogram for 100-6000 *m/z*. Lower inset: close-up of the reconstructed ion electropherogram (RIE). Remark: The 'double' peaks for 1, 5, and 9 originate from isotopic traces of other ions and/or fragment ions at the respective *m/z*.

Finally, the pulled tip was cleaned outside with ethanol and coated with a thin film of epoxy glue onto which, in the wet state, some gold power was dusted for electrical contact (modified 'fairy dust' procedure).

### 2.3.2. *µLC-TOF-MS Operating Conditions*

The µLC column was connected *via* a *Valco* injection valve model *C6W* (*Valco Instruments*, Houston, TX) equipped with a 0.3 µl (external) fused-silica loop to a *Jasco PU-980 Intelligent* HPLC pump. Before the TOF-MS experiments, the column was conditioned with mobile phase for at least 30 min. A peptide mixture containing Val-Tyr-Val, methionine enkephalin (Tyr-Gly-Gly-Phe-Met), and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) was separated isocratically with a mobile phase composed of 50% ACN and 50% 20 mM ammonium acetate, pH 5.

The electrospray voltage applied to the stainless steel capillary was set to +3.0 kV. The data acquisition parameters were as follows: 800 sums, 6.25 spec/second, 5 kHz, threshold 2041.

## 2.4. *CE*

### 2.4.1. *Capillary Pre-Treatment*

A 1-m long piece of 25 µm i.d. × 360 µm o.d. fused silica was mechanically shaped as described by *Barnidge et al.* [16]. The conductive coating procedure with polyimide was followed by curing at 200° for 30 min [14]. The method described by *Moseley et al.* [15] was used to deactivate the inner wall of the capillary surface with APS.

### 2.4.2. *CE-TOF-MS Operating Conditions*

A *BERTAN* model *ARB 30* (Hicksville, New York, USA) high-voltage power supply was used to provide the CE-voltage of –30 kV. The CE column was mounted through the steel capillary on the xyz-positioning stage according to *Fig. 1*. The column inlet was inserted into a vial containing the CE background electrolyte together with a platinum electrode from the HV power supply at the same height level as the emitter tip. A standard 9-peptide mixture, composed of methionine enkephalin (Tyr-Gly-Gly-Met), leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), oxytocin, bradykinin (fragment 1–5), bombesin, leutinising hormone releasing hormone (LHRH), [Arg8]-vasopressin, bradykinin and substance P, was diluted with 80% ACN and 20% 10 mM acetic acid and separated using a background electrolyte containing 50% ACN and 50% 10 mM

acetic acid, pH 3. Injections were made through a 10-cm hydrodynamic lift of the column inlet relative to the emitter tip for 5 s. The electrospray voltage was set to +3.3 kV, resulting in a net separation voltage of 33.3 kV. The data acquisition parameters were 400 sums, 12.5 spec/second, 5 kHz, threshold 2040.

## 3. Results and Discussion

### 3.1. *μLC-ESI/TOF-MS*

A peptide mixture composed of Val-Tyr-Val, Met-Enk, and Leu-Enk was baseline-separated as shown in *Fig. 2*. The absolute amount of each peptide injected referred to 75 pg, since 300 nl of a 1:4000 dilution of a 1 mg/ml stock solution were injected onto the column.

Applying mobile phase in the constant-pressure mode at 200 kg/cm$^2$ resulted in a flow rate of about 50 nl/min. For five consecutive runs, retention factors ($k' = (t_R - t_0)/t_0$) were averaged to be 0.6 (rmsd: 6.3%) for Val-Tyr-Val, 0.63 (rmsd: 7.3%) for Met-Enk, and 0.7 (rmsd: 5.9%) for Leu-Enk. The beginning of the baseline dip from water was considered equal to $t_0$. Since the peaks were slightly tailing (asymmetry factors between 1.17 and 1.37), the equation of *Foley* and *Dorsey* was applied to calculate the number of theoretical plates [18]. Even when using the width at 10% of the maximum height, outstanding plate numbers ($N_{0.1h}$/m) were obtained: 2'177'550 for Val-Tyr-Val (rmsd: 22.4%), 824'520 for Met-Enk (rmsd: 9.6%) and 497 645 plates/meter for Leu-Enk (rmsd: 5.1%). The variations in retention factor and column efficiency can be explained by a minor loss of packing material due to instability in the glass microfibre frit in retaining all of the 3-μm particles under the high pressure applied. In future experiments, we will test a porous teflon frit as described by *Robins* and *Guido* [5g].

