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Short communication

On-line coupling of solid-phase extraction with mass spectrometry for the analysis of biological samples III. Determination of prednisolone in serum

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Abstract

Solid-phase extraction (SPE) was directly coupled to mass spectrometry (MS) to assess the feasibility of the system for the rapid determination of prednisolone in serum. A C_{18} stationary phase allowed washing of the cartridge with 25% methanol. Elution was performed by switching the methanol percentage from 25% in the washing step to 50% during elution. The high flow-rates during the extraction (5.0 ml/min) combined with ion-trap MS detection resulted in a total analysis time of 4 min. Some tailing of the prednisolone peak was observed. However, the tailing was found acceptable, since by this elution procedure most matrix compounds were prevented from eluting from the cartridge. Some matrix interference was still observed with a triple-quadrupole MS, even in the multiple reaction monitoring mode. This resulted in a detection limit (LOD) of about 10 ng/ml. The matrix interference and the LOD were similar for atmospheric pressure chemical ionisation and atmospheric pressure photo ionisation. Applying an ion-trap MS in the MS–MS mode resulted in cleaner chromatograms. Due to extensive fragmentation of prednisolone, the LOD was not lower than about 5 ng/ml prednisolone in serum, and a limit of quantitation of about 10 ng/ml (relative standard deviation <15%) was observed.

Keywords: Prednisolone

1. Introduction

Prednisolone is a glucocorticoid derived from hydrocortisone. Its main therapeutic application is due to its immunosuppressive effects [1]. For this reason, prednisolone is often administered prior to, during and after organ transplants in order to decrease the risk of organ rejection. Prednisolone is a hormone-like compound and its side-effects are therefore also hormone-related. A balance must be found between the side effects and the chance of a successful organ transplant. The rapid determination of prednisolone in serum at concentrations down to the low ng/ml level is thus required. Most methods

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used for the determination of prednisolone in serum or plasma apply liquid–liquid extraction [2–5] or off-line solid-phase extraction (SPE) [6,7]. Such techniques are time-consuming and error-prone steps such as evaporation and reconstitution of the eluate are required. Modern developments in the coupling of liquid chromatography with mass spectrometry (MS) [8,9] have offered tremendous potential for high-throughput analysis. On-line coupling of SPE with LC–MS is well established [10–13]. Furthermore, the potential of SPE coupled directly with MS was also shown [14,15].

In this study, we investigated the potential of SPE–MS^n for the rapid analysis of prednisolone in serum down to the low ng/ml level. Steroids are easily fragmented to various fragments simultaneously by thermal degradation as well as by collision-induced dissociation (CID) [16–21], which may result in limited sensitivity, and the latter aspect should thus be carefully investigated. To achieve low ng/ml levels, an ion-trap MS and a triple-quadrupole MS were compared. The latter type of MS was used applying atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photo ionisation (APPI). A detailed description of the APPI mechanism can be found in the literature [22]. APPI may ionise analytes, and in particular hormones, more efficiently than APCI [22–24], thus potentially allowing the determination of lower concentrations of prednisolone.

2. Experimental

2.1. Chemicals and instrumentation

All on-line SPE–MS experiments were performed with a Prospekt sample handler (Spark, Emmen, The Netherlands) using one six-port valve, the cartridge-switching device, and a solvent delivery unit (SDU). Activation, conditioning, sampling, trapping, and washing were done using the SDU. The effluent was connected to waste during these steps. All steps of the SPE procedure were carried out using a forward-flush mode. A cartridge was replaced after single use. A second flow stream from a Series 1100 gradient pump (Hewlett-Packard, Waldbronn, Germany) was used for the elution which was connected to the mass spectrometer via the cartridge. During the optimisation of the SPE procedure two polymeric (PLRP-S and Resin GP) and a C_{18} stationary phase (all from Spark-Holland) were used. After optimisation, HySphere-9 (C_{18}, 10×2 mm, particle size 7 μm) cartridges were applied.

