Journal of Chromatography, 647 (1993) 367–374
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2794

Off-line coupling of capillary electrophoresis with matrix-assisted laser desorption mass spectrometry

P.A. van Veelen and U.R. Tjaden*

Division of Analytical Chemistry, Leiden-Amsterdam Center for Drug Research, P.O. Box 9502, 2300 RA Leiden (Netherlands)

J. van der Greef

Division of Analytical Chemistry, Leiden-Amsterdam Center for Drug Research, P.O. Box 9502, 2300 RA Leiden (Netherlands) and Department of Structure Elucidation and Instrumental Analysis, HVV-TNO, P.O. Box 360, 3700 AJ Zeist (Netherlands)

A. Ingendoh and F. Hillenkamp

Institute for Medical Physics and Biophysics, University of Münster, W-4400 Münster (Germany)

ABSTRACT

Capillary electrophoresis was coupled off-line with matrix-assisted laser desorption mass spectrometry. Special attention was paid to minimizing loss of the achieved electrophoretic resolution, which was realized by appropriate voltage programming during the sampling of the analytes migrating out of the capillary. Different modes of coupling these techniques are discussed in terms of resolution, sensitivity and ease of handling.

INTRODUCTION

Capillary electrophoresis (CE) has since 1981 grown into a separation technique characterized by speed, high efficiencies and small sample volumes [1]. Detection usually takes place on-capillary using UV absorbance or laser-induced fluorescence (LIF), or, more recently, by on-line coupling to a mass spectrometer equipped with a continuous-flow fast atom bombardment (FAB) or electrospray (ES) interface.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has since 1988 rapidly developed into a very sensitive and fast MS technique capable of analysing high molecular masses, up to 300 000, by irradiation of small sample spots.

The characteristics of both methods makes coupling in principle very attractive. On-line coupling is not as easy to achieve as, for example, with ES-MS, which, as a liquid introduction system, is easily coupled. Therefore, the off-line coupling of CE and MALDI-MS has been investigated, with the obvious advantage that both techniques can be optimized separately. On-line LC–laser desorption MS of some small compounds ($M_r < 300$) without use of a matrix was reported in 1984 [3]. Until now the off-line coupling of CE with desorption MS methods, such as plasma desorption (PD) MS and MALDI-MS, has been achieved by fraction collection of peptides and proteins, using a porous glass

* Corresponding author.

0021-9673/93/$06.00

joint to complete the electrical circuit at the cathodic end of the column [4,5].

We here report the off-line coupling of CE and MALDI-MS by collecting the effluent on a moving belt-like system, after which the trace is subsequently scanned by the laser beam. The main objective of the study was to investigate whether the high resolution obtained in the CE system could be maintained using this off-line coupling procedure. The various aspects of this approach will be discussed in terms of resolution, sensitivity and ease of use. The results using this approach are compared with those achieved with fraction collection.

EXPERIMENTAL

Chemicals

All chemicals, unless otherwise stated, were purchased from Merck (Darmstadt, Germany). The buffers used (sodium phosphate, ammonium phosphate, ammonium acetate and ammonium hydrogencarbonate) were all at 7 m*M* concentration and pH 8.5. The β-endorphins were a gift from Organon (Oss, Netherlands), and used without further purification.

Capillary electrophoresis

CE was performed in untreated fused-silica capillaries with an internal diameter of 75 μm (SGE, Milton Keynes, UK). The capillary length was 1 m in all cases. The on-capillary UV detection was realized by creating a window made by burning off the coating of the capillary over a length of 5 mm, at a distance of 54 cm from the anodic end of the capillary. Effluent collection at the end of the capillary was done by appropriate timing. Before starting an electrophoresis run, the capillary was flushed with the buffer solution for 30 mins.

