ELSEVIER

Journal of Chromatography A, 717 (1995) 325–333

JOURNAL OF CHROMATOGRAPHY A

Separation of desulphoglucosinolates by micellar electrokinetic capillary chromatography based on a bile salt

Charlotte Bjergegaard[a], Søren Michaelsen[b], Peter Møller[a], Hilmer Sørensen[a,*]

[a]*Chemistry Department, Royal Veterinary and Agricultural University, 40-Thorvaldsensvej, DK-1871 Frederiksberg C, Denmark*

[b]*Novo Nordisk A/S, Enzyme Research, Microbial Metabolism, Novo Allé, DK-2880 Bagsværd, Denmark*

Abstract

Micellar electrokinetic capillary chromatography (MECC) has been developed as a promising method for the determination of 40 desulphoglucosinolates. A sodium cholate based MECC method was found to be efficient for the qualitative and quantitative analysis of desulphoglucosinolates produced in an on-column, enzymatic step from the corresponding intact glucosinolates. Separation conditions and sensitivity of the method have been optimised with respect to different parameters, including capillary types, where the 75-μm I.D. capillary increased the sensitivity 2.5 times over that of a 50-μm capillary. With use of a high-sensitivity optical cell assembly (Z-cell), the sensitivity was further increased ten times, resulting in detection of picogram amounts, or concentration levels corresponding to 10^{-6} *M*. Repeatability with a 75-μm capillary was good, with the relative standard deviation varying between 0.2% and 0.9% for relative migration times and for relative normalised areas between 1.0% and 3.0%. Linearity of the optimised method gave correlation coefficients between 0.99 and 0.9999 for the 50-μm capillary and 0.99 and 0.9997 for the 75-μm capillary. Separation efficiency expressed as number of theoretical plates (N/m) was in the range of 250 000–300 000 for the 50-μm capillary and 210 000–250 000 for the Z-cell. Limitations and possibilities of the MECC method here presented are discussed with respect to analyses of glucosinolates occurring in a wide range of cruciferous seed, vegetative plant parts including cabbage varieties, feed and food.

1. Introduction

Glucosinolates constitute a well-defined group of more than a hundred naturally occurring compounds which has attracted special attention due to their importance in relation to quality of food and feed [1,2]. The effect of glucosinolates, and especially their degradation products, may be of positive or negative character, e.g., anti-nutritive, toxic and off-flavour effects [3–5], as well as anticarcinogenic properties [6,7], and functions in herbivore host-plant recognition [8]. The various physiological effects, which can be related to glucosinolates and products thereof [2,9], result in the need for fast, inexpensive and reliable quantitative methods of analysis.

Qualitative and quantitative determination of glucosinolates has until recently been performed by the use of gas chromatography (GC) and high-performance liquid chromatography (HPLC) [1,10–12], and during the last few years, HPLC of desulphoglucosinolates has been the reference method of the EU [13]. Capillary electrophoresis (CE) for the analysis of intact and desulphoglucosinolates was introduced four years ago [14,15]. The method, based on micel-

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00535-8

lar electrokinetic capillary chromatography (MECC) with cetyltrimethylammonium bromide (CTAB) as surfactant, proved to be superior to both GC and HPLC for intact glucosinolates with respect to separation capacity as well as cost. The separation of the desulphoglucosinolates, obtained after on-column enzymatic desulphation of crude glucosinolate extract [1,12,13], was, however, suboptimal compared with the separation of the intact glucosinolates and various transformation products of glucosinolates [16,17]. Determination of glucosinolates is in several cases hampered by interfering compounds, which may occur in some vegetative plant parts, food and feed. Methods based on desulphoglucosinolates are in such cases a powerful supplement to ones based on intact glucosinolates, as has also been concluded for methods based on HPLC [1,12]. The limitations of desulphoglucosinolate techniques are related to on-column desulphation [1,12], and this comprises thus both HPLC [1,13] and HPCE–MECC [16]. Determinations based on desulphoglucosinolates have, however, due to their isolation, some advantages in analytical specificity compared with the determination of the intact glucosinolates, and there is thus a need for this more specific technique based on a more efficient MECC separation system for the compounds than obtainable with both HPLC [13] and the MECC system based on CTAB [14,15].