Methanol was of HPLC grade (Lab Scan, Dublin, Ireland). Glacial acetic acid was of analytical-reagent grade (Merck, Darmstadt, Germany). Water was obtained from an Elgastat Maxima system (Salm and Kipp, Breukelen, The Netherlands). Aqueous solutions were passed through a 0.45-μm RC 55 membrane filter (Schleicher & Schuell, Dassel, Germany) prior to use. Prednisolone (Ph.Eur., Genfarma, Maarssen, The Netherlands) was dissolved in methanol (1 mg/ml) and stored in the dark at −20 °C. Spiking of samples was performed by addition of a small volume of the stock solution that is adequately diluted with buffer to fetal calf serum (PAA Laboratories, Linz, Austria).

2.2. SPE procedure

The final SPE procedure for the C_{18} cartridges was as follows: activation was performed with 2.5 ml methanol, and conditioning with 3.75 ml diluted acetic acid (0.5%, pH about 3) at a flow-rate of 5.0 ml/min. A 500-μl sample was loaded onto the cartridge with diluted acetic acid (0.75 min at 2.0 ml/min) to minimize possible carry-over, followed by a washing step with 3.75 ml of 25:75 methanol–diluted acetic acid (0.5%) at a flow-rate of 5.0 ml/min. The elution was started with 25:75 methanol–acetic acid (0.5%), and subsequently within 0.1 min the methanol percentage was increased to 50%. This percentage was maintained for 1.15 min. A flow-rate of 1.0 ml/min was used during elution. Quantitation was performed by the use of external calibration, i.e. by comparing the signal of spiked buffer or spiked serum with known quantities of prednisolone added to the same matrix.

2.3. Mass spectrometry

An LCQ Classic ion-trap MS (Thermoquest, San Jose, CA, USA) equipped with an APCI source was

used. The vaporiser temperature was set at 350 °C. The sheath gas and auxiliary gas (both nitrogen) were 34 and 3 (arbitrary units), respectively. The discharge current was set at 5.00 μA and the capillary voltage was 15.00 V. The temperature of the heated capillary was 170 °C, and the tube lens offset was set at 40.00 V. All scans were recorded in the full-scan mode with three microscans over the range of m/z 295 to 370 using the positive-ion mode. The maximum injection time was set at 300 ms. Helium was applied as cooling gas and collision gas. Extracted ion chromatograms in all MS modes were obtained for $[M+H]^+$ (m/z 361) or fragment ions ±0.5 Th. The isolation width during MS^n experiments was 2.0 Th. The collision energy applied during MS–MS experiments was 20%.

An API3000 triple-quadrupole MS (MDS-Sciex, Concord, Canada) was used with both an APCI and an APPI [22] source. The settings used during single-MS analysis and multiple-reaction monitoring (MRM) are presented in Table 1. In the single-MS mode a Q1-scan was performed from m/z 100 to 400. In the MRM mode m/z 361.1 was fragmented and the products at m/z 307.0 and 325.0 were monitored. When applying APCI, nitrogen was used as curtain gas and auxiliary gas, and zero air was used as nebulising gas. During APPI experiments, only nitrogen was used. The lamp protection gas was set at about 1 l/min, and a lamp current of 0.75 mA was used. Toluene was used as dopant, which was added to the auxiliary gas line via a T-piece. A flow-rate of 50 μl/min was used.

Table 1
Settings of the triple-quadrupole MS during Q1-scan and MRM experiments

Nebulizer gas (arb)	15	Q0 (V)	−10.00
Curtain gas (arb)	10	IQ1 (V)	−11.00
CAD (arb)	0 (4[a])	ST (V)	−16.00
Needle current (μA)	2.00[b]	RO1 (V)	−11.0
Temperature (°C)	450	IQ2 (V)	−18.0
Orifice (V)	20	RO2 (V)	−100 (−25.0[a])
Ring (V)	50	ST3 (V)	−120 (−45.0[a])
CEM	2500	RO3 (V)	−102 (−27.0[a])
		DF (V)	−100

[a] Numbers in brackets are settings for MRM experiments.
[b] For APPI experiments—source offset voltage = 2000 V.

