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Short communication

Determination of glucosinolates in rapeseed and *Thlaspi caerulescens* plants by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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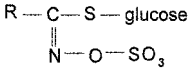
Abstract

Liquid chromatography–atmospheric pressure chemical ionization mass spectrometry was used to identify glucosinolates in plant extracts. Optimization of the analytical conditions and the determination of the method detection limit was performed using commercial 2-propenylglucosinolate (sinigrin). Optimal values for the following parameters were determined: nebulization pressure, gas temperature, flux of drying gas, capillar voltage, corona current and fragmentor conditions. The method detection limit for sinigrin was 2.85 ng. For validation of the method the glucosinolates in reference material (rapeseed) from the Community Bureau of Reference Materials (BCR) were analyzed. The method was applied for the determination of glucosinolates in *Thlaspi caerulescens* plants.

Keywords: Thlaspi caerulescence; Plant materials; Glucosinolates; Sulfur compounds; Carbohydrates

1. Introduction

Glucosinolates are sulfur-containing natural compounds that are produced by the secondary metabolism of plants from the order Capparales, which among other families includes the Brassicaceae. Up to date, more than 100 different compounds have been identified [1]. The general structure of glucosinolates is characterized by a –C≡N group, a sulfate, and a β-D-glucopyranosyl.



According to the chemical structure of the side chain (–R), among others, alkenyl-, phenyl- and indolylmethylglucosinolates can be distinguished.

The interest in glucosinolates arises from several observations: these compounds have antinutritional effects in cattle, they are involved in plant defense mechanisms against depredation, and some of these compounds appear to be protective against chemical carcinogens [1]. Several investigations also suggest important functions of glucosinolates in sulfur metabolism [2] and, by their relation to auxins, in growth regulation of plants [1].

While the methods for the quantification of the total glucosinolate content based either on enzymic release of sulfate or glucose or on X-ray fluorescence spectroscopy are relatively simple and well established, the analysis of individual glucosinolates is more conflicting [3]. The combination of high-performance liquid chromatography (HPLC) separation and mass spectrometry (MS) methods of detection

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has been proven to be very useful. Among others, thermospray LC–MS [4,5], thermospray LC–MS–MS [6] and negative-ion electrospray MS [7] have been used. Relatively large sample sizes are usually required for the determination of individual glucosinolates. The recent development of atmospheric pressure chemical ionization (APCI) MS may provide higher sensitivity and may substantially improve the determination of individual glucosinolates in small samples. In this study we optimized experimental conditions for APCI–MS detection of glucosinolates and determined the detection limit using commercial sinigrin. The method was further validated by the identification of individual glucosinolates in reference plant material (rapeseed) and in vegetative tissue of *Thlaspi caerulescens* (Brassicaceae). Previous investigations have found differences in the total glucosinolate contents of *Thlaspi caerulescens* populations that differed in Zn tolerance [8] and a role for glucosinolates in defense against fungal infection in metalophytes has been suggested [9]. However, as far as we know this is the first report on the identification of individual glucosinolates in this metal hyperaccumulator.

2. Experimental

2.1. Preparation of standards and plant samples

Commercially available sinigrin (allylglucosinolate) from horseradish (Sigma–Aldrich, St. Louis, MO, USA) was used for the optimization of the analytical technique and for the determination of the method detection limit. Sinigrin was also used as internal standard in samples of *Thlaspi caerulescens*. As a small concentration of sinigrin was present in extracts of rapeseeds sinigrin could not be used as an internal standard in this material. Nevertheless, some rapeseed samples were also spiked with sinigrin; not for quantification purpose, but for enhancing a reference peak in the chromatogram.