The samples were injected using a PRINCE (PRogrammable INjector for CE) purchased from Lauerlabs (Emmen, Netherlands). Injections were made hydrodynamically by applying a pressure of 10 mbar for 10 s (8 nl) in most cases. In some cases larger volumes of up to 48 nl were injected (60 mbar for 10 s). The PRINCE system allows easy and fast switching between several voltages and/or pressures in a well-defined way, which makes the collection and deposition procedures very reproducible.

The high-voltage power supply (up to 30 kV) was obtained from Lauerlabs, and was set at 30 kV during the CE separations, with a resultant current of 15 μA, which was measured post capillary using a Model 8062 A multimeter (Fluke, Tilburg, Netherlands). Deposition of the effluent took place directly on a laser desorption target, made of stainless steel, which acted as the cathode. During the analyte collection and deposition, normally 5 kV were applied.

Addition of a sheath flow was accomplished by a Model 22 syringe infusion pump (Harvard Apparatus, South Natick, MA, USA), with the following assembly. The cathodic end of the separation capillary was inserted into a 300 μm I.D. fused-silica capillary via a T-piece. The matrix solution was added through the annular space between both capillaries at a flow-rate of 0.3 μl/min.

UV detection was done using a Kratos 757 Spectroflow absorbance detector (ABI, Maarssen, Netherlands), in which a home-made detection cell with an illuminated volume of about 7 nl was installed.

Mass spectrometry

The mass spectrometer used was a VISION 2000 reflector-type time-of-flight laser desorption instrument (Finnigan MAT, Bremen, Germany) equipped with a Q-switched (5 ns) frequency-tripled Nd-YAG laser (Speser 600, Spektrum, Berlin, Germany), operating at a wavelength of 355 nm. The laser beam was focused at the sample surface to a spot diameter of 70 μm with irradiances between 10^6 and 10^7 W/cm^2, close to the threshold for obtaining ions in all cases. The ions generated were accelerated to a potential of 5 kV in the ion source and post-accelerated to a potential of 10 kV for detection with a secondary electron multiplier. Registration of the analogue signal was performed with a transient recorder (LeCroy 9450) with a 5 ns per channel resolution. The acquired spectra consisted of the sum of twenty single acquisitions. They were internally calibrated using the known masses of the matrix peaks.

The VISION 2000 offers the opportunity to

observe the sample by a camera mounted on the end of a microscope with a lateral resolution of approximately 10 μm. Thus control of the sample and selection of different sites of it during the measurement is possible. Every spot on the target can be reached for irradiation by means of an $x-y$ manipulator.

The matrix used was 2,5-dihydroxybenzoic acid (DHB) at a concentration of 10 g/l in water [6]. It was added in an approximately 1000-fold molar excess to the analytes. After deposition on a stainless-steel target, it was air dried and introduced into the mass spectrometer.

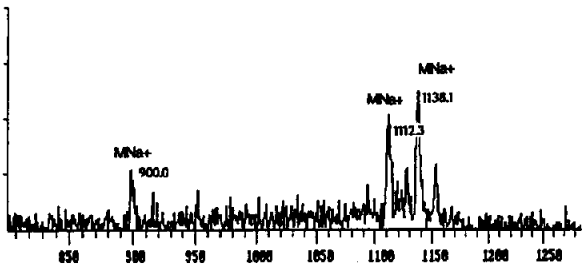

Fig. 1. Matrix-assisted laser desorption mass spectrum obtained from 10 fmol per component of the β-endorphin mixture using DHB as a matrix. The concentration of the loaded sample solution was $2 \cdot 10^{-9}$ M.