3. Results and discussion

3.1. Optimisation of the SPE procedure

When applying SPE, steroid- or hormone-like compounds are commonly extracted from the sample by use of an apolar extraction phase [25–27]. Therefore, the apolar C_{18} phase and two polymeric phases (PLRP-S and Resin GP) were investigated for the extraction of prednisolone. The sample was loaded onto the cartridge after which the stationary phase was washed with buffer at pH 8.5, 7 or 3. These pH values should ensure that the system would be compatible with MS (use of volatile buffers, e.g. ammonium acetate), while a good buffer capacity (pH 8.5 and 3) was still maintained or a pH near that of serum was applied (pH 7). After washing, a 10-min gradient from 0 to 100% methanol, buffered at the same pH, was used for the elution. Diode-array detection was applied during the optimisation of the SPE procedure. The peak shape and the position for the analyte with regard to the matrix were the criteria for the selection of an appropriate stationary phase. With the polymeric phases, hardly any separation between the analyte and the matrix could be obtained.

With the C_{18} stationary phase, no separation was observed at pH 8.5. Decreasing the pH to 7 or 3 did not move the prednisolone peak due to the fact that prednisolone is a neutral species. However, the retention of the co-extracted matrix components was increased, resulting in more distinction between prednisolone and the matrix compounds. The best results were obtained at pH 3. It was possible to wash the cartridge with 25:75 methanol–diluted acetic acid (0.5%) at a flow-rate of 5.0 ml/min without breakthrough of the analyte from the extraction phase. This ensured that the early eluting matrix compounds, i.e. polar components, were removed. Furthermore, a high recovery (comparison of spiked samples and aqueous solutions) was obtained for the analyte (that is, for spiked samples no losses of analyte were observed during the sampling and washing step). Subsequently, the elution was performed starting at 25% methanol and increasing this to 50% within 0.1 min. No further increase in the percentage of methanol was used so that most of

the co-extracted and apolar matrix components that were not removed during the wash step were retained by the stationary phase, and could therefore not interfere with the MS detection. After each analysis the cartridge was discarded. The final extraction procedure is presented in Table 2. The total analysis time was about 4 min. The first optimisation was performed applying DAD, and subsequently the results of single-MS analysis were compared with those of DAD analysis. Obviously, applying MS may result in the detection of matrix compounds not observed with DAD. Thus, further evaluation of the total SPE–MS procedure will be necessary.

3.2. SPE–MS system

3.2.1. Use of ion-trap MS

An ion-trap MS was applied for the SPE–MS system. The vaporiser temperature was set at 350 °C, which gave adequate evaporation and no memory effect was observed. However, extensive fragmentation was observed due to the thermolability of the analyte and/or the easy CID of prednisolone. The fragmentation pattern and a mass spectrum in the MS mode are shown in Fig. 1A and B. The fragmentation of prednisolone implied that the parent ion m/z 361 was only about 54% of the total abundance. With MS–MS experiments, no formation of the fragments with m/z 329 and 301 were observed, suggesting that these ions were indeed formed by thermal degradation. Three fragments, i.e. m/z 343, 325 and 307, were formed after CID of the parent ion $[M+H]^+$ (Fig. 1C). Fragment m/z 343 gave the highest signal, but the summation of the extracted ions of m/z 307 and 325 resulted in the best signal-to-noise (S/N) ratio, even though only about 15% of the signal of the product ion was converted into these fragments.