For the analysis of glucosinolates in plant material, certified reference rapeseeds (CRM 367) from the European Community Bureau of Reference (BCR, Brussels, Belgium) and shoots of 5-week-old *Thlaspi caerulescens* plants that had been grown in hydroponics under controlled environmental conditions [10] were used. The glucosinolates were extracted from either 500 mg (fresh mass) of rapeseeds or from 100 mg (dry mass) of the freeze dried plant material with 70% aqueous methanol in a boiling water bath for 5 min. After cooling and centrifugation, the proteins of the supernatant were precipitated with a solution containing lead acetate and barium acetate [11]. In the reference material, the extraction method rendered a 100% recovery of the certified total glucosinolate content, as determined by the myrosinase method [3]. Methods for sample preparation for glucosinolate separation by HPLC using either intact [12] or desulfatated [13] glucosinolates have been reported. Both methods may render similar results [3]. However, the high concentration of organic acids present in the *Thlaspi caerulescens* samples [10] made a clean-up by an enzyme-specific release advisable. For this enzymatic desulfatation the extract solution was loaded onto a DEAE-Sephadex A-25 column and the glucosinolates were treated in the column with aryl sulfatase (H-1 type from Sigma, St. Louis, MO, USA) following the method of Michinton et al. [13]. The eluate was freeze–dried and stored at −80°C. For HPLC analysis, the residue was dissolved in water and passed through a 0.45-μm filter.

2.2. LC–APCI-MS analysis

2.2.1. Chromatographic conditions

A liquid chromatograph equipped with a diode array detector Model HP 1090 (Hewlett-Packard, CA, USA) was used.

For the analysis 25-μl samples were injected onto a LiChrospher 60 RP Select B column (Merck, Darmstadt, Germany). The mobile phases used for the elution of samples were acetonitrile and water containing 0.5% acetic acid. The gradient elution was performed as follows: from 100% A (water) and 0% B (LC-grade acetonitrile) to 88% A and 12% B in 65 min, at a flow-rate of 1 ml/min.

2.2.2. Mass spectrometric analysis

LC–APCI-MS in the positive mode was used for the determination of glucosinolates. A HP 1100 mass spectrometer (Hewlett-Packard) equipped with an APCI interface and APCI spray chamber was used. In order to optimize glucosinolate detection the

following parameters were investigated: nebulizer pressure, vaporizer temperature, drying gas temperature, corona current, capillar voltage, drying gas flow and fragmentor conditions. For determining the detection limit for sinigrin by the flow injection analysis (FIA) method, 25-μl samples of sinigrin at concentrations ranging from 0.011 to 114.24 ng/μl were used.

3. Results and discussion

Results from the optimization of APCI-MS parameters for sinigrin analysis are shown in Table 1. Best peak height and shape was obtained with relatively low values for nebulization pressure, gas temperature, capillar voltage and fragmentor voltage. A relatively high flow-rate of the drying gas, high corona current, and a high vaporization temperature were required for best resolution. Applying these optimum conditions the linear relation between peak area and the quantity of injected sinigrin followed the equation

$$\text{Peak area} = 90.65\ (\text{ng sinigrin}) - 3582.7$$

$$(R^2 = 0.9972)$$

The APCI-MS detection limit for sinigrin determined by FIA was 2.85 ng. The detection limit using column injection would be somewhat higher. Nonetheless, the high sensitivity of the method will allow the determination of low glucosinolate concentrations in small plant samples.

To validate the method methanol extracts of the reference rapeseeds (Fig. 1) and from *Thlaspi caerulescens* (Fig. 2) were analyzed. Examples of total ion chromatograms (Fig. 1), of absorbance spectra at 230 nm (Fig. 1) and at 254 nm (Fig. 2), and of the specific ion chromatogram of mass 280 (Fig. 1) are shown. Positive APCI-MS in combination with the retention times allowed the identification of a total of 20 glucosinolates (Table 2). The side chains that define the different groups of glucosinolates rendered the characteristic diagnostic ions:

$[R]^+$ (A) $[R\text{-}C{=}NOH]^+$ (B) $[R\text{-}C(\text{-}S){=}NOH+2H]^+$ (C) $[M+H]^+$ (D)

The intensity of the diagnostic ions observed with APCI-MS were different from those reported by others using thermospray MS. As a rule fragmentation by APCI rendered higher molecular mass fragments than the more aggressive thermospray technique and for many glucosinolates the most intense m/z signal was obtained for the molecular ion ($M+H^+$). This high intensity of high-molecular-mass ions facilitates the identification of glucosinolates in the selected ion monitoring (SIM) mode (Fig. 2) and, in combination with the high sensitivity, renders the APCI-MS technique very useful for the detection of individual glucosinolates in small plant samples.