RESULTS AND DISCUSSION

The molecular masses of the three investigated β-endorphins are listed in Table I. As a first step, the detection limit of the compounds was determined by using the normal preparation, *i.e.* by dripping a dilute solution, both in water and in buffer solution, onto the target and adding some matrix solution [6]. The detection limits were determined in a mixture, since suppression effects do not play a significant role in MALDI-MS. The detection limits for all compounds of interest were 10 fmol, determined from a pure water solution as well as from the buffer solution. A spectrum is shown in Fig. 1. As can be seen, only the sodium and potassium cationized molecules were detected. Apparently, the peptides have a great affinity for these cations. The absolute amount of 10 fmol was loaded on the target in a 0.5-μl droplet, corresponding to a concentration of 20 pmol/ml. When capillary electrophoresis is used as a separation system it must be considered, owing to the very small injection volumes of about 10–50 nl, that the above-mentioned concentration corresponds to an absolute amount of material between 200 and 1000 amol, which is far below the detection limit. This means that higher concentrations are necessary to obtain any signal.

We used MALDI-MS for qualitative determination, *i.e.* to reveal the identity of the analyte.

A typical electropherogram of three β-endorphins using on-capillary UV detection is shown in Fig. 2. The plate numbers of the different peaks are between 100 000 and 150 000. This electropherogram was recorded using a 7 mM sodium phosphate buffer (pH 8.5). This relatively high pH was used to prevent analyte adsorption to the wall since at this pH both the wall and the analytes have a negative charge.

Since this buffer is not very suitable for combination with MS, several other buffers containing ammonium acetate, ammonium hydrogencarbonate and ammonium phosphate were studied. The separations based on ammonium hydrogencarbonate appeared to be rather poor, while

TABLE I

LIST OF THE AMINO ACID SEQUENCES AND THE MOLECULAR MASSES OF THE THREE β-ENDORPHIN FRAGMENTS USED

Peptide	Amino acid sequence	Average mass (MH^+)	Average mass (MNa^+)	Average mass (MK^+)
β-End 8–17	EKSQTPLVTL	1116.3	1138.3	1154.3
β-End 6–15	TSEKSQTPLV	1090.2	1112.2	1128.2
β-End 6–13	TSEKSQTP	877.9	899.9	915.9

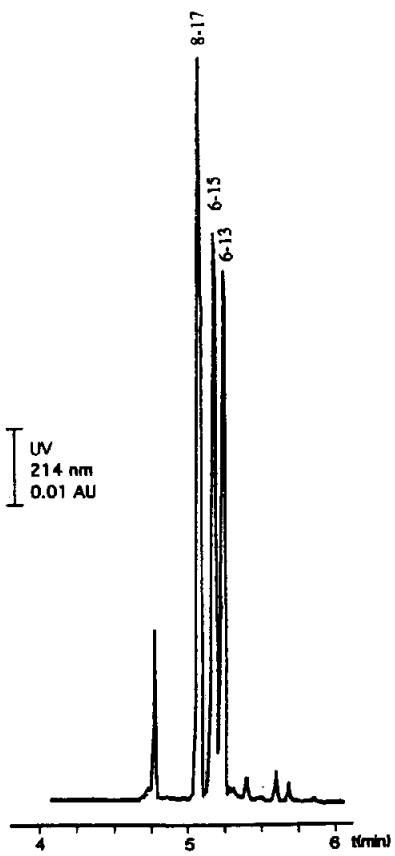

Fig. 2. On-capillary UV detection trace of the separation of three β-endorphins. $V_{inj} = 8$ nl; $[C] = 300$ μg/ml; $L_{cap} = 1$ m (54 cm to detector), I.D.$_{cap} = 75$ μm; Buffer = 7 m*M* ammonium phosphate. Plate numbers are between 100 000 and 150 000.

with ammonium phosphate the same results as with sodium phosphate could be obtained.

In the case of PD-MS, buffers which have ammonium as a cation are not troublesome [7], even if they are not volatile. This is in contrast with ES-MS, in which non-volatile buffers can contaminate the source. Since in PD-MS and MALDI-MS only a negligible fraction of the sample is consumed, the enormous excess of non-volatile material does not cause contamination problems. Therefore, it was decided to continue with the ammonium phosphate buffer.