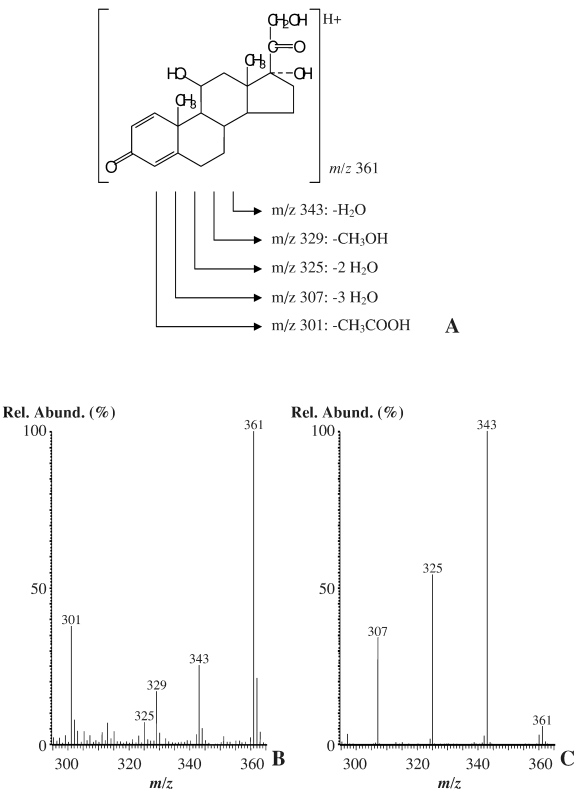

Fig. 1. (A) Structure and fragmentation pathway of prednisolone in the ion-trap MS; (B) mass spectrum (MS mode); (C) mass spectrum (MS–MS mode, fragmentation of m/z 361).

The use of the ion-trap MS in the single-MS mode (Fig. 2A) showed severe matrix interferences, which

Table 2
Optimised SPE procedure for C_{18} cartridges

	Solvent composition	Flow-rate (ml/min)	Volume (ml)	Time (min)
Activation	Methanol	5.0	2.5	0:00–0:50
Conditioning	Acetic acid (0.5%)	5.0	3.75	0:50–1:25
Sampling	Serum (500 μl)[a]	2.0	1.5	1:25–2:00
Washing	75:25 (v/v) acetic acid–methanol	5.0	3.75	2:00–2:75
Elution	50:50 (v/v) acetic acid–methanol	1.0	1.25	2:75–4:00

[a] Sampled with excessive amount of acetic acid (0.5%).

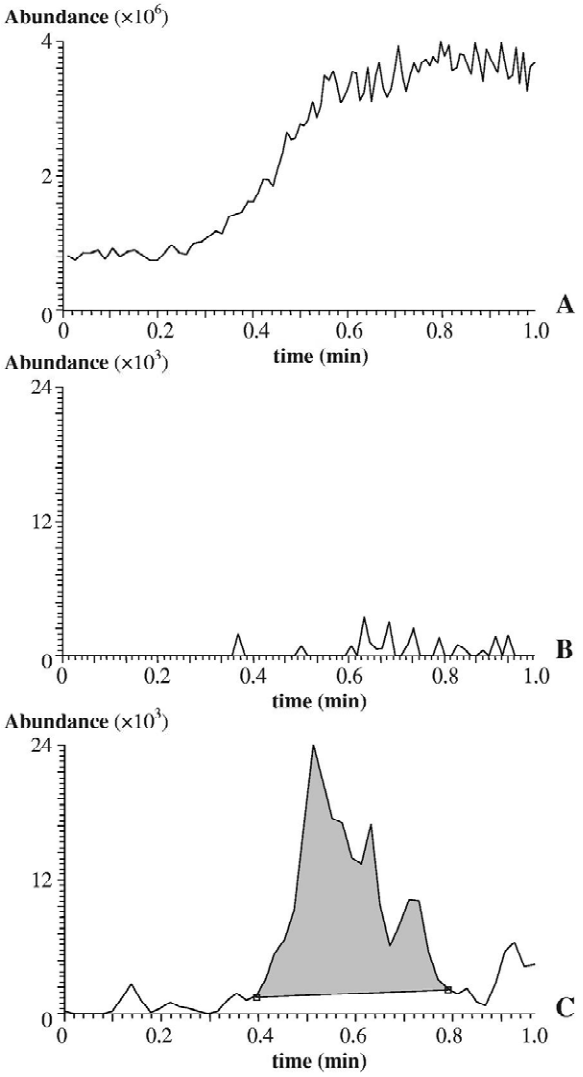

Fig. 2. (A) SPE–MS with an ion-trap MS using the APCI interface for extraction of blank serum (total ion count, m/z 295–370); (B) SPE–MS–MS of blank serum and summation of extracted ions m/z 307 and 325, (C) SPE–MS–MS of serum spiked with 10 ng/ml prednisolone.

