Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1050 (2004) 173–178
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of beauvericin and four other enniatins in grain by liquid chromatography–mass spectrometry

Silvio Uhlig*, Lada Ivanova

National Veterinary Institute, P.O. Box 8156 Dep., N-0033 Oslo, Norway

Received 19 March 2004; received in revised form 10 August 2004; accepted 13 August 2004

Abstract

A method is described using LC–MS–MS for the detection of five different enniatins in grain. The method involves extraction of the fungal secondary metabolites using acetonitrile–water and quantification using LC–MS–MS with atmospheric pressure chemical ionisation, without further treatment of sample extracts. The selected ion reaction of $[M + NH_4]^+$ to $[M + H]^+$ was utilised in the specific detection of the compounds. Mean recoveries (n = 5–12) of enniatins from spiked grain samples over a period of six months were 99–115%, 86–131%, 97–113%, 73–100% and 78–114% for beauvericin, enniatin A, A1, B and B1, respectively. The limits of detection were 3.0 μg/kg for beauvericin, enniatin A, B and B1 and 4.0 μg/kg for enniatin A1, which corresponds to on-column detection limits of 7.5 pg and 10 pg, respectively.

Keywords: Depsipeptides; Food; *Fusarium*; Mycotoxin; Validation

1. Introduction

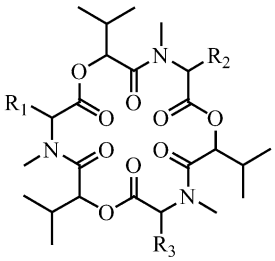
Enniatins are secondary fungal metabolites that have been known to science for several decades [1]. They represent six-membered cyclic depsipeptides, which are commonly composed of three alternating D-α-hydroxyisovaleryl and three *N*-methyl-L-amino acid residues (Fig. 1). Individual enniatins are distinguished by the nature of the *N*-methylamino acid residue and more than 10 distinct enniatins have been either isolated or synthesised so far [2–8].

Enniatins are cationophoric compounds that posses insecticidal activity [9,10] and were found to be potent and specific inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT) activity [11]. Beauvericin, which is the only enniatin that is composed of *N*-methyl-L-phenylalanyl residues, was subject to more profound toxicological investigations in recent years. It was discovered that the metabolite was produced by *Fusarium subglutinans* [12] and *F. proliferatum* strains [13] that were isolated from maize and cultivated in the laboratory. It is toxic to mammalian and human cell lines, influences the contraction of smooth muscle and induces apoptosis [14–16]. Recent reports of the production of beauvericin by *F. avenaceum* [17], the most important grain-infecting fungus of the genus *Fusarium* in Norway [18], as well as findings of high concentrations of beauvericin and three other enniatins in Finnish grain, prompted us to develop a screening method for the determination of these fungal metabolites in grain.

The development of LC–MS provided the analytical chemist with a powerful tool for the fast and selective determination of many compounds. Fragmentation of the analytes of interest, either by collision-induced dissociation in the ion source or in a collision cell/ion trap, in many circumstances produces specific daughter ions, which allow for the removal of serious matrix interferences. It is therefore often possible to minimise or even omit sample preparation. Few methods have been published that deal with the analysis of beauvericin in corn [19–21] and meat [22]. They employ HPLC with

* Corresponding author. Tel.: +47 23216264; fax: +47 23216201.
E-mail address: silvio.uhlig@vetinst.no (S. Uhlig).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.031



Compound	
Beauvericin	R_1=R_2=R_3= -$CH_2C_6H_5$
EnniatinA	R_1=R_2=R_3= -$CH(CH_3)CH_2CH_3$
Enniatin A1	R_1=R_2= -$CH(CH_3)CH_2CH_3$, R_3= -$CH(CH_3)_2$
Enniatin B	R_1=R_2=R_3= -$CH(CH_3)_2$
Enniatin B1	R_1=R_2= -$CH(CH_3)_2$, R_3= -$CH(CH_3)CH_2CH_3$

Fig. 1. Simplified drawing of the structure of the enniatins.

either UV or mass spectrometric detection after electrospray or thermospray ionisation. One paper deals with the simultaneous analysis of beauvericin, enniatin A, A1, B and B1 together with fusaproliferin from spiked water samples using LC–MS [23]. However, since the compounds possess low water solubility the latter study may be considered of low practical importance.

