ELSEVIER

Journal of Chromatography A, 707 (1995) 327–333

JOURNAL OF CHROMATOGRAPHY A

Determination of isoflavones using capillary electrophoresis in combination with electrospray mass spectrometry

M.A. Aramendia*, I. García, F. Lafont, J.M. Marinas

Department of Organic Chemistry and Mass Spectrometry Service, Faculty of Sciences, University of Córdoba, Avda S. Alberto Magno s/n, E-14004 Córdoba, Spain

First received 27 December 1994; revised manuscript received 7 March 1995; accepted 8 March 1995

Abstract

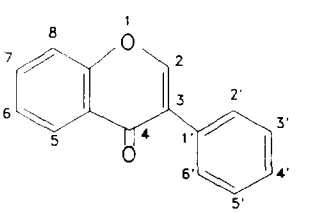
Various isoflavones were separated on an uncoated fused-silica capillary electrophoresis (CE) column (110 cm × 75 μm I.D.) using 25 m*M* ammonium acetate buffer and UV and electrospray ionization mass spectrometric (ESI-MS) detection. CE–ESI-MS with negative-ion electrospray ionization has been shown to be a suitable technique for the determination of this type of natural compound. The modest sample loading of the CE technique can be circumvented by coupling it with MS; in addition, the mass-resolving capability and high sensitivity of MS for structural analysis of mixtures can thus be exploited. Furthermore, ESI-MS allows one not only to determine the molecular mass of isoflavones, but also the presence of various functional groups according to observed losses from the $[M-H]^-$ ion during collision-induced dissociation by adjusting some MS parameters.

1. Introduction

Isoflavonoids make up a large group of naturally occurring substances in plants. In contrast to their near ubiquitous occurrence in higher plants of other flavonoids, isoflavonoids are primarily confined to one group of plants, the sub-family Lotoideae of the Leguminosae [1].

One distinct feature of isoflavonoids is that they possess biological activity. In contrast to other flavonoids, most of which are harmless substances [2], isoflavonoids have oestrogenic, insecticidal, pesticidal and antifungal properties. Isoflavones are by far the most common type of isoflavonoid. Their structures are based on the 3-phenylbenzopyrone group (see formula) and differ in the extent of hydroxylation, methylation and glycosylation. Isoflavones are weak oestrogens; their presence in forage legumes has been recognized to result in animal infertility. Formononetin, genistein and biochanin A are the principal oestrogenic isoflavones; their degradation products consist mainly of simple phenols such as *p*-ethylphenol [3].

Available analytical techniques for flavonoid determination include HPLC [4], TLC and GC, most of which, however, are laborious and time consuming.



* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00343-6

Electron impact mass spectrometry is a powerful tool for determining isoflavonoid structures. In fact, it has been succesfully applied to all types of flavonoid [5]. In contrast, chemical ionization MS using methane as the reactant gas has been applied to only a few flavonoids and produces few diagnostic fragments except for flavonones and dihydroflavonols [6]. Most flavonoids yield intense peaks for the molecular ion M^+ and indeed this is often the base peak. Derivatization is therefore unnecessary unless GC–MS is to be carried out, in which case trimethylsilylation or permethylation [7] provides the required volatility.

Capillary electrophoresis (CE) is a powerful technique that affords rapid, high-resolution separations (10^4–10^6 theoretical plates) while requiring only a few femtomoles of sample. A broad range of compounds, including peptides, nucleotides, surfactants, environmental pollutants and natural products, are amenable to separations by CE. The technique is applicable to a wide range of analytes present in buffered aqueous solution as charged species. The utility of CE, however, is greatly enhanced by MS detection, particularly with electrospray ionization (ESI), a soft ionization technique that can be used to produce ions even from thermally labile, non-volatile, polar compounds [8–11].