The present study introduces the use of a cholate-based micellar system for the analysis of desulphoglucosinolates. The developed MECC system was evaluated with respect to qualitative performance of the method, including discussion of factors of importance for sensitivity, and in this connection introduction of a special Z-cell detection system. Results for repeatability, linearity and separation efficiency of the method of analysis are also presented.

2. Experimental

2.1. Apparatus

The apparatus used was an ABI Model 270 A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) with a 1000 mm × 0.05 mm or 0.075 mm I.D. fused-silica capillary. Detection was performed by on-column measurements of UV absorption (230 nm) at a position 760 mm from the injection end of the capillary. For investigations on improvements of the sensitivity, a high-sensitivity optical cell assembly was used, comprising a fused-silica capillary (1000 mm × 0.075 mm I.D.) with an optical cell (Z-cell) placed 780 mm from the injection end. The path length for the optical cell is specified at 3 mm. Data processing was carried out by use of an IBM-compatible 486 DX, 50 MHz personal computer with Turbochrom 3.3 (PE Nelson, Perkin-Elmer, Beaconsfield, UK).

2.2. Samples and reagents

Glucosinolates (potassium salts) from the collection in this laboratory were used [1,8,12]. The compounds were extracted from various plants and isolated and purified as desulphoglucosinolates, as described elsewhere [1,18]. Identification and determination of glucosinolate purity were based on paper chromatography, high-voltage electrophoresis, UV and NMR spectroscopy, and HPLC [1,11].

The names and structures of the desulphoglucosinolates used in this study are presented in Figs. 1 and 2 and Table 1, together with numbers used in the other figures and tables.

Boric acid and sodium cholate were from Sigma (St. Louis, MO, USA). All chemicals were of analytical reagent grade.

2.3. Procedure

The different test mixtures of the desulphoglucosinolates were prepared by mixing the isolated and purified single compounds.

The separation buffer was prepared with 250 m*M* sodium cholate and 200 m*M* boric acid, and the pH was adjusted to 8.5. Filtration of buffers was performed through a 0.20-μm membrane filter prior to use.

Washing of the capillary was performed with 1.0 *M* NaOH for 2 min and with separation buffer for 5 min before each analysis. Buffers

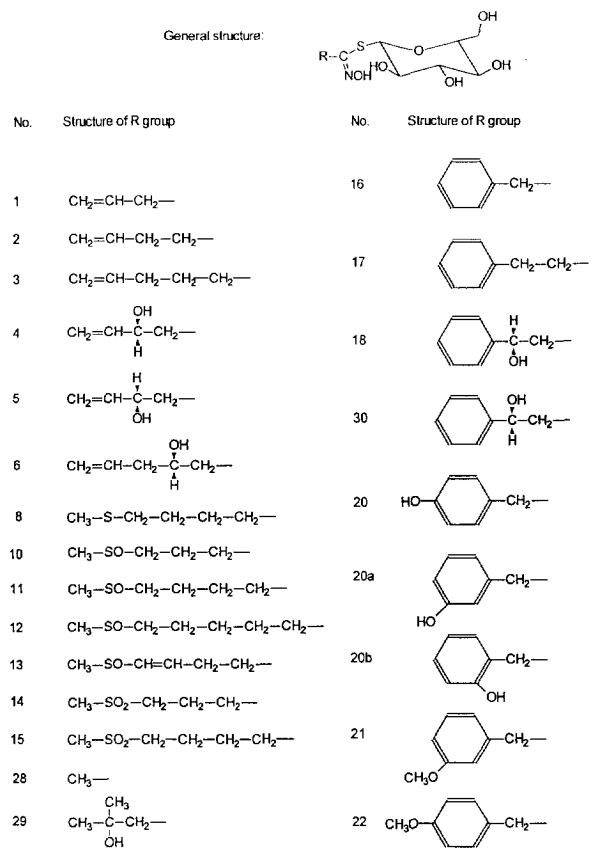

Fig. 1. Numbers and structures of aliphatic and aromatic desulphoglucosinolates used in the MECC analyses. Trivial names of the individual desulphoglucosinolates are indicated in Table 1.

were changed by auto buffer vial change on the ABI 270A-HT instrument after five analyses.