This paper reports the development and validation of a rapid and selective LC–MS–MS method for the simultaneous determination of beauvericin, enniatin A, A1, B and B1 in oats, barley and wheat.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol (Rathburn, Walkerburn, UK) were of LC quality, water was purified and deionised using a Purite Analyst HP water purifier (Oxon, UK). Beauvericin (ca. 99% purity), a mixture of enniatin A, A1, B and B1 (homologue composition 3%, 20%, 19% and 54%, respectively, ca. 97% purity), as well as ammonium formate were purchased from Sigma (St. Louis, MO, USA). Stock solutions of 500 μg/ml and 1000 μg/ml of beauvericin and enniatin mixture, respectively, were prepared by dissolution of the crystalline material in methanol and stored at −26 °C.

2.2. Extraction procedure

Grain samples (oats, wheat and barley, 500 g) were milled on a Retsch ZM 100 laboratory mill (Haan, Germany). Ten gram sub-samples of milled grain were homogenised (Ultra Turrax T25, Janke & Kunkel, Staufen i.Br., Germany) with 80 ml of acetonitrile–water (84:16, v/v) for 3 min and thereafter placed on an orbital shaker at 175 min^{-1} for 30 min. Extracts were filtered through S&S $595^{1/2}$ folded filters (Schleicher & Schüll, Dassel, Germany). Approximately 1 ml was transferred to HPLC vials, sealed tightly and analysed without further purification.

2.3. LC–MS–MS

The enniatins were separated on a 150 mm × 3.9 mm Symmetry C_{18} (5 μm) column (Waters, Milford, USA). A model P4000 pump and a Model AS3000 autosampler (TSP, San Jose, CA, USA) were used for providing a mobile phase flow of 1 ml/min and for the injection of 20 μl extract aliquots, respectively. The mobile phase for isocratic elution was water–methanol–acetonitrile (15:40:45, v/v/v, containing 15 mM ammonium formate). After elution of the enniatins (3.7–6.2 min), the column was flushed with methanol–acetonitrile (50:50, v/v) for 10 min. The HPLC was coupled to a LCQ ion trap mass spectrometer operating with an atmospheric pressure chemical ionisation (APCI) interface (Finnigan MAT, San Jose, CA, USA). The MS was operated in selected ion reaction monitoring mode (SRM). The ion injection time was set to 300 ms with a total of three microscans. The APCI interface was operated with a vaporisation temperature of 350 °C and a sheath gas rate of 20 units (approximately 200 ml/min). No auxiliary gas was applied to the interface. A corona discharge voltage of 6 kV and a heated capillary temperature of 150 °C were used. Tuning of ionisation and MS–MS collision energy was carried out while continuously infusing a tuning solution containing either beauvericin or enniatin A, A1, B and B1, dissolved in methanol, at a flow rate of 1.5 μl/min into the mobile phase. The capillary voltage as well as the tube lens offset and the octapole offsets were optimised prior to the commencement of sample analysis using a methanolic solution of 70 μg/ml beauvericin and direct infusion as above. Typical values for the capillary voltage and the tube lens offset were in the range 30–40 V and 10–30 V, respectively.

2.4. Quantification

Enniatins were quantified using external calibration curves, which were constructed for each compound by plotting the amount (μg/kg) against the signal height.

As a consequence of the different concentrations of enniatin A, A1, B and B1 in the standard material and hence in the stock solution, the calibration range was for practical reasons not the same for all five analytes. Calibration curves were plotted using pure standards as well as oats matrix-assisted standards in all cases (all in acetonitrile–water, 84:16, v/v). However, for the purpose of quantification oats matrix-assisted standard curves were used for beauvericin, enniatin A and A1 in the concentration ranges 7.9–512 μg/kg, 3.2–30 μg/kg and 8.0–160 μg/kg, respectively. Oats matrix-assisted standards were prepared as follows: Oats extract (600 μl, considered blank for beauvericin as well as enniatin A and A1, but containing low amounts of enniatin B and B1), which was obtained as described above, was evaporated at 60 °C to dryness using a gentle stream of nitrogen. Six

Table 1
MS method including scan events, parent and daughter ion masses as well as relative collision energy

Scan segment	Scan event	Compound	MS^1, $[M + NH_4]^+$ (m/z)	MS^2, $[M + H]^+$ (m/z)	Relative collision energy (%)
1		Enniatin B	657	640	23
2	1	Enniatin B1	671	654	22
2	2	Beauvericin	801	784	25
3		Enniatin A1	685	668	24
4		EnniatinA	699	682	26

hundred μl of the appropriate standard in acetonitrile–water (84:16, v/v) was then added and the residue dissolved by vortexing. For quantification of enniatin B and B1 calibration curves from pure standards were used in the concentration range 7.7–760 μg/kg and 14–2160 μg/kg, respectively.