In this work, we explored the use of on-line capillary electrophoresis and mass spectrometry with electrospray ionization for the separation and characterization of selected isoflavones. Collision-induced dissociation (CID) reactions in the intermediate pressure region between the ESI source and the single quadrupole mass analyser for this type of compound are described.

2. Experimental

2.1. Mass spectrometer and electrospray interface

All experiments were performed on a VG Platform single-quadrupole mass spectrometer (Fisons Instruments) equipped with an electrospray ionization source. A diaxial electrospray probe was used for direct infusion of samples. The instrument was operated in the negative-ion mode at a probe tip voltage of −3.5 kV. The extraction cone voltage was varied from −25 to −75 V. Nitrogen was used as both the electrospray nebulizing gas and the drying gas. The nitrogen pressure was set at 6 bar and the flow-rates of the drying gas and nebulizing gas were set at 200 and 10 l h^{-1}, respectively, for maximum sensitivity. The source temperature was maintained at 70°C. Samples were introduced into the source by direct injection via a Rheodyne Model 7125 injection valve with a 10-μl loop; the solvent flow (10 μl min^{-1}) was delivered by an LKB 2150 HPLC pump. The mass spectrometer was scanned from m/z 90 to 325 at a rate of 2 s per scan.

A triaxial electrospray probe which incorporates a sheath tube (0.005 in. I.D. PEEK tube) allowing additional solvent to be transported to the probe tip and mixed coaxially with the sample flow (at the end of the CE capillary) before spraying, was used in the CE–ESI-MS mode. This make-up fluid performs two functions: (a) to supplement the CE flow by the extent required for electrospray ionization and (b) to make electrical contact between the CE buffer and the probe tip. The probe allows the CE system to be interfaced to a standard electrospray source using the 375 μm O.D. fused-silica column employed in the CE experiment. Approximately 1 cm of the fused-silica external polyamide coating was removed from the outlet side at the probe tip. The source was operated at −3.5 kV (in the negative-ion mode). A coaxial sheath liquid consisting of water–2-propanol (80:20) at a flow-rate of 10 μl min^{-1} was used as the make-up fluid for CE–ESI-MS. Nitrogen was used as both the drying gas (50 l h^{-1}) and the ES nebulizing gas (10 l h^{-1}). In this case, mass spectral data were acquired using selected-ion recording (SIR mode; 0.2 s dwell time, 0.2 mass unit span) for the $[M-H]^-$ ion.

Mass calibration was carried out with a mixture of NaI and CsI in 2-propanol–water (50:50).

2.2. Capillary electrophoresis

A P/ACE 5500 CE system (Beckman Instruments) equipped with a P/ACE diode-array detector was used for CE and CE–MS experiments. A 110 cm × 75 μm I.D. × 375 μm O.D. uncoated fused-silica capillary column was used. The polyamide coating was removed 20 cm from the capillary inlet to create a window for UV absorbance measurements. This column was used for CE–UV and CE–UV–ESI-MS analyses. The untreated capillary was washed first with 100 m*M* NaOH and then with Milli-Q-purified water and the electrolyte. Samples were injected at a low pressure (0.5 bar) for 5 s (injection volume ca. 3 nl). Bulk flow in the CE capillary was minimized by adjusting the height of the inlet (anode) relative to the ES probe. A voltage of 30 kV was applied to the anode; since the electrospray needle acted as the cathode (−3.5 kV), the overall potential difference was 33.5 kV, so samples were electrophoresed at 305 V cm^{-1}, which resulted in very stable currents of ca. 35 μA. UV detection was performed at 260 nm, with scanning between 190 and 400 nm.