The separation parameters for the sodium cholate system were: a voltage of 15 kV and a temperature of 60°C. Detection was performed at 230 nm. Injection by vacuum was performed from the positive end of the capillary for 1 s.

2.4. Calculations

Calculations of relative migration times (RMT) (relative to an internal standard, trigonellinamide), normalised areas (NA), relative normalised areas (RNA) and resolution (R_s) were performed as described elsewhere [15,16]. RMT was calculated relative to trigonellinamide, whereas RNA was calculated relative to sinigrin (**1**) in test mixture A and relative to glucolimnanthin (**21**) in test mixture B. The types of compounds included in the test mixtures and in the samples analysed are shown in connection with Tables 1–5 and Figs. 1–5. The numbers of theoretical plates (N) were calculated by the Foley–Dorsey approximation, assuming an exponentially modified Gaussian distribution as the skewed peak model. The equation for N is:

$$N = \frac{41.7(\mathrm{MT}/W_{0.1})^2}{B/A + 1.25}$$

where MT is the migration time for the peak, $W_{0.1}$ is the peak width at 10% of peak height, and B/A is an empirical asymmetry ratio.

The linearity of the method was determined from linear regression analysis based on least-squares estimates. Repeatability was estimated from the means and relative standard deviations (R.S.D.).

3. Results and discussion

Analysis of desulphoglucosinolates may be complicated by the procedure used for isolation and purification, resulting in incomplete recovery of certain compounds from the column system [1,12]. This is a problem when the glucosinolates have acidic or readily oxidisable groups in the side chain, and those with an acylated thioglucose part create special problems with their transformation into desulphoglucosinolates [1]. However, techniques based on desulphoglucosinolates will lead to a more specific analysis, an advantage if the glucosinolates occur in vegetative plant parts, food and feed with appreciable amounts of interfering compounds, justifying the development of a specific method for these compounds.

The structural variations in the desulphoglucosinolates considered reside in their side chains. Desulphoglucosinolates are compounds without charge if their side chains are without protolytical active groups [1], and separation in a MECC system should therefore mainly be based on hydrophobic interaction between the micellar phase and preferably with possibilities of sepa-

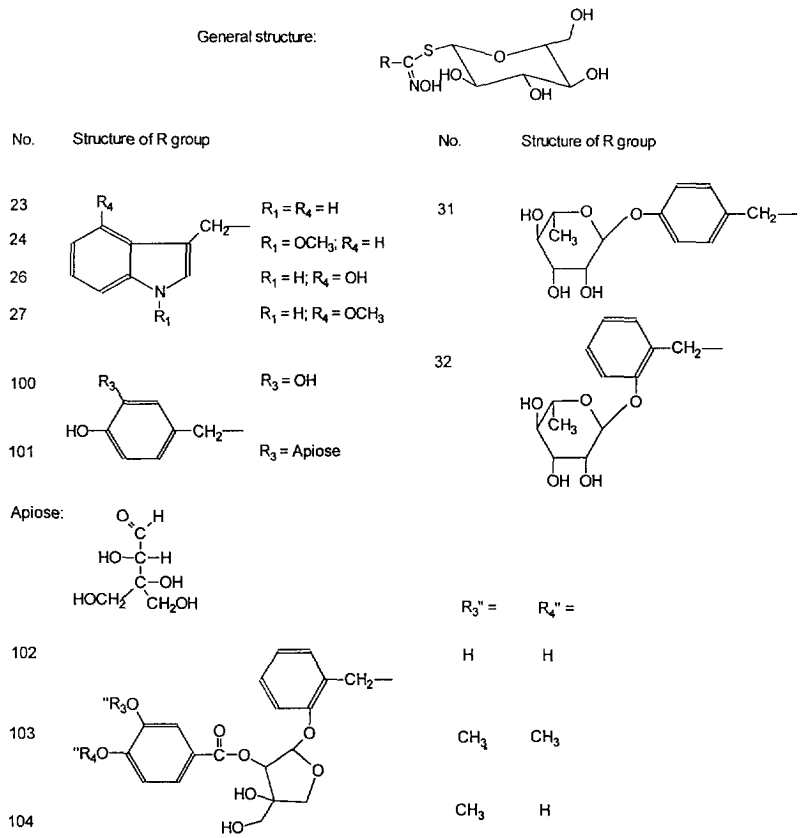

Fig. 2. Numbers and structures of indolyldesulphoglucosinolates and glycosylated desulphoglucosinolates used in the MECC analyses. Trivial names of the individual desulphoglucosinolates are indicated in Table 1.