Off-line coupling of the CE-separated analytes with MALDI-MS was first studied using fraction collection, which can take place in several ways. The use of a porous glass joint close to the end of the capillary, which serves to decouple the high voltage from the capillary outlet [4,5], enables the collection of fractions without disturbing the CE process. The fractions can subsequently be analysed by mass spectrometry.

Our first approach was to perform the electrophoresis until the separated analytes reached the very end of the capillary, then switch off the high voltage and subsequently apply a pressure to the anodic end of the capillary, thereby pushing out the separated fractions for fraction collection.

The result is depicted in Fig. 3 for different pressures. The situation at the cathodic end of the capillary is mimicked by performing the analogous experiment in front of the UV window. It can be seen that the resolution was strongly reduced when pressures between 60 and 30 mbar were applied, because of the laminar-flow profile generated in the capillary. At 20 mbar, and especially at 10 mbar, there is hardly any loss of resolution. The high voltage only

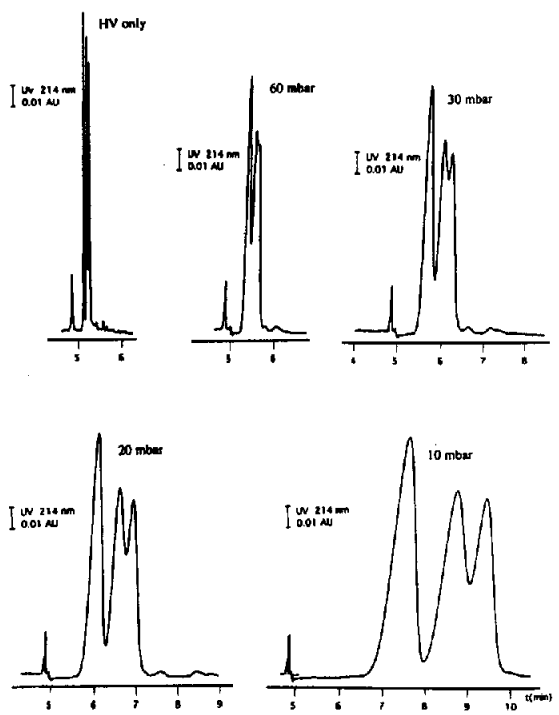

Fig. 3. The effect of pressures applied, for pushing out the effluent at the capillary outlet, after switching the high voltage off, is shown for several pressures. The high voltage-only picture is the reference electropherogram run under pure CE conditions.

electropherogram, *i.e.* without pressure, has been added as a reference electropherogram in the figure. An additional helpful effect of the application of low pressure is the very low flow-rate in the capillary, leading to an expanded time-scale, which is favourable for collecting such very close peaks. The volumes of the various peaks remain the same, and are difficult to handle. The various fractions were therefore collected in small vials containing 1 μl of matrix solution. This solution was subsequently loaded onto a MALDI-MS target for further analysis. A major disadvantage of this fraction collection procedure is the dilution factor, which is approximately 5.

Using this method, the resolution is not seriously affected when the low pressure is applied for only several minutes. When the porous glass joint approach [4,5] is used, the analytes move out of the capillary after passing the electrical decoupling point in a laminar-flow mode. When 30 kV is applied over the separation capillary, an electroosmotic flow is generated similar to a hydrodynamic flow obtained with a pressure drop of 90 mbar (see Table II). This implies that during electrophoresis in the post-porous joint part of the capillary a laminar flow is induced, which causes a dramatic loss of resolution (see also Fig. 3). The required resolution for our problem and for all closely separated compounds will therefore be lost and the method in such cases will be less useful.