### 3.2. CE-TOF-MS

*Fig. 3* shows a CE-ESI/TOF-MS run using a mechanically shaped emitter on a 1-m long aminopropylsilane(APS)-coated capillary. For six consecutive runs, rmsd values of the migration time were determined to be about 5%. The efficiency of the separation was enough to resolve all but one of the peptides (see lower inset of *Fig. 3*) although shorter CE columns have given us much higher efficiencies (data not shown) [19]. The upper inset of *Fig. 3* shows a total ion electropherogram for all ions between 100–6000 *m/z*. This total ion electropherogram demonstrates that, for this particular column, there was little or no interference from the CE background electrolyte during the analysis.

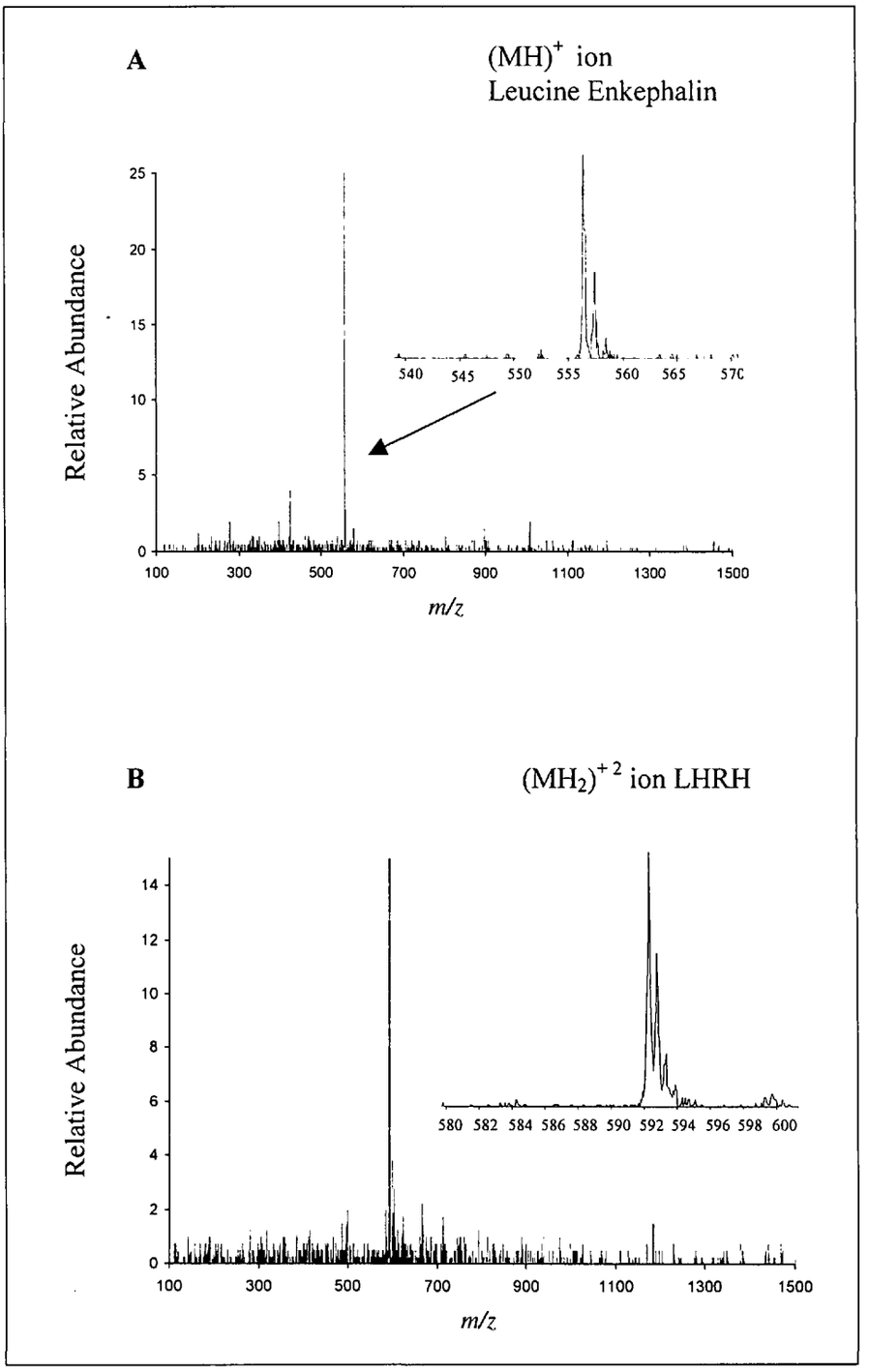


Fig. 4. *Single mass spectra taken at peak maximum of A) Leu-Enk, B) LHRH*

The fact, however, that this is not always the case and that other CE columns occasionally give higher backgrounds was studied in more detail. The results suggest that the APS coating can vary from column to column resulting in variable column efficiency and EOF. Further development of stable deactivated columns for CE is therefore needed.