ration of stereoisomeric glucosinolates. The MECC system introduced here, using the negatively charged cholate as the surfactant, should theoretically provide a good system for the separation of the uncharged desulphoglucosinolates. Cholate creates a kind of 'inverse micelles', having a negatively charged core and a hydrophobic uncharged surface, which is a micellar structure giving priority to separation based on hydrophobic interaction [16].

3.1. Migration order

The migration order of a wide spectrum of desulphoglucosinolates was determined, in order to demonstrate the significance of the small differences in structure necessary for acceptable separation of the compounds (Fig. 3). It appears that the behaviour of desulphoglucosinolates in the cholate system are based mainly on differences in hydrophobicity, as clearly demonstrated by the migration order of the homologous series such as **1–2–3**, **10–11–12**, and **16–17**. Generally, the aliphatic desulphoglucosinolates appeared early in the electropherogram, followed by the aromatic compounds, and with the indolyl-derivatised desulphoglucosinolates and desulphoglucosinolates having more than one aromatic group in the side chain migrating with the lowest velocity (Fig. 4). The importance of the steric hindrance for the interaction of desulphoglucosinolates with the micelles was seen by the separation of the epimers **18** and **30**. Also, the structural isomers, such as **20–20b**, **21–22** and

Table 1
Trivial names of desulphoglucosinolates used in the MECC cholate analyses

Number	Trivial name[a]	Number	Trivial name[a]
1	Sinigrin	**21**	Glucolimnanthin
2	Gluconapin	**22**	Glucoaubrietin
3	Glucobrassicanapin	**23**	Glucobrassicin
4	Progoitrin	**24**	Neoglucobrassicin
5	Epiprogoitrin	**26**	4-Hydroxyglucobrassicin
6	Napoleiferin	**27**	4-Methoxyglucobrassicin
8	Glucoerucin	**28**	Methylglucosinolate
10	Glucoiberin	**29**	2-Hydroxy-2-methyl-propylglucosinolate
11	Glucoraphanin	**30**	Glucosibarin
12	Glucoalyssin	**31**	*p*-Rhamnopyranosyloxybenzylglucosinolate
13	Glucoraphenin	**32**	*o*-Rhamnopyranosyloxybenzylglucosinolate
14	Glucocheirolin	**100**	3,4-Dihydroxybenzylglucosinolate
15	Glucoerysolin	**101**	3-Apiosyloxy-4-hydroxybenzylglucosinolate
16	Glucotropaeolin	**102**	3-[2″-(3,4-Dihydroxybenzoyl)apiosyloxy]-4-hydroxybenzylglucosinolate
17	Gluconasturtiin		
18	Glucobarbarin	**103**	3-[2″-(3,4-Dimethoxybenzoyl)apiosyloxy]-4-hydroxybenzylglucosinolate
20	Sinalbin		
20a	Glucolepigramin	**104**	3-[2″-(3-Methoxy-4-hydroxybenzoyl) apiosyloxy]-4-hydroxybenzylglucosinolate
20b	*o*-Hydroxybenzylglucosinolate		

The trivial names are as for the corresponding intact glucosinolates. Numbers as in Figs. 1 and 2. Separation conditions as described in the Experimental section.
[a] The semisystematic names were given for **20b**, **28**, **29**, **31**, **32** and **100–104**.

27–24 were well separated, demonstrating the high separation capacity of the MECC system (vide infra). Glycosylated desulphoglucosinolates would generally be expected to appear earlier in the electropherogram than the corresponding non-glycosylated compounds due to the lower hydrophobicity caused by the sugar moiety. As seen for, e.g., **20b–32** and **100–101** (Fig. 5), this was, however, not the case. An increased size of the glycosylated compounds, counteracting the change in hydrophobicity, may be the explanation for this observation, and derivatives of these glycosides have increased affinity to the micelles and thereby increased MT, as seen for the compounds **102–104**.