TABLE II

ELUENT FLOW THROUGH THE SEPARATION CAPILLARY AS A RESULT OF THE APPLIED ELECTRICAL FIELD OR AS A RESULT OF DIFFERENT APPLIED PRESSURES AT THE ANODIC END OF THE CAPILLARY

Pressure (mbar)	Voltage (kV)	Flow (nl/s)
0	30	7.3
10	0	0.8
20	0	1.6
30	0	2.3
50	0	3.9
60	0	4.7
90	0	7.0

Another way of maintaining resolution during fraction collection is by lowering the high voltage from, for example, 30 to 5 kV, when the analytes have reached the capillary outlet. In principle, the contact to the target, which is at earth potential, should be maintained all the time. However, it appeared in practice this is not necessary when the applied high voltage does not exceed 10 kV. The contact could be interrupted several times, for changing the collection vials, without disturbing the resolution of the electrophoresis. Collection in this way is quite convenient, although still very small volumes must be handled. The time scale can be elongated to any desired size, analogous to the case in which small pressures are applied.

The last method investigated was deposition of the effluent on a moving belt-like system, followed by scanning the deposited electropherogram with the laser beam in the ion source of the mass spectrometer. Deposition of CE effluents on a moving belt system has been reported [8,9]. In this case the effluent is deposited on a blotting membrane and further analysed by protein chemical methods. In our case the moving belt-like system was a moveable laser desorption target, which was part of the electrical circuit.

Deposition of the effluent can be done in a continuous as well as in a stepwise manner, when either pressure or a lowered high voltage is applied, as described above. It appeared that application of a high voltage yields the best results. Therefore, this method was used in combination with the moveable target system.

Continuous deposition of the effluent on a moving target is in principle the best way of maintaining resolution. By appropriate tuning of the target speed, the total electropherogram can be transferred to the target without loss of resolution. Care must be taken not to dilute the analytes over a too large surface area.

In practice, it is easier to deposit the effluent by moving the belt in a stepwise manner, and break the contact at every step for a very short period. As discussed above, the procedure does not cause the electrophoresis performance to deteriorate, and can be tuned just as well as in case of continuous deposition. Since the time-scale can be optimized as desired by choosing the

appropriate voltage, as in the case of fraction collection, this stepwise mode is similar to the continuous mode. An advantage of stepwise deposition is the resulting higher surface density of the collected analytes, which is favourable for MALDI-MS. A severe problem that also occurs in direct deposition onto a laser desorption target is the tiny amount of effluent (approximately 1 nl/s at 5 kV). This problem has been solved by application of a sheath flow-at a rate of 0.3 μl/min. This sheath flow contained the matrix needed for MALDI-MS. Depositing new droplets every 30 s yielded drops of about 180 nl. After drying the droplets, nice crystals were observed through the camera mounted on the laser desorption instrument. This way of applying the sample to the target yields the same results as after the normal preparation, in which usually larger drops are deposited, and more time is available for mixing of analyte and matrix, and also for good crystallization. Using even smaller sheath flows, down to 0.1 μl/min, yielded very small droplets. However, crystals of poor quality were obtained, with consequently poor signals in the MALDI-MS experiments.

The results of the stepwise deposition method are presented in Figs. 4 and 5. Absolute amounts of 2.4 pmol and 144 fmol per analyte were injected into the CE capillary. The corresponding electropherograms are also shown (see