2.3. Sample preparation

Standards including daidzein (7,4′-dihydroxyisoflavone, M_r 254.2), formononetin (7-hydroxy - 4′ - methoxyisoflavone, M_r 28.3), pseudobaptigenin (7-hydroxy - 3′,4′ - dioxomethyleneisoflavone, M_r 282.3), biochanin A (5,7-dihydroxy - 4′ - methoxyisoflavone, M_r 284.3), genistein (4′,5,7 - trihydroxyisoflavone, M_r 270.2), isoliquirtigenin (4,2′,4 - trihydroxychalcone, M_r 256.3) and biochanin A 7-glucoside (M_r 446.4) were all obtained from Sigma. Standard solutions for direct ES experiments were prepared at a 0.1 m*M* concentration in water–2-propanol (80:20) containing 0.3% ammonia. For CE–ESI-MS, 1 m*M* standards were dissolved in a buffer solution containing 25 m*M* aqueous ammonium acetate at pH 9.5 (adjusted with ammonia solution). All organic solvents (HPLC grade) and samples were filtered through 0.2-μm pore-size nylon membrane filters (Millipore).

3. Results and discussion

3.1. Electrospray

Figs. 1–5 show the ESI mass spectra for individual isoflavones dissolved in water–2-propanol containing 0.3% ammonia, recorded at different extraction cone voltages (−25, −50 and −75 V). The extraction cone voltages serves primarly to focus ions into the mass analyser. However, above a typical cone voltage of 40 V (absolute value), sample ions can gain sufficient energy to undergo CID reactions with neutral molecules in the intermediate pressure region and thus provide useful information about fragment ions [12–15].

In a basic medium, substances are detected as anions because the acid–base equilibrium is

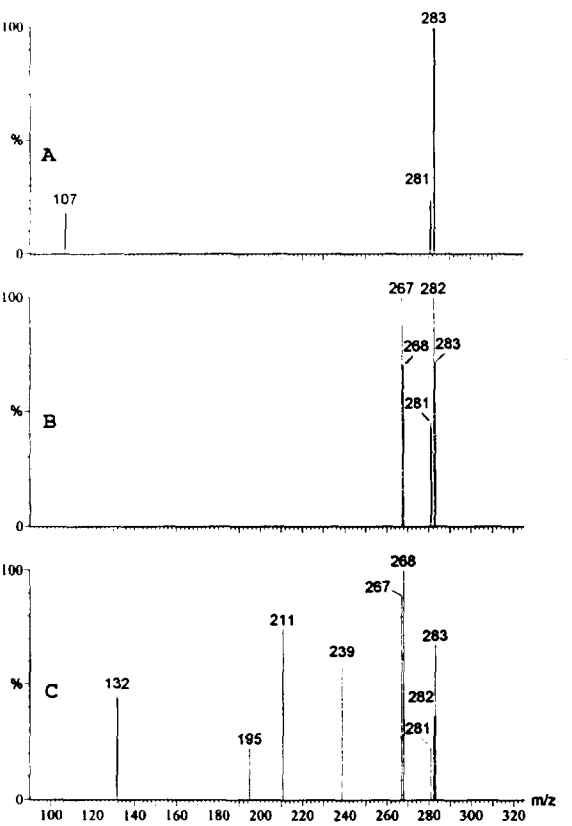


Fig. 1. ESI mass spectra for biochanin A at different extraction cone voltages: (A) −25; (B) −50; (C) −75 V.