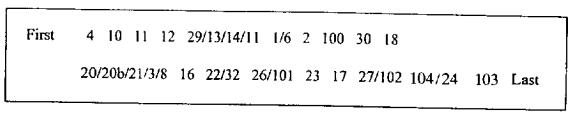

Fig. 3. Migration order of desulphoglucosinolates analysed in the MECC cholate system. Numbers as in Fig. 1. Separation conditions as described in the Experimental section.

3.2. Repeatability

Two test mixtures A and B, described in the Experimental section, were used for determination of the repeatability of MT, RMT, NA and RNA using traditional 50-μm and 75-μm I.D. capillaries. As revealed from the results (Table 2), the method performed satisfying, with R.S.D. values generally below 1% for RMT, except for test mixture B (50-μm capillary). Some adjustments of the method are, however, still needed in order to obtain reproducible peak areas, although use of RNA instead of NA in test mixture A improved the results partly. No systematic effect of the capillary diameter on R.S.D. values could be observed.

3.3. Linearity

Correlation coefficients (r^2) from linear regression analysis by the least-squares method of NA and various concentrations of desulphog-

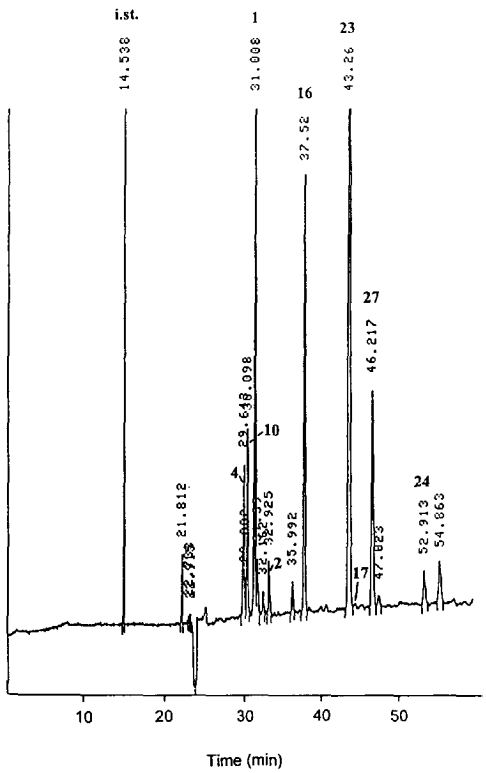

Fig. 4. Electropherogram of desulphoglucosinolates isolated from Savoy cabbage. Numbers as in Fig. 1. The peak labelled i.st. is the internal standard (trigonellinamide). Separation conditions as described in the Experimental section.

lucosinolates analysed in the MECC cholate system (test mixtures A and B) are given in Table 3. Correlation coefficients were in general high, ranging from 0.9876 to 0.9999 for the 50-μm capillary and from 0.9866 to 0.9997 for the 75-μm capillary, respectively. The good linearity is, together with acceptable repeatability of NA/RNA values, a precondition for quantitative analyses, which also provides the use of an internal standard, and knowledge of the relative response factors for the actual system, as is the case for HPLC [1,12,13]. The internal standard chosen should optimally be related in structure to desulphoglucosinolates, and a good choice would be a well-defined desulphoglucosinolate absent from the sample of interest. Trigonellinamide used in the present study should thus only be considered a reference compound in the calculation of RMT. Preliminary studies of the relative response factors indicate the same course of values as found in HPLC, using UV (230 nm) as the detection system [12,13,19].

3.4. Sensitivity

Introduction of the high-sensitivity optical cell assembly has opened up the possibility of improved sensitivity without changing the UV detection system used in the present method. Results from the registration of NA for test mixture A obtained with the high-sensitivity optical cell assembly (capillary 75 μm I.D., path length 3 mm), a standard 75-μm capillary and a standard 50-μm capillary are presented in Table 4. A ten-fold increment in response was obtained compared with results with the corresponding 75-μm standard capillary, whereas the NA values were increased by a factor of about 2.5 upon changing the internal diameter of the standard capillaries from 50 to 75 μm.