made the detection of concentrations below about 50 ng/ml impossible. The application of MS–MS resulted in clean chromatograms after extraction of blank serum (Fig. 2B) and an improved LOD (5 ng/ml; determined as three times the level of spikes in the chromatogram) was observed (Table 3). A good reproducibility and linearity were obtained. A representative chromatogram of spiked serum is depicted in Fig. 2C. Some tailing of the prednisolone peak was observed. Preliminary validation results showed good linearity and reproducibilities over the investigated concentration range. The limit of quantitation was about 10 ng/ml (relative standard deviation <15%). Comparing the signals after extraction from buffer and from serum showed higher signals for the latter. At 10 ng/ml, the signal after extraction from serum was about two times as high as the signal after extraction from buffer. This decreased to a factor of about 1.5 at 20 ng/ml and was about 1.2 at higher concentrations (30–550 ng/ml). A post-cartridge continuous infusion of the analyte while extracting blank serum [28], showed a similar ion enhancement effect. This matrix effect will be further explored in the near future.

Table 3
Analytical data of the SPE–MS^2 systems for the determination of prednisolone in serum using an ion-trap MS (MS–MS mode) and a triple-quadrupole MS (MRM mode)

	Ion-trap MS APCI	Triple-quadrupole MS	
		APCI	APPI
LOD (ng/ml)	5	10	10
Linearity[a] (R)	0.9944	0.9962	0.9959
Range (ng/ml)	10–550[b]	10–275[b]	10–275[b]
RSD (%)[c]	8.8	9.9	10.2

[a] Weighted regression $1/x$.
[b] Maximum concentration investigated.
[c] At 20 ng/ml, $n=6$.

3.2.2. Use of triple-quadrupole MS

Another type of MS, a triple-quadrupole instrument, was used with an APCI and APPI source. The vaporiser temperature was set at 450 °C for adequate evaporation of the eluate. With lower temperatures, memory effects were observed. The high temperature caused severe fragmentation of prednisolone due to thermal degradation. Therefore, the scan range for the triple-quadrupole MS (Q1 scan) was enlarged in comparison with analysis with the ion-trap MS (single MS) to be able to detect all fragments formed. Still, similar chromatograms as with the ion-trap MS were obtained in the single-MS mode (Q1 scan) upon analysis of serum samples (Fig. 3A). However, applying MS–MS (MRM mode), monitoring the same fragments as with the ion-trap MS

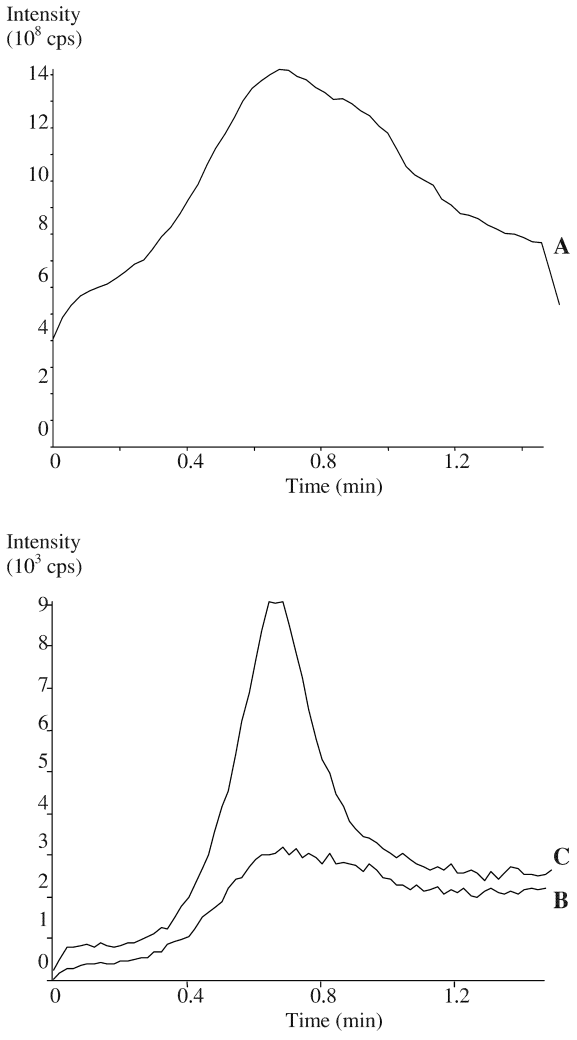

Fig. 3. (A) SPE–MS with a triple-quadrupole MS using the APCI interface for extraction of blank serum (Q1 scan m/z 100–400); (B) SPE–MS–MS of blank serum (MRM mode, transition of m/z 361 to m/z 325 and 307); (C) SPE–MS–MS of serum spiked with 10 ng/ml prednisolone (offset 500 cps).