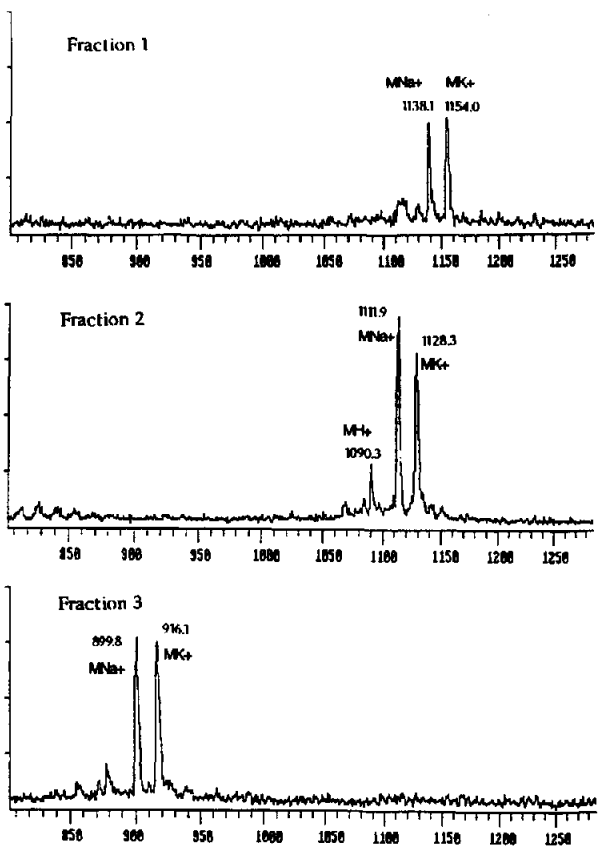

Fig. 4. Mass spectra of the separated compounds collected on the moveable target system, after changing the high voltage from 30 to 5 kV. As can be seen, the several fractions are not contaminated with components of other fractions. $V_{inj} = 8$ nl, $[C] = 300$ μg/ml, corresponding to 2.4 pmol per component. The corresponding electropherogram is shown in Fig. 2.

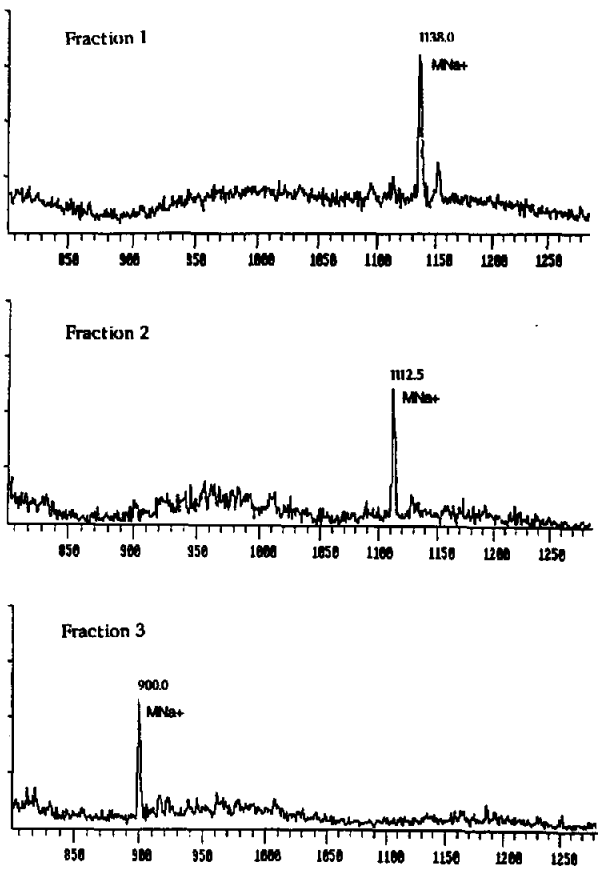

Fig. 5. Mass spectra of the separated compounds collected on the moveable target system, after changing the high voltage from 30 to 5 kV. As can be seen, the several fractions are not contaminated with components of other fractions. $V_{inj} = 48$ nl, $[C] = 3$ μg/ml, corresponding to 144 fmol per component. The corresponding UV electropherogram is shown in Fig. 6.

Figs. 2 and 6). The absolute amounts injected corresponds to concentrations of 3000 μg/ml (8 nl injected) and 3 μg/ml (48 nl injected), respectively. As can be seen, an injection of 48 nl already decreases the resolution in the electropherogram. Higher injection volumes cannot therefore be used when maximum resolution is needed. At lower analyte concentrations (see Figs. 1 and 5) only the sodium adducts are observed and not the potassium adducts, although the same buffer is used. So far, we cannot explain this phenomenon.