2.5. Method validation

Spike recovery experiments were carried out in order to validate the method. Spiking solutions were prepared in methanol and appropriate amounts added to either oats, barley or wheat matrix the day before extraction. Assessed parameters included method linearity, accuracy as spike recovery and precision as repeatability as well as reproducibility (day-to-day repeatability). The limit of detection and the limit of quantification was determined at 3× signal-to-noise (S/N) and 10× S/N, respectively, from spiked samples.

3. Results and discussion

3.1. LC–MS–MS analysis

The investigations of the positive and negative ion mass spectral characteristics of the enniatins lead to the development of the final LC–MS method for the determination of the enniatins in grain. The ionisation efficiency, and hence the sensitivity of the method, was not dependent on the interface (electrospray ionisation or APCI), at least not for the mobile phase conditions investigated. The APCI interface was finally chosen because it reportedly is less prone to ion suppression than electrospray ionisation [24]. In the positive APCI mode, all enniatins afforded similar ion patterns. The predominant signals were those from $[M + H]^+$ and $[M + NH_4]^+$ ions, together with trace signals from $[M + Na]^+$ and $[M + K]^+$ adducts. The most important parameter for the intensity ratio $[M + H]^+/[M + NH_4]^+$ was the temperature of the heated capillary, a higher capillary temperature favouring formation of $[M + H]^+$. At a temperature of 150 °C, the intensity ratio $[M + H]^+/[M + NH_4]^+$ was about 20/80. Upon negative ionisation, all enniatins afforded $[M - H]^-$ ions of approximately one hundred times less intensity compared to the ions that were produced in the positive mode. Operating the ion-trap mass analyzer in the MS–MS mode increased the selectivity of the method, minimised the background signal and thereby improved method performance. Application of the appropriate energy to the $[M + NH_4]^+$ ions afforded $[M + H]^+$ ions, which were used for quantification (Table 1). The comparatively high content of ammonium in the mobile phase (15 mM) ensured a constant signal height for $[M + NH_4]^+$. When a certain fragmentation energy level was exceeded, the molecule broke up into several fragments. In our experiments, the enniatins fragmented in the same way as reported for beauvericin by Sewram et al. [21]. However, in addition to the reported fragments, beauvericin as well as the other enniatins afforded $[M + H - 28]^+$ ions. The intensity of the corresponding signal was lower for beauvericin compared to the other enniatins and is likely due to loss of CO after opening of the cyclic molecule α to the carbonyl carbon. This transition may be used for further verification. After each LC–MS analysis, the chromatographic column was flushed with acetonitrile–methanol (50:50, v/v) for 10 min, which was necessary since extracts were not purified prior to analysis. This lead to the elution of non-polar compounds and prevented the increase of the background signal after analysis of several samples.

It has been shown that in cases where the gas-phase basicity (positive ionisation) or acidity (negative ionisation) of co-eluting compounds is larger than that of the analyte of interest the analyte signal may be significantly suppressed [25]. There are two ways of controlling matrix effects. Ideally, an isotope-labelled internal standard is added to the sample, which, apart from its mass, is chemically identical to the analyte. The internal standard will co-elute with the analyte and it may then be assumed that both experience the same suppression effects. Another possibility is the preparation of standards in the presence of sample matrix. In other instances, when co-eluting compounds are not prominent, matrix-assisted standards may be used in order to “saturate” active surfaces of the system, which may lead to more accurate results. It is for this reason, matrix-assisted standards were employed in this study. In contrast to another study [26], the matrix effects from different grain species were similar. However, even if the matrix effect was constant during a run, it varied between runs and even changed in sign (Table 2). The reason for this behaviour is not clear, but it is assumed that it is caused by a change of the activity of surfaces when samples of a different nature had been run on the days between the analyses of grain samples. In consequence of this, spike recovery experiments and, ideally, a control sample should always be incorporated into the analytical routine. The difference between the slope of matrix-assisted calibration curves and those plotted using pure standards was commonly 0–10%. However, the highest observed difference between the two calibration curves