(that is m/z 325 and 307), did not completely eliminate the matrix interference (Fig. 3B). The LOD was now about 10 ng/ml (Table 3). A representative chromatogram is depicted in Fig. 3C. With APPI, similar results were observed as with APCI with respect to sensitivity, linearity and reproducibility (Table 3). The matrix interference was at the same level and no improved sensitivity was observed. Upon the extraction of prednisolone from buffer, in which no chemical noise interfered with the determination, a lower LOD was observed with APPI in comparison with APCI. About a factor of five more sensitivity was observed with APPI than with APCI, which indicates that APPI may indeed give better sensitivity if no chemical noise deteriorates the detection. These results were in accordance with the results of other studies [22,23].

The determination of the LOD (three times the blank level) after extraction from serum and detection with the triple-quadrupole MS was rather ambiguous due to the presence of the matrix. In this study, constant signals were obtained with the blank serum. However, with real-life samples, often no blank sample is available, thus making detection at such low levels more unpredictable. The presence of matrix interference from blank serum may be due to similar fragmentation pathways of co-extracted compounds, since many endogenous compounds such as steroid hormones have similar fragmentation patterns [16,17]. The loss of H_2O is rather easy to establish from any hydroxylated compound. With hormone-like compounds, the loss of two or more H_2O molecules is very common [16,20].

The difference in matrix interference between the ion-trap MS (MS–MS mode) and the triple-quadrupole MS (MRM mode) is probably due to the difference of the MS–MS principles. In the ion-trap MS, only the precursor ion is accelerated sufficiently to be fragmented. Fragments are readily stabilised in the centre of the trap, thereby decreasing the potential for further fragmentation. In the triple quadrupole MS, more fragmentation is commonly observed [17,18], since all ions are accelerated. This implies that an ion that is already fragmented will still be accelerated towards the end of the second and the third quadrupole. Thus, a formed fragment (product ion) may thereby collide and fragment further in the second and the third quadrupole. This has the consequence that not only the analyte is more easily fragmented (as observed during MS–MS experiments), but also the matrix components. In a triple quadrupole MS, the higher level of interference can thus be due to product ions (ions should already be formed in the source or in the first quadrupole to

m/z 361) and their consecutive fragments, which is less common in an ion-trap MS.

4. Conclusions

The separation of prednisolone from endogenous compounds present in serum was difficult to obtain, and some matrix interference was still observed in the MRM mode. The present SPE–MS–MS system applying the ion-trap MS showed an increase in selectivity with regard to the triple quadrupole MS. The LOD is in the low ng/ml range, but is still rather high due to the thermal degradation and easy CID of prednisolone. The total analysis time was about 4 min, which was due to high flow-rates (up to 5 ml/min, except for the elution) during the SPE procedure. The use of even higher flow-rates (up to 10 ml/min), as well as the application of two cartridges in parallel may further enhance the throughput. In terms of speed, the SPE–MS–MS system seems very promising for analysis of prednisolone, although the sensitivities that can be obtained may be critical for some applications. Furthermore, a complete validation with real-life samples is still required.

In general, SPE–MS^n has shown good potential for high-throughput bioanalysis. The development of a rapid SPE–MS system implies fast extraction, while obtaining good selectivity at the front of the analysis (SPE) as well as at the end (MS). One should always carefully monitor the effects of matrix, and in particular those compounds that co-elute with the analyte of interest. The use of an internal standard may help to improve the reproducibility when required.