The results for the 300 and 30 μg/ml solutions could be easily obtained, while for the 3 μg/ml solutions some problems were met. In this concentration range obtaining the MALDI mass spectra was more difficult, but successful in every case, mainly because the sample could be observed in the mass spectrometer and because of the possibility of selecting different sites during operation.

As can be seen from the electropherograms, the limit for UV detection is almost reached for the lowest concentrations used. Comparison of the data shows that MALDI-MS offers about the same detection limits as UV detection.

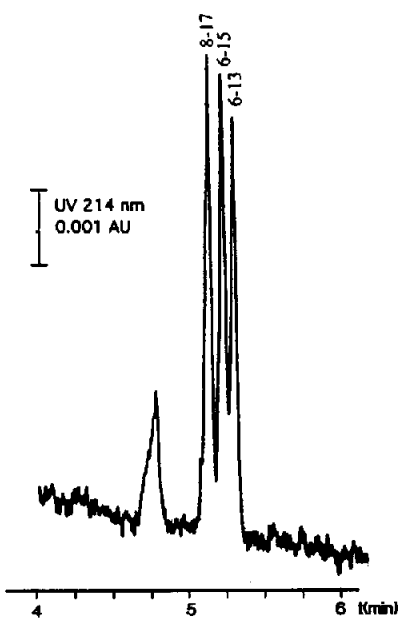

Fig. 6. On-capillary UV detection trace of the separation of three β-endorphins. $V_{inj} = 48$ nl; $[C] = 3$ μg/ml. The corresponding mass spectral are depicted in Fig. 5.

Future research will be devoted to improving detection limits as well as to applying our findings to analyse more intricate electrophoretic separations by MALDI-MS.

CONCLUSIONS

Several modes of performing off-line CE–MALDI-MS have been investigated. Deposition of the effluent on a moving belt-like system in a stepwise manner, using a sheath flow of matrix solution and subsequent scanning of the laser desorption target yielded the best results in terms of resolution, detection limit and ease of handling. Absolute detection limits are of the order of 100 fmol for some β-endorphins, corresponding to low μg/ml concentrations.

It has been shown that the high separation efficiency obtained in CE can be maintained easily in the off-line MALDI-MS detection system. MALDI-MS is shown to yield detection limits of the same order of magnitude as UV detection.

ACKNOWLEDGEMENTS

We thank N.J. Reinhoud for many helpful discussions and practical advice. We wish to thank Professor S. Hjertén for the fruitful discussions on this topic. These investigations were supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Technology Foundation (STW).

REFERENCES

1 J.W. Jörgenson and K.,D. Lukacs, *J. Chromatogr.*, 218 (1981) 209.
2 M. Karas and F. Hillenkamp, *Anal. Chem.*, 60, (1988) 2299.
3 T.P. Fan, E.D. Hardin and M.L. Vestal, *Anal. Chem.*, 56 (1984) 1870.
4 R. Takigiku, T. Keough, M.P. Lacey and R.E. Schneider, *Rapid Commun. Mass Spectrom.*, 4 (1990) 24.
5 T. Keough, R. Takigiku, M.P. Lacey and M. Purdon, *Anal. Chem.*, 64 (1992) 1594.
6 K. Strupat, M. Karas and F. Hillenkamp, *Int. J. Mass Spectrom. Ion Process.*, 111 (1991) 89.
7 M. Mann, H. Rahbek-Hielsen and P. Roepstorff, in A.

Hedin and B.U.R. Sundqvist (Editors), *Ion Formation from Organic Solids V, Proceedings of the 5th IFOS Conference, Lövånger, Sweden, June 18–21, 1989*, Wiley, Chichester, 1990, p. 47.

8 K.-O. Eriksson, A. Palm and S. Hjertén, *Anal. Biochem.*, 201 (1992) 211.

9 M. Albin, S.M. Chen, A. Louie, C. Pairaud, J. Colburn and J. Wiktorowicz, *Anal. Biochem.*, 206 (1992) 382.