Table 2
Slopes of calibration curves from pure and oats matrix-assisted standards showing the day-to-day variation of the matrix effect

Beauvericin, 7.9–512 μg/kg		Enniatin A, 3.2–30 μg/kg		Enniatin A1, 8.0–160 μg/kg		Enniatin B, 7.7–152 μg/kg		Enniatin B1, 14–432 μg/kg	
CH_3CN–water	Oats matrix	CH_3CN–water	Oats matrix	CH_3CN–water	Oats matrix	CH_3CN–water	Oats matrix	CH_3CN–water	Oats matrix
0.81	1	0.90	1	0.98	1	1.09	1	0.97	1
0.91	1	0.97	1	1.02	1	1.23	1	1.29	1
0.96	1	1.03	1	1.03	1	1.11	1	1.09	1
0.95	1	1.08	1	1.07	1	1.26	1	0.95	1
0.74	1	0.80	1	0.91	1	0.96	1	1.01	1

The slope of the calibration curve from oats matrix-assisted standards is normalised to 1.

Table 3
Mean spike recovery data determined for beauvericin and four other enniatins from the method linearity test

Compound	Spike level (μg/kg)	*n*	Recovery (%)	R.S.D (%)	R^2	Residual (μg/kg)
Beauvericin	9.6	1	68			0.3
	29	1	78			−1.6
	45	6	87 (71–103)	18	0.971	−9.7 to 10
	90	1	94			3.4
	128	1	90			−2.0
Enniatin A	4.2	1	104			2.4
	8.1	1	104			1.6
	12	6	91 (82–100)	10	0.961	−2.3 to 0.6
	21	1	97			−2.3
	30	1	121			2.5
Enniatin A1	10	1	119			3.6
	45	1	98			6.3
	80	6	79 (73–85)	7	0.944	−8.5 to 1.6
	120	1	100			18.3
	160	1	82			−3.2
Enniatin B	76	1	83			31
	133	1	86			31
	190	6	64 (55–74)	13	0.971	−30 to 17
	380	1	81			1.7
	570	1	86			13
Enniatin B1	27	1	84			36
	122	1	77			3.0
	216	6	86 (74–97)	13	0.973	−38 to 31
	378	1	95			−13
	540	1	107			29

95% confidence limits are given in parenthesis (from the *t*-distribution). The squared linear correlation coefficient and residuals are from the plot of theoretical against found concentration.

was about 30% (Table 2). In other studies, peak area reductions of up to 40% were detected when mycotoxins were quantified in food mixtures [27] or 19–42% when shellfish poisoning toxins were quantified in scallops using LC–APCI-MS [28]. However, matrix effects were absent when type A-trichothecenes were analysed in grain using LC–APCI-MS [29]. The favourable ionisation properties of the enniatins allowed the direct analysis without concentration of sample extracts, thus potentially interfering matrix components were likewise not concentrated and the matrix effect, was kept below 10% in the majority of sample runs.

3.2. Method validation

The LC–MS–MS method was validated from spike recovery experiments. Fig. 2 shows a LC–MS–MS profile of a spiked oats sample acquired using SRM. The validation ranges for different analytes complied with the relative ratio of the fungal metabolites in Norwegian grain [30]. Beauvericin, enniatin A and A1 were quantified using oats matrix-assisted standard curves while enniatin B and B1 were quantified using pure standards. The matrix effect was most pronounced at low concentrations and was of minor importance for high concentrations (>100 μg/kg). Since almost exclusively high amounts of the latter compounds could be found in natural contaminated samples it was assessed as unnecessary to use matrix-assisted standards for their calibration. However, in order to keep track of the matrix effect, matrix-assisted standards were always run for enniatin B and B1 in the concentration ranges 7.7–152 μg/kg and 14–432 μg/kg. Overall recoveries were 86%, 97%, 87%, 72% and 88% for beauvericin, enniatin A, A1, B and B1, respectively, during the method linearity test (Table 3). Residuals show that data points were distributed randomly around the linear line of