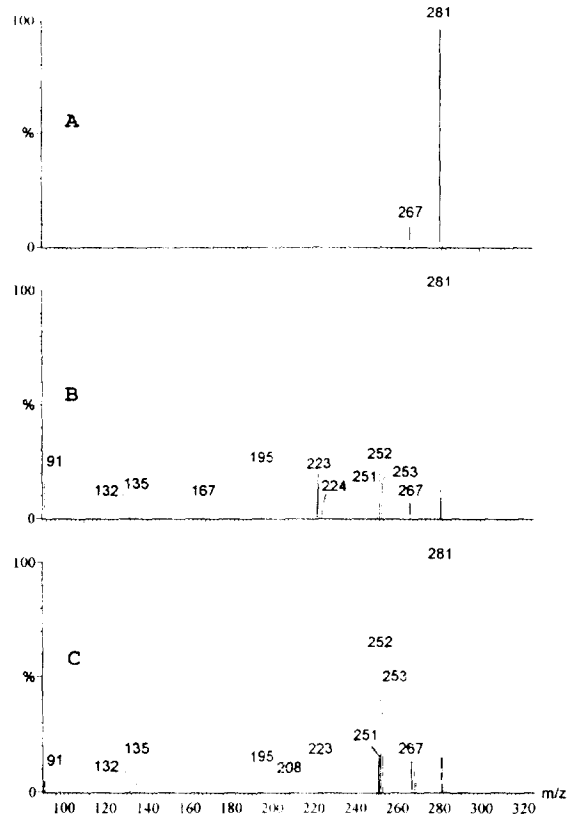


Fig. 2. ESI mass spectra for pseudobaptigenin at different extraction cone voltages: (A) −25; (B) −50; (C) −75 V.

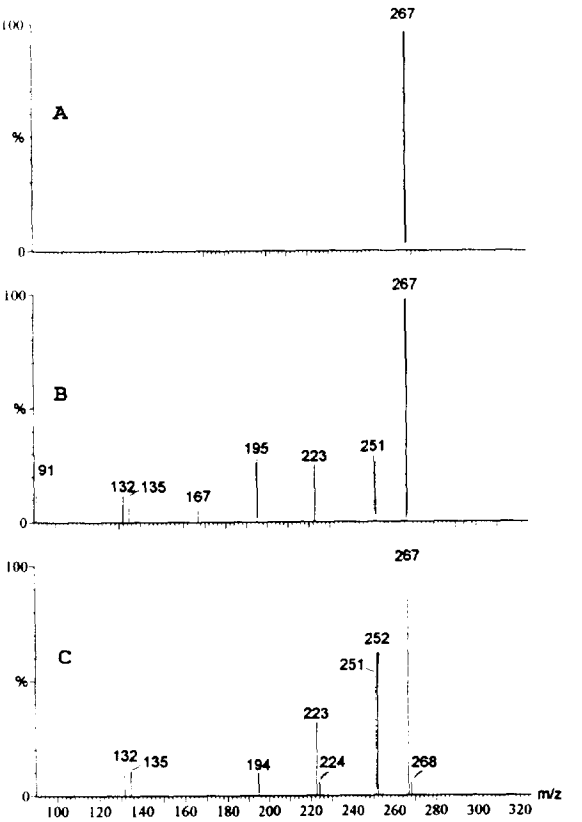


Fig. 3. ESI mass spectra for formononetin at different extraction cone voltages: (A) −25; (B) −50; (C) −75 V.

shifted to the basic form, so the spectrum exhibits the $[M-H]^-$ peak. At low extraction cone voltages, the mass spectrum of each isoflavone tested consisted exclusively of $[M-H]^-$ ion as the base peak; fragment peaks from CID reactions appear at high extraction voltages values (−50 and −75 V) and provide structural information about molecules. Biochanin A exhibited an $[M-H]^-$ ion peak at m/z 283 as the base peak at an extraction cone voltage of −25 V (Fig. 1); however, raising the extraction cone voltage produced new peaks at m/z 268 ($[M-H-CH_3]^-$), m/z 239 ($[M-H-CH_3-COH]^-$) and m/z 211 ($[M-H-CH_3-COH-CO]^-$). Pseudobaptigenin (Fig. 2) produced an $[M-H]^-$ ion at m/z 281 as the base peak and additional peaks at m/z 267 ($[M-H-CH_3]^-$), m/z 252 ($[M-H-OCH_3]^-$), m/z 223 ($[M-H-OCH_3-CO]^-$) and m/z 195 ($[M-H-OCH_3-CO-CO]^-$). Formononetin exhibited an $[M-H]^-$ base peak at m/z 267 (Fig. 3) and other peaks at m/z 252 ($[M-H-CH_3]^-$), m/z 223 ($[M-H-CH_3-COH]^-$) and m/z 195 ($[M-H-CH_3-COH-CO]^-$). Biochanin A 7-glucoside (Fig. 4) provided no peak at −25 V; at a higher voltage, however, it produced several peaks, viz., one at m/z 283 (biochanin A) due to glucosidic bond breakdown, another at m/z 255 ($[M-H-CO]^-$) and a very small $[M-H]^-$ peak at m/z 445. On the other hand, both daidzein and genistein (Fig. 5) gave fewer peaks than the other compounds studied, exhibiting practically the $[M-H]^-$ peak at extraction cone voltages between −25 and −75 V.