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References

[1] T.M. Brody, J. Larner, K.P. Minneman, in: Human Pharmacology. Molecular to Clinical, Mosby-Year Book, St. Louis, 1998, p. 626.
[2] F.J. Frey, B.M. Frey, L.Z. Benet, Clin. Chem. 25 (1979) 1944.
[3] M.H. Cheng, W.Y. Huand, A.I. Lipsey, Clin. Chem. 34 (1988) 1897.
[4] W.J. Jusko, N.A. Pyszczynski, M.S. Bushway, R. D'Ambrosio, S.M. Mis, J. Chromatogr. 658 (1994) 47.
[5] S.A. Döppenschmitt, B. Scheidel, F. Harrison, J.P. Surmann, J. Chromatogr. 674 (1995) 237.
[6] H. Hirata, T. Kasama, Y. Sawai, R.R. Fike, J. Chromatogr. 658 (1994) 55.
[7] N. Shibata, T. Hayakawa, K. Takada, N. Hoshino, T. Minouchi, A. Yamaji, J. Chromatogr. B 706 (1998) 191.
[8] A.P. Bruins, T.R. Covey, J.D. Henion, Anal. Chem. 59 (1987) 2642.
[9] T. Yasuda, M. Tanaka, K. Iba, J. Mass Spectrom. 31 (1996) 879.
[10] N.G. Knebel, S. Grieb, S. Leipenheimer, M. Locher, J. Chromatogr. B 748 (2000) 97.
[11] R.A.M. van der Hoeven, A.J.P. Hofte, M. Frenay, H. Irth, U.R. Tjaden, J. Van der Greef, A. Rudolphi, K.-S. Boos, G. Marko Varga, L.E. Edholm, J. Chromatogr. A 762 (1997) 193.
[12] E.R. Brouwer, D.J. van Iperen, I. Liska, H. Lingeman, U.A.Th. Brinkman, Int. J. Environ. Anal. Chem. 47 (1992) 257.
[13] E.H.R. van der Wal, E.R. Brouwer, H. Lingeman, U.A.Th. Brinkman, Chromatographia 39 (1994) 239.
[14] M.W.J. van Hout, C.M. Hofland, H.A.G. Niederländer, G.J. de Jong, Rapid Commun. Mass Spectrom. 14 (2000) 2103.
[15] G.P. Bowers, S.C. Clegg, S.C. Hughes, A.J. Harker, S. Lambert, LC–GC 15 (1997) 48.
[16] M. Mulholland, T.J. Whelan, H. Rose, J. Keegan, J. Chromatogr. A 870 (2000) 135.
[17] M. Fiori, E. Pierdominici, F. Longo, G. Brambilla, J. Chromatogr. A 807 (1998) 219.
[18] J.-P. Antignac, B. le Bizec, F. Monteau, F. Poulain, F. André, Rapid Commun. Mass Spectrom. 14 (2000) 33.
[19] M. van de Wiele, K. de Wasch, J. Vercammen, D. Courtheyn, H. de Brabander, S. Impens, J. Chromatogr. A 904 (2000) 203.
[20] P.W. Tang, W.C. Law, T.S.M. Wan, J. Chromatogr. B 754 (2001) 229.
[21] K. Fluri, L. Rivier, A. Dienes-Nagy, C. You, A. Maître, C. Schweizer, M. Saugy, P. Mangin, J. Chromatogr. A 926 (2001) 87.
[22] D.B. Robb, T.R. Covey, A.P. Bruins, Anal. Chem. 72 (2000) 3653.
[23] D.B. Robb, A.P. Bruins, T.R. Covey, Atmospheric pressure photoionization (APPI): a new ionization technique for LC/MS, in: Proceedings of 48th Conference of Mass Spectrometry and Allied Topics, Long Beach, CA, USA, 2000.

[24] D.B. Robb, A.P. Bruins, H.A.M. Peters, P.L. Jacobs, Atmospheric pressure photoionization (APPI) for high sensitivity LC/MS in bioanalysis, in: Proceedings of 48th Conference of Mass Spectrometry and Allied Topics, Long Beach, CA, USA, 2000.
[25] R. Draisci, L. Palleschi, E. Ferreti, L. Lucentini, P. Cammarata, J. Chromatogr. A 870 (2000) 511.
[26] X.-Y. Xiao, D.V. McCalley, J. McEvoy, J. Chromatogr. A 923 (2001) 195.
[27] S. Noé, J. Böhler, E. Keller, A.W. Frahm, J. Pharm. Biomed. Anal. 18 (1996) 471.
[28] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 19 (1999) 1175.