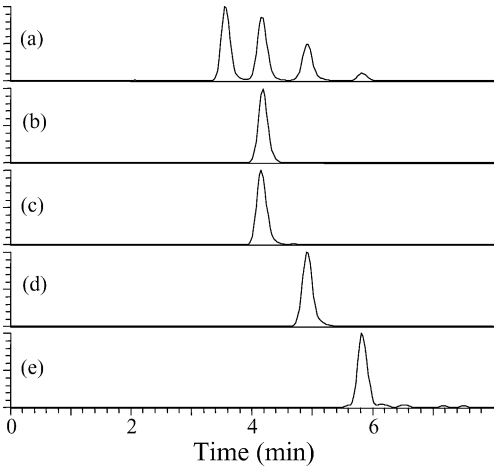


Fig. 2. LC–MS–MS chromatogram of an oats sample spiked at 45, 4.2, 28, 26 and 76 μg/kg beauvericin, enniatin A, A1, B and B1, respectively. Upper to lower traces: (a) total ion chromatogram from SRM, (b) and (c) mass separation of beauvericin and enniatin B1, respectively, (d) enniatin A1 and (e) enniatin A.

regression. Reproducibility was assessed from further spike recovery experiments, which were carried out over a period of six months without any regard to grain species (Table 4). Four analysts were involved in the experiments. Overall recoveries were 115%, 86%, 104%, 86% and 80% for beauvericin, enniatin A, A1, B and B1, respectively. Relative standard deviations were found acceptable, keeping in mind that instrument repeatability for repetitive injections was around 10%. The limits of detection and quantification were excellent, even if the sample extracts were not concentrated and emphasise the high ionisation potential of the enniatins (Table 5). The limits of detection of the compounds in pure standard solutions were approximately half the limits of detection in

Table 4
Mean spike recovery data determined for beauvericin and four other enniatins accumulated over the period of six months

Compound	Spike level (μg/kg)	*n*	Recovery (%)	R.S.D (%)
Beauvericin	9.6	5	99 (62–136)	30
	48	11	105 (92–119)	19
	128	8	115 (104–126)	11
Enniatin A	4.2	8	131 (109–153)	20
	12	8	104 (89–118)	17
	30	8	86 (78–94)	11
Enniatin A1	10	6	97 (82–111)	15
	28	8	113 (99–127)	14
	80	7	104 (91–118)	14
Enniatin B	76	7	100 (80–119)	21
	190	11	73 (63–84)	21
	570	6	86 (65–108)	23
Enniatin B1	27	6	109 (87–131)	19
	76	8	114 (90–137)	25
	216	8	96 (88–105)	10
	540	12	78 (74–82)	8
	1620	6	84 (72–97)	14

95% confidence limits are given in parenthesis (from the *t*-distribution).

Table 5
The concentration and mass (on-column) limit of detection and the concentration limit of quantification for spiked grain samples using the LC–MS–MS methodology

	cLOD (μg/kg)	mLOD (pg)	cLOQ (μg/kg)
Beauvericin	3.0	7.5	10
Enniatin A	3.0	7.5	10
Enniatin A1	4.0	10	13
Enniatin B	3.0	7.5	10
Enniatin B1	3.0	7.5	10

spiked samples. The squared correlation coefficient (R^2) for the seven or eight-point calibration plots was between 0.97 and 0.99. The goodness-of-the-fit was not dependent on the analyte but rather on the daily performance of the instrument. In our recent study, we have chosen to force the calibration plot through the origin. This might not be the ordinary approach and even excludes the validation of the regression line's intercept with the *y*-axis. However, forcing the calibration plot through the origin distributed individual residuals evenly around the line of regression.

4. Conclusions

This study has successfully shown the potential of LC–APCI-MS to rapidly screen levels of five closely-related enniatins in grain. The methodology enabled the simultaneous detection of the depsipeptides at low detection limits without the need for extract clean-up and concentration.

Acknowledgements

We thank the Norwegian Research Council for financially supporting this work as a part of project nr. 141221/130. The authors also wish to thank Dr. Thomas Rundberget and Dr. Tone Asp for their helpful suggestions during the preparation of this manuscript.