Excepting *p*-hydroxybenzylglucosinolate (sinalbin), all glucosinolates listed in Table 2 were present in the rapeseeds (Fig. 1). In addition to the glucosinolates previously reported in this reference material [14], 4-hydroxybutyl-, 5-hydroxypentyl-, and 7-methylsulfinylheptyldesulfoglucosinolates were tentatively identified according to ion mass distribution (Table 2) and retention times. The presence of 4-hydroxybutyldesulfoglucosinolate has previously been reported in *Arabidopsis thaliana* [4], where the thermospray LC–MS spectrum was domi-

Table 1
Optimization of parameters for sinigrin analysis by APCI-MS

Parameter (units)	Assayed range	Number of assayed intervals	Optimum value
Nebulization pressure (p.s.i.g.)[a]	40–60	5	45
Gas temperature (°C)	280–350	8	290
Drying gas flow-rate (l/min)	3–8	6	6
Capillar voltage (V)	2000–6000	9	2000
Corona current (μA)	2–10	5	8
Fragmentor voltage (V)	60–130	8	70
Vaporization temperature (°C)	300–500	5	500

[a] 1 p.s.i. = 6894.76 Pa.

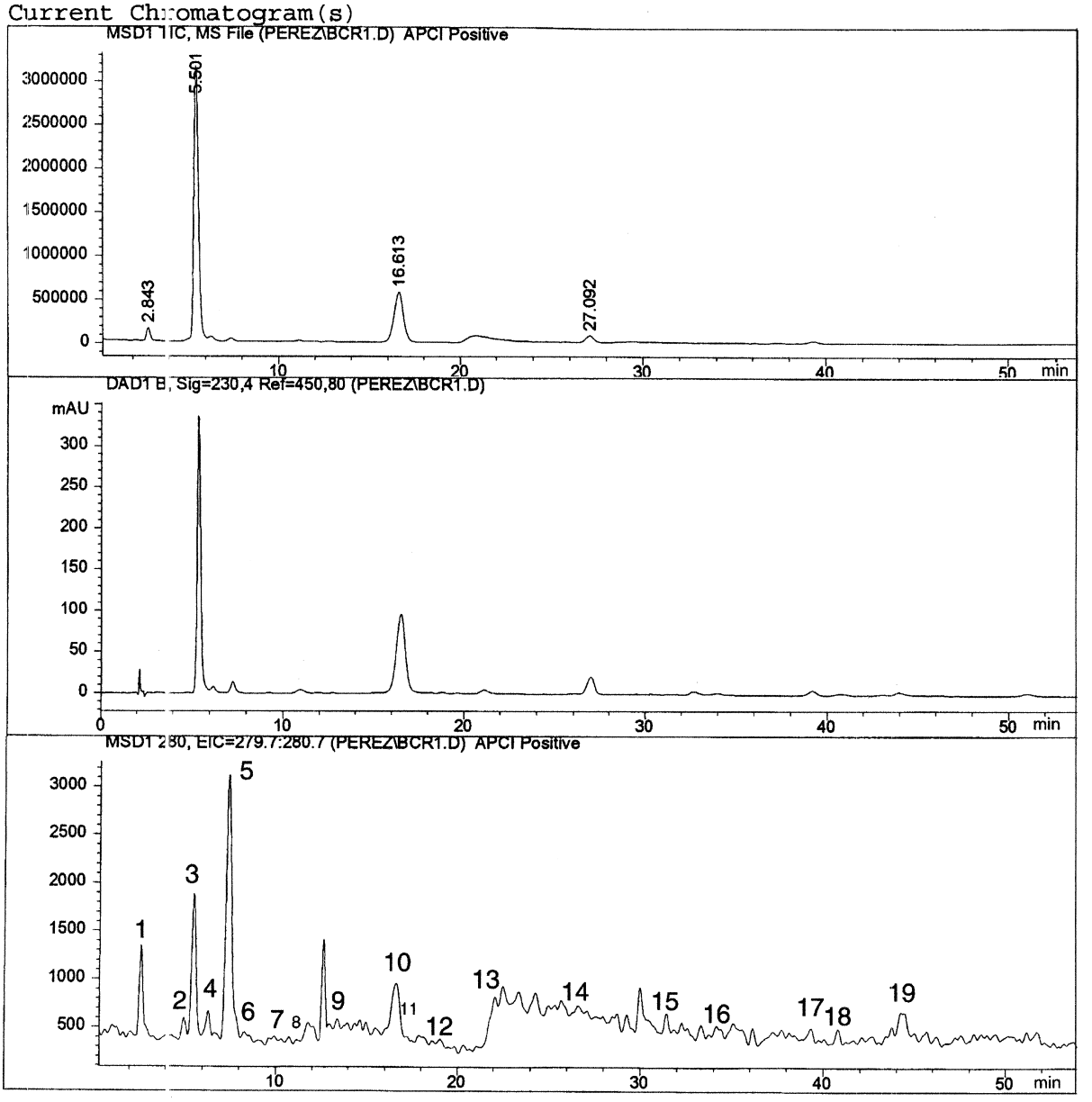

Fig. 1. Total ion chromatogram, absorbance spectrum (230 nm), and ion specific spectrum at mass 280 from glucosinolate extracts of rapeseed (BCR reference material). Peaks are numbered according to Table 2.

nated by the ion m/z 117. In contrast, the LC–APCI-MS spectrum in our study showed highest intensity of the B+2 ion (m/z 118) and relatively high intensity (58%) of m/z 294; this mass may correspond to the molecular ion with the loss of a hydroxyl group (M−17, 294).

SIM allowed one to identify five different desulfoglucosinolates in *Thlaspi caerulescens*: 3-methylsulfinylpropyl- (glucoiberin), *p*-hydroxybenzyl- (sinalbin), 3-butenyl-, 4-hydroxyindolylmethyl-, and 4-metoxyindolylmethyldesulfoglucosinolates (Fig. 2). Sinalbin has previously been detected in