As a general rule, compounds with a methoxy group (biochanin A and formononetin) exhibit one fragment ion at m/z $[M-H-CH_3]^-$ when a high extraction cone voltages is used.

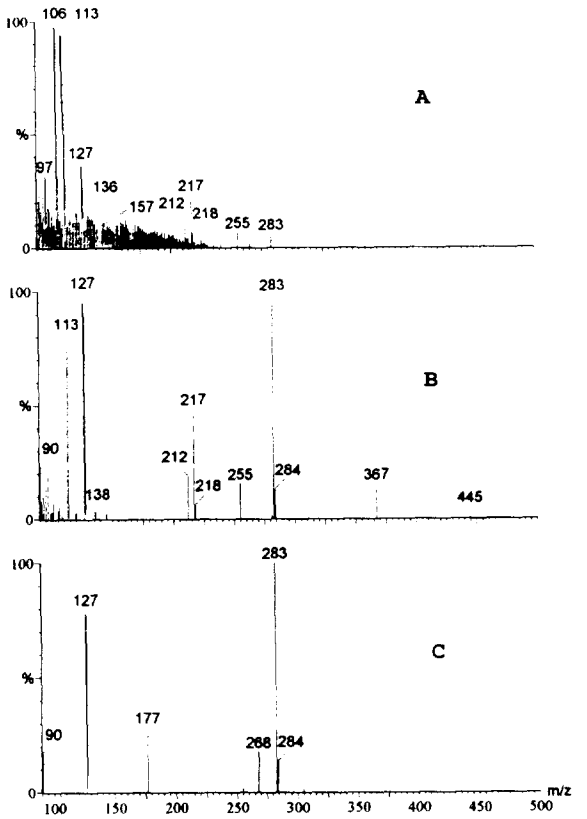


Fig. 4. ESI mass spectra for biochanin A 7-glucoside at different extraction cone voltages: (A) −25; (B) −50; (C) −75 V.

These results are typical for the negative ES ionization of small molecules, i.e., major ions result from $[M-H]^-$, with essentially no fragment ions; also, only under stronger ES conditions no molecular fragments appear.

3.2. CE–UV and CE–MS

Figure 6 shows the electropherogram for an isoflavone mixture (1 m*M*) containing about 3 fmol of each compound after 20 cm of capillary using UV detection at 260 nm. Each analysis was complete within 4 min, although higher resolution could have been achieved by using a longer distance between the capillary inlet and the UV window. On the other hand, biochanin A, formononetin and pseudobaptigenin could not be resolved under these conditions.