References

[1] E. Gäumann, St. Naef-Roth, L. Ettlinger, Phytopath. Z. 16 (1949) 289.
[2] B.S. Deol, D.D. Ridley, P. Singh, Aust. J. Chem. 31 (1978) 1397.
[3] S. Gupta, C. Montillor, Y.S. Hwang, J. Nat. Prod. 58 (1995) 733.
[4] M. Krause, A. Lindemann, M. Glinski, T. Hornbogen, G. Bonse, P. Jeschke, G. Thielking, W. Gau, H. Kleinkauf, R. Zocher, J. Antibiot. 54 (2001) 797.
[5] H. Tomoda, H. Nishida, X.H. Huang, R. Masuma, Y.K. Kim, S. Omura, J. Antibiot. 45 (1992) 1207.
[6] A. Visconti, L.A. Blais, J.W. Apsimon, R. Greenhalgh, J.D. Miller, J. Agric. Food Chem. 40 (1992) 1076.
[7] Y.C. Lin, J. Wang, X.Y. Wu, S.N. Zhou, L.L.P. Vrijmoed, E.B.G. Jones, Aust. J. Chem. 55 (2002) 225.
[8] C. Nilanonta, M. Isaka, R. Chanphen, N. Thong-Orn, M. Tanticharoen, Y. Thebtaranonth, Tetrahedron 59 (2003) 1015.
[9] R. Benz, J. Membr. Biol. 43 (1978) 367.

[10] J.F. Grove, M. Pople, Mycopathologia 70 (1980) 103.
[11] H. Tomoda, X.H. Huang, J. Cao, H. Nishida, R. Nagao, S. Okuda, H. Tanaka, S. Omura, H. Arai, K. Inoue, J. Antibiot. 45 (1992) 1626.
[12] R. Krska, M. Lemmens, R. Schuhmacher, M. Grasserbauer, M. Pronczuk, H. Wisniewska, J. Chelkowski, J. Agric. Food Chem. 44 (1996) 3665.
[13] R.D. Plattner, P.E. Nelson, Appl. Environ. Microbiol. 60 (1994) 3894.
[14] R. Lemmens-Gruber, B. Rachoy, E. Steininger, K. Kouri, P. Saleh, R. Krska, R. Josephs, M. Lemmens, Mycopathologia 149 (2000) 5.
[15] L. Macchia, R. Di Paola, F. Fornelli, S. Nenna, A. Moretti, R. Napoletano, A. Logrieco, M.F. Caiaffa, M.F. Tursi, A. Bottalico, Abstracts of the International Seminar on *Fusarium*, Martina Franca, Italy, 1995.
[16] D.M. Ojcius, A. Zychlinsky, L.M. Zheng, J.D. Young, Exp. Cell Res. 197 (1991) 43.
[17] A. Logrieco, A. Moretti, G. Castella, M. Kostecki, P. Golinski, A. Ritieni, J. Chelkowski, Appl. Environ. Microbiol. 64 (1998) 3084.
[18] B. Kosiak, M. Torp, E. Skjerve, U. Thrane, Acta Agric. Scand. Sect. B Soil Plant Sci. 53 (2003) 168.
[19] R.D. Josephs, R. Krska, R. Schuhmacher, M. Grasserbauer, Fresenius J. Anal. Chem. 363 (1999) 130.
[20] R. Krska, R. Schuhmacher, M. Grasserbauer, P.M. Scott, J. Chromatogr. A 746 (1996) 233.
[21] V. Sewram, T.W. Nieuwoudt, W.F. Marasas, G.S. Shephard, A. Ritieni, J. Chromatogr. A 858 (1999) 175.
[22] R.A. Thakur, J.S. Smith, J. Agric. Food Chem. 45 (1997) 1234.
[23] S.M. Monti, V. Fogliano, A. Logrieco, R. Ferracane, A. Ritieni, J. Agric. Food Chem. 48 (2000) 3317.
[24] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[25] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) 972.
[26] P. Zöllner, A. Leitner, D. Berner, M. Kleinova, J. Jodlbauer, B.X. Mayer, W. Lindner, LC–GC Eur. 16 (2003) 163.
[27] T. Rundberget, A.L. Wilkins, J. Chromatogr. A 964 (2002) break 189.
[28] S. Ito, K. Tsukada, J. Chromatogr. A 943 (2002) 39–46.
[29] E. Razzazi-Fazeli, B. Rabus, B. Cecon, J. Bohm, J. Chromatogr. A 968 (2002) 129.
[30] S. Uhlig, M. Torp, B. Heier, Food Chem., submitted for publication.