The *Table* gives the monoisotopic masses for the nine peptides found in the standard mixture along with efficiency values, migration times, and peak widths measured as full width at half height (FWHH) for the data presented in *Fig. 3*. The *Table* also gives the estimated amount of peptide eluting from the column for one scan taken at the peak maximum for a 89-pl injection of a 5 μg/ml solution, resulting in a total amount of 444 fg injected of each peptide.

For this experiment, the sum value was set at 200, resulting in a collection time of 40 ms for each mass spectrum with a scan

Table. *List of Peptide Masses and Chromatographic Data for CE-ESI/MS Using a 1-m Long APS Column with Mechanically Shaped Emitter*

| Analyte | Monoisotopic Mass | Number of Theor. Plates | Migration Time [min] | FWHH [s] | amol at $Peak_{Max}$ |
|---|---|---|---|---|---|
| 1 MetEnk $(MH)^+$ | 574.23 | 226,203 | 4.04 | 1.2 | 49 |
| 2 LeuEnk $(MH)^+$ | 556.28 | 419,979 | 4.13 | 0.9 | 67 |
| 3 Oxytocin $(MH)^+$ | 1009.46 | 536,696 | 4.15 | 0.8 | 41 |
| 4 Bradykinin[1–5] $(MH)^+$ | 573.31 | 326,398 | 4.45 | 1.1 | 53 |
| 5 Bombesin $(MH_2)^{2+}$ | 810.90 | 279,217 | 4.49 | 1.2 | 17 |
| 6 LHRH $(MH_2)^{2+}$ | 591.79 | 420,182 | 4.59 | 1.0 | 28 |
| 7 [Arg8]-Vasopressin $(MH_2)^{2+}$ | 543.73 | 359,468 | 4.67 | 1.1 | 28 |
| 8 Bradykinin $(MH_2)^{2+}$ | 530.78 | 237,384 | 4.83 | 1.4 | 23 |
| 9 Sub P $(MH_2)^{2+}$ | 674.37 | 508,622 | 5.05 | 1.0 | 25 |

range of 1–6'000 Da. *Fig. 4A* shows the mass spectrum for the singly charged ion of leucine enkephalin taken from the peak maximum. The amount of peptide eluting from the column during the collection time of the spectrum is approximately 67 amol determined by calculating the mass flow for a *Gaussian* peak. The *Figure* shows that, even at low amol levels, the mass spectrum produced can readily provide the charge state of the ion and the monoisotopic mass of the analyte. *Fig. 4B* displays the result for a single mass spectrum collected from the peak maximum for leutinising hormone releasing hormone (LHRH) representing approximately 28 amol of peptide. In this instance, a centroid for the doubly charged ion can be easily assigned with an estimated resolution of 3'250 for the monoisotopic peak of LHRH. Each of these mass spectra depicts the sensitivity attainable with CE-ESI/TOF-MS performed using an APS-modified CE capillary with a mechanically shaped emitter coated with gold particles for sheathless electrospray ionization.

## 4. Conclusions

Microseparation techniques can favourably be coupled to an orthogonal time-of-flight mass spectrometer as detector to fully take advantage of the high-speed data acquisition rate, high sensitivity and mass resolution provided for monitoring peaks of small band width and low concentration. Also, the integrated emitter design for sheathless electrospray ionisation has been demonstrated to be an easy and powerful tool for promoting the transfer of liquid phase at low flow rate (range: nl/min to few µl/min) into charged droplets and ions to enter the mass spectrometer for analysis. Thereby, the high resolution and efficiency of the separation is maintained. The methodology described can also be used for CEC-ESI/TOF-MS, which is presently under further investigation in our laboratory.

Financial support from the *Swedish Natural Science Research Council*, project K-1439-326 and from the *Swedish Foundation for Strategic Research* is gratefully acknowledged.