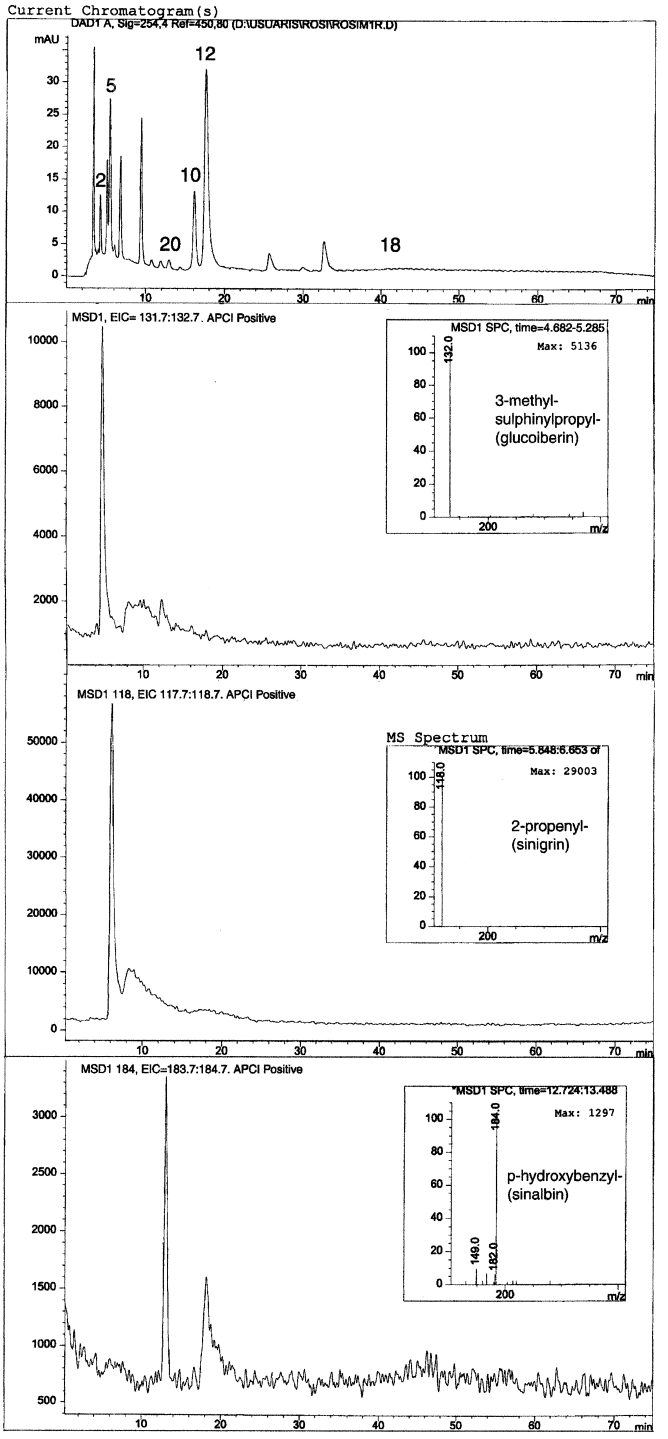

Fig. 2. Absorbance spectrum (254 nm) and examples from selective ion monitoring (mass 132, 118 and 184) of glucosinolate extracted from of *Thlaspi caerulescens* shoots. Inserts are mass spectra from glucoiberin, sinigrin (internal standard) and sinalbin. Peaks are numbered according to Table 2.

Table 2
Intensities of molecular ions and diagnostic ions for the desulfoglucosinolates investigated (m/z, %)

Peak No.	Type of side chain	Diagnostic ions					
	Methylsulfinylalkyl side chain	B	B−16	C	C−14	D	D−14
2	3-Methylsulfinylpropyl- (glucoiberin)	148 (9)	132 (100)	182 (3)	168 (0)	344 (1)	330 (0)
6	4-Methylsulfinylbutyl- (glucorafanin)	162 (0)	146 (16)	196 (0)	182 (43)	358 (0)	344 (100)
9	6-Methylsulfinylhexyl- (glucoalissin)	A 147 (18)	A−15 132 (18)	224 (0)	190 (0)	386 (100)	372 (0)
13	7-Methylsulfinylheptyl-	A−1 160 (10)	A−43 118 (80)	238 (0)	224 (0)	D−1 399 (100)	386 (3)
	Methylthioalkyl side chain	A	B	B−2	B+2	C+2	D
15	4-Methyltiobutyl- (erucin)	103 (15)	146 (7)	144 (27)	148 (2)	182 (20)	342 (100)
	Hydroxyalkyl side chain	B	B+1	B+2	B+18	D	D−18
1	4-Hydroxybutyl-	116 (0)	117 (0)	118 (100)	136 (0)	312 (0)	294 (58)
7	5-Hydroxypentyl-	130 (7)	131 (8)	132 (100)	148 (10)	326 (0)	308 (3)
	Alkenyl side chain	C	C−1	C−2	C+1	D	D+14