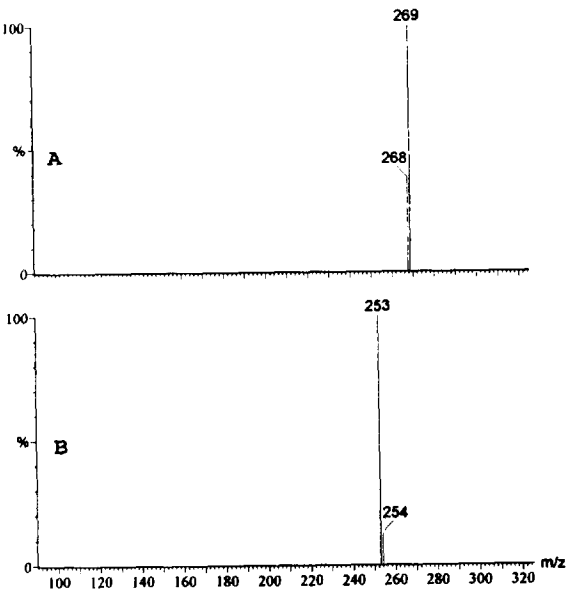


Fig. 5. ESI mass spectra for (A) genistein and (B) daidzein at an extraction cone voltage of −75 V.

The SIR chromatograms in Fig. 7 show the final separation of the mixture achieved after 110 cm of capillary. The elution sequence was established by mass spectral interpretation and the known CE migration behaviour and was found to be (1) genistein, (2) daidzein, (3) formononetin, (4) pseudobaptigenin and biochanin

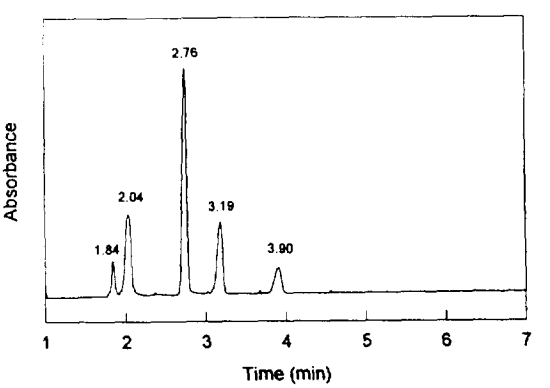


Fig. 6. Electropherogram for an isoflavone mixture with UV detection at 260 nm. Buffer, 25 m*M* NH_4OAc (pH 9.5); run voltage, 33.5 kV; current, 35 μA; sample concentration, 0.1 m*M*; injection, 5 s. Peaks: genistein (1.89 min), daidzein (2.04 min), pseudobaptigenin, formononetin and biochanin A (2.76 min), isoliquirtigenin (3.19 min) and biochanin A 7-glucoside (3.90 min).

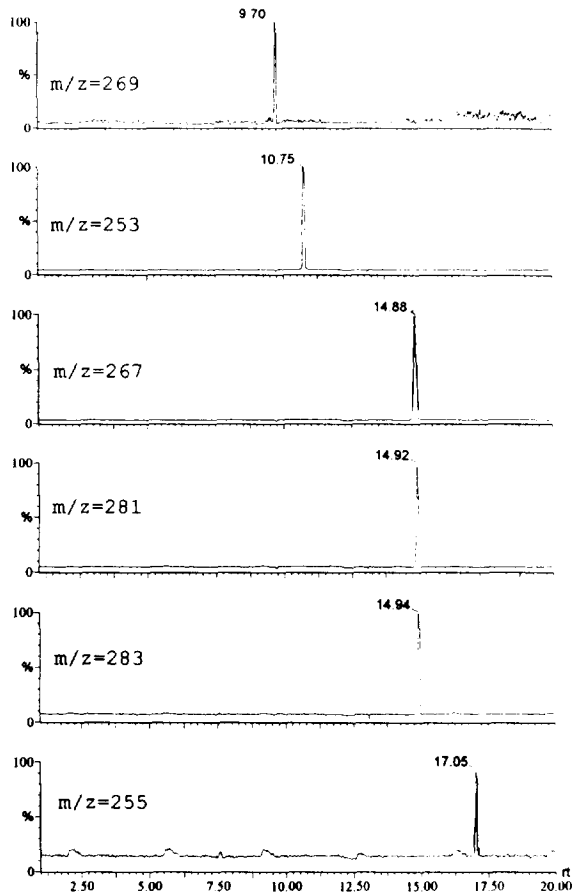


Fig. 7. CE–ESI mass chromatograms for a mixture containing about 3 fmol of each isoflavone.