Received: June 22, 1999

[1] I.M. Lazar, G. Naisbitt, M.L. Lee, *Analyst* **1998**, *123*, 1449.
[2] R.B. Cole, 'Electrospray Ionisation Mass Spectrometry', John Wiley & Sons, 1997.
[3] W.S. Wilm, M. Mann, *Anal. Chem.* **1996**, *68*, 1.
[4] a) V.L. McGuffin, C.E. Evans, S. Chen, *J. Micro Sep.* **1993**, *5*, 3; b) I.Halasz, R. Endele, J. Asshauer, *J. Chromatogr.* **1975**, *112*, 37.
[5] a) B. Hyllbrant, N. Tyrefors, B. Långström, K.E. Markides, *J. Micro Sep.* **1999**, *11(5)*, 353; b) M.T. Davis, T.D. Lee, *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 194; c) R.E. Moore, L. Licklider, D. Schumann, T.D. Lee, *Anal. Chem.* **1998**, *70(23)*, 4879; d) R.J. Dijkstra, B.L.M. Van Baar, C.E. Kinetz, W.M.A. Niessen, U.A.T. Brinkman, *RCMS* **1998**, *12*, 5; e) P.A. D'Agostino, L.R. Provost, J.R. Hancock, *J. Chromatogr. A* **1998**, *808*, 177; f) J.J. Dalluge, B.C. Nelson, J.B. Thomas, M.J. Welch, L.C. Sander, *RCMS* **1997**, *11*, 1753; g) R.H. Robins, J.E. Guido, *RCMS* **1997**, *11*, 1661.
[6] a) J.F. Banks, *J. Chromatogr. A* **1997**, *786*, 67; b) A.P.L.Wang, X. Guo, L. Li, *Anal. Chem.* **1994**, *66(21)*, 3664.
[7] a) F. Hsieh, E. Baronas, C. Muir, S.A. Martin, *RCMS* **1999**, *13*, 67; b) Y. Hirabayashi, A. Hirabayashi, H. Koizumi, *RCMS* **1999**, *13*, 712; c) J.F. Kelly, L. Ramaley, P. Thibault, *Anal. Chem.* **1997**, *69(1)*, 51; d) M. Mazereeuw, A.J.P. Hofte, U.R. Tjaden, J. van der Greef, *RCMS* **1997**, *11*, 981.
[8] a) I.M. Lazar, A.L. Rockwood, E.D. Lee, J.C.H. Sin, M.L. Lee, *Anal. Chem.* **1999**, *71*, web release date: May 19; b) J.F. Banks, T. Dresch, *ibid.* **1996**, *68*, 1480.
[9] a) M.E. Palmer, M.R. Clench, L.W. Tetler, D.R. Little, *RCMS* **1999**, *13*, 256; b) see ref. [1].
[10] a) I.M. Lazar, E.D. Lee, A.L. Rockwood, M.L. Lee. *J. Chromatogr. A* **1998**, *829*, 279; b) J.T. Wu, M.G. Qian, M.X. Li, K. Zheng, P. Huang, D.M. Lubman, *ibid.* **1998**, *794*, 377.
[11] a) V. Spikmans, S.J. Lane, U.R. Tjaden, J. van der Greef, *RCMS* **1999**, *13*, 141; b) R.N. Warriner, A.S. Craze, D.E. Games, S.J. Lane, *RCMS* **1998**, *12*, 1143; c) J. Ding, T. Barlow, A. Dipple, P. Vouros, *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 823; d) G.A. Lord, D.B. Gordon, P. Myers, B.W. King, *J. Chromatogr. A* **1997**, *768*, 9; e) S.J. Lane, R. Boughtflower, C. Paterson, M. Morris, *RCMS* **1996**, *10*, 733; f) K. Schmeer, B. Behnke, E. Bayer, *Anal. Chem.* **1995**, *67(20)*, 3656.
[12] a) P. Huang, X. Jin, V. Chen, J.R. Srinivasan, D.M. Lubman, *Anal. Chem.* **1999**, *71(9)*, 1786; b) G. Choudhary, C. Horvath, J.F. Banks, *J. Chromatogr. A* **1998**, *828*, 469; c) P. Huang, J.T. Wu, D.M. Lubman, *Anal. Chem.* **1998**, *70(14)*, 3003.
[13] I.M. Lazar, B. Xin, M.L. Lee, E.D. Lee, A.L. Rockwood, J.C. Fabbi, H.G. Lee, *Anal. Chem.* **1997**, *69(16)*, 3205.
[14] D.R. Barnidge, S. Nilsson, K.E. Markides, *Anal. Chem.*, submitted.
[15] M.A. Moseley, J.W. Jorgenson, J. Shabanowitz, D.F. Hunt, K.B.J. Tomer, *J. Am. Soc. Mass Spectr.* **1992**, *3*, 289.
[16] D.R. Barnidge, S. Nilsson, K.E. Markides, H. Rapp, K. Hjort, *RCMS* **1999**, *13*. 994.
[17] U. Selditz, S. Nilsson, K.E. Markides, in preparation.
[18] J.P. Foley, J.G. Dorsey, *Anal. Chem.* **1983**, *55(4)*, 730.
[19] S. Nilsson, poster presentation at Analysdagarna 1999, June 14–17, in Uppsala.