5	2-Propenyl- (sinigrin)	118 (100)	117 (0)	116 (0)	119 (0)	280 (11)	294 (5)
10	3-Butenyl- (gluconapin)	132 (100)	131 (2)	130 (1.5)	133 (0)	294 (13)	308 (2)
14	4-Pentenyl- (glucobrassicanapin)	146 (100)	145 (0)	144 (1)	147 (7)	308 (7)	294 (0)
	Hydroxyalkenyl side chain	C	C−1	C−2	C−18	D	D−16
3	2-Hydroxy-3-butenyl- (progoitrin)	148 (100)	147 (2)	146 (6)	130 (52)	310 (22)	294 (1)
4	2-Hydoxy-3-butenyl- (epi-progoitrin)	148 (100)	147 (2)	146 (6)	130 (52)	310 (22)	294 (1)
8	2-Hydroxy-4-pentenyl- (gluconapoliferin)	B+2 130 (18)	B+16 148 (36)	C−16 146 (49)	144 (100)	D−1 323 (7)	D−14 310 (25)
	Aromatic side chain	B+1	B−1	B−2	B−16	C	D
11	Benzyl- (tropaeolin)	135 (0)	133 (0)	132 (0)	118 (16)	168 (0)	330 (100)
20	*p*-Hydroxybenzyl- (sinalbin)	154 (4)	149 (2)	148 (16)	134 (0)	184 (100)	346 (4)
17	2-Phenylethyl- (gluconasturtiin)	149 (1)	150 (0)	151 (0)	167 (0)	182 (100)	344 (19)
	Indolylmethyl side chain	A−1	A	A+13	C−1	D	Indolyl
16	3-Indolylmethyl- (glucobrassicin)	130 (100)	131 (5)	144 (12)	207 (69)	369 (14)	118 (0)
12	4-Hydroxyindolylmethyl-	147 (9)	148 (19	160 (33)	224 (0)	386 (100)	118 (22)
18	4-Metoxyindolylmethyl-	A−2 160 (100)	A−32 130 (10)	A−14 148 (4)	238 (0)	399 (53)	118 (12)
19	1-Metoxyindolylmethyl-	160 (0)	130 (5)	148 (5)	238 (0)	399 (80)	118