A and (5) isoliquirtigenin. Biochanin A 7-glucoside did not appear because we used ESI conditions (extraction cone voltage −25 V) where each compound provided a single mass peak $[M-H]^-$, so this compound, as noted earlier, gave no signal. Therefore, although this isoflavone mixture cannot be readily resolved using conventional CE with UV detection, it can be resolved by CE–MS. The signal reproducibility was found to be very good (the variation in peak areas was less than 10% for the same sample).

The success of the CE–ESI-MS analysis of isoflavones relies on many factors, including the ESI interface and buffer composition. Analyte detectability and sensitivity vary widely with the buffer concentration, the optimum analytical signal for CE–ESI-MS being obtained when volatile buffers are utilized at the lowest possible concentration (10–25 m*M*); in fact, higher concentrations reduce the analyte ionization efficience in the electrospray process.

4. Conclusions

Combined CE–ESI-MS allows the efficient separation and identification of isoflavones with higher specificity than CE (with UV detection) alone. The reported results demonstrate the utility of this coupled technique for the qualitative analysis of natural compound mixtures such isoflavones, with high reproducibility and sensitivity. In addition, capillary electrophoresis, with its exceptionally low flow-rate (nl min^{-1}), is easier to interface to MS than is liquid chromatography, since no flow splitting is required. One other advantage of ESI is the ability to induce CID reactions in the intermediate pressure region of the source by increasing the extraction cone voltage; this permits the recognition of certain functional groups in unknown compounds and confirmation of target analytes by using the intensity ratios of several diagnostic fragment ions.

Acknowledgements

The authors acknowledge funding of this research by the Consejería de Educación y Ciencia de la Junta de Andalucía and the Dirección General de Investigación Científica y Técnica (DGICyT) in the framework of Project PB92-0816. They also thank Dr. M. Tena for providing the isoflavone standards.

References

[1] M. Jay, P. Lebreton and R. Letoublon, Boissiera, 19 (1971) 219.
[2] W.B. Whalley (Editor), The Pharmacology of Plant Phenolics, Academic Press, London, 1959.

[3] J.B. Harborne, T.J. Mabry and H. Mabry (Editors), The Flavonoids, Chapman and Hall, London, 1975.
[4] S.M. Lunte, J. Chromatogr., 384 (1987) 371.
[5] A. Weissberger and E.C. Taylor (Editors), Mass Spectrometry of Heterocyclic Compounds, Wiley-Interscience, New York, 1971.
[6] D.G.I. Kingston and H.M. Fales, Tetrahedron, 29 (1973) 4083.
[7] E.D. Pellizzari, C.M. Chuang, J. Kuc and E.B. Williams, J. Chromatogr., 40 (1969) 285.
[8] T. Braun and S. Zsindely, Trends Anal. Chem., 11 (1992) 307.
[9] J.A. Loo, H.R. Udseth and R.D. Smith, Anal. Biochem., 179 (1989) 204.
[10] J.A. Olivares, N.T. Nguyen, C.R. Yonker, and R.D. Smith, Anal. Chem., 59 (1987) 130.
[11] C.M. Whitehouse, A.N. Dreyer, M. Yamashita and J.B. Fenn, Anal. Chem., 57 (1985) 675.
[12] D.R. Doerge, S. Bajic and S. Lowes, Rapid Commun. Mass Spectrom., 7 (1993) 462.
[13] S. Pleasance, P. Blay, M.A. Quilliam and G.J. O'Hara, J. Chromatogr., 558 (1991) 155.
[14] K.L. Duffin, T. Wachs and J.D. Henion, Anal. Chem., 64 (1992) 61.
[15] D.R. Doerge, S. Bajic and S. Lowes, Rapid Commun. Mass Spectrom., 7 (1993) 1126.