yellow mustard (*Brassica hirta*) where the intact compound, without previous sulfatase treatment, was characterized by m/z 424, as found by electrospray MS [7]. The APCI-MS spectrum of desulfosinalbin from *Thlaspi* was dominated by m/z 184 corresponding to the molecular ion without the glucose molecule (Table 2). APCI offers much more fragmentation than electrospray; this may be an advantage for compound identification. However, further work comparing both techniques for glucosinolate analysis in different plant samples are required.

4. Conclusions

Optimization of experimental parameters of LC–APCI-MS allowed one to establish the detection limit for sinigrin at 2.85 ng. The method proved to be very useful for the separation and identification of desulfoglucosinolates in small samples not only of reference seeds with a certified high total glucosinolate content, but also in vegetative parts of *Thlaspi caerulescens*.

The *Thlaspi cearulescens* plants were character-

ized by the presence of 3-methylsulfinylpropyl- (glucoiberin), 3-butenyl- (gluconapin), and *p*-hydroxybenzyl- (sinalbin) glucosinolates and by glucosinolates with a indolylmethyl side chain.

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References

[1] R.M. Wallsgrove, R.N. Bennett, K. Doughty, in: B.K. Singh (Ed.), Plant Amino Acids, Biochemistry and Biotechnology, Marcel Dekker, New York, 1999, p. 523.
[2] E. Schnug, in: L.J. De Kok, I. Stulen, H. Rennenberg, C. Brunold, W.E. Rauser (Eds.), SPB Academic, The Hague, 1993, p. 179.
[3] R.K. Heaney, G.R. Fenwick, in: P.G. Waterman (Ed.), Methods in Plant Biochemistry, Methods for Glucosinolate Analysis, Vol. 8, Academic Press, San Diego, CA, 1993, p. 531.
[4] L.R. Hogge, D.W. Reed, E.W. Underhill, G.W. Haughn, J. Chromatogr. Sci. 26 (1988) 551.
[5] F.A. Mellon, J.R. Chapman, J.A.E. Pratt, J. Chromatogr. 394 (1987) 209.
[6] C.E.M. Heeremans, R.A.M. Van der Hoeven, W.M.A. Niessen, J. Chromatogr. 472 (1989) 219.
[7] C.L. Zrybko, E.K. Fukuda, R.T. Rosen, J. Chromatogr. A 767 (1997) 43.
[8] W. Mathys, Physiol. Plant. 40 (1977) 130.
[9] W.H.O. Ernst, in: H. Rennenberg, C.H. Brunold, L.J. de Kok, I. Stulen (Eds.), Sulfur Nutrition and Assimilation in Higher Plants, SPB Academic, The Hague, 1990, p. 131.
[10] R.P. Tolrà, Ch. Poschenrieder, J. Barceló, J. Plant Nutr. 19 (1996) 1541.
[11] W. Thies, Fette Seifen Anstrichm. 78 (1976) 231.
[12] B. Björkqvist, A. Hase, J. Chromatogr. 435 (1988) 501.
[13] I. Minchinton, J. Sang, D. Burke, R.J.W. Truscott, J. Chromatogr. 247 (1982) 141.
[14] J.P. Wathelet, P.J. Wagstaffe, A. Boenke, The Certification of the Total Glucosinolate and Sulphur Contents of Three Rapeseeds (Colza), CRMs 190, 366 and 367; BCR Information Reference Materials, Commission of the European Communities, Brussels, 1991.