3.5. Separation efficiency

The resolution and number of theoretical plates per meter of capillary were calculated for the mixture used in the sensitivity test (vide supra). As shown in Table 5, R_s ranged from 2.2–6.1, whereas N/m generally exceeded 200 000, with the highest values obtained for the 50-μm capillary. Although not in the same range as obtained for the intact glucosinolates [15] (R_s: 10.1–35.8; N/m: 296 000–566 000), this may be considered as satisfactory, especially compared with typically no more than 20 000 N/m in HPLC analyses of both intact and desulphoglucosinolates. Surprisingly, the high-sensitivity optical cell assembly showed better separation efficiency than the standard 75-μm capillary, and this, combined with the high sensitivity of the system, makes it an evident choice for, e.g., low-concentration samples.

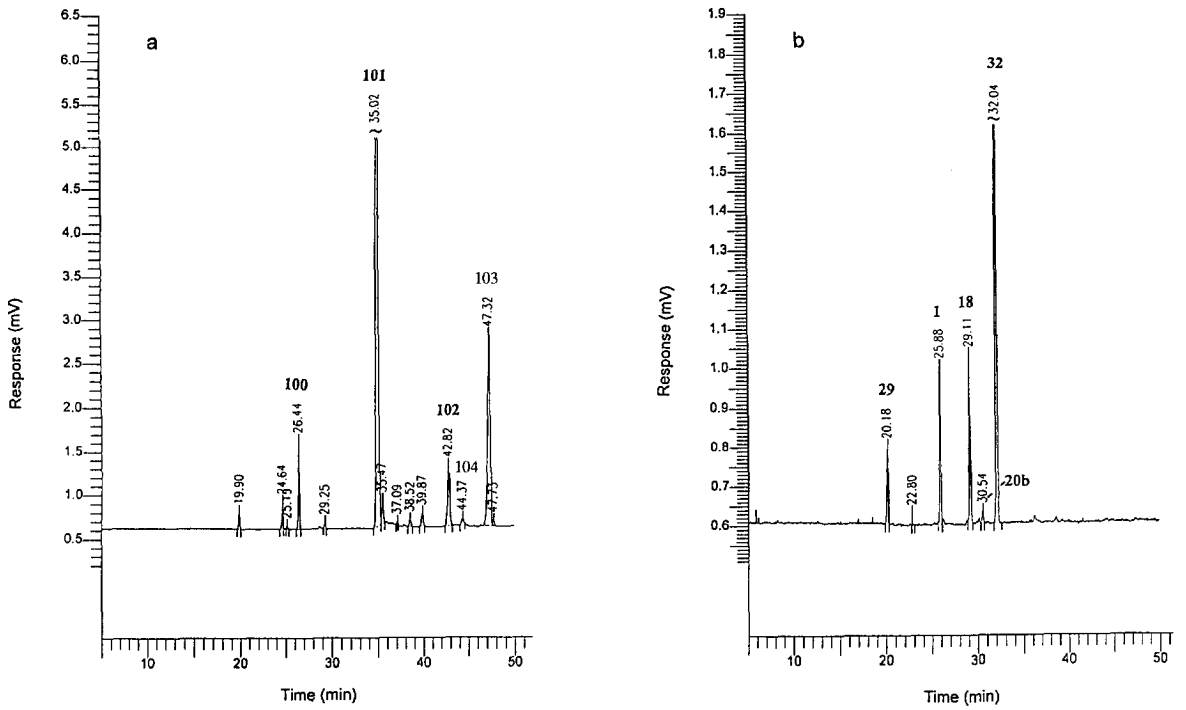

Fig. 5. Electropherogram of desulphoglucosinolates isolated from (a) *Hesperis matronalis* and (b) *Reseda lutea*. Numbers as in Fig. 1. Separation conditions as described in the Experimental section.

Table 2
Relative standard deviation (%) of migration times (MT), relative migration times (RMT), normalised peak area (NA) and relative normalised peak area (RNA), as an expression of repeatability of the MECC cholate method for determination of desulphoglucosinolates ($n = 5$–11)

Test mixture	Number	Relative standard deviation (%)							
		50-μm Capillary				75-μm Capillary			
		MT	RMT[a]	NA	RNA[b]	MT	RMT[a]	NA	RNA[b]
A	**1**	0.87	0.63	4.27	–	3.90	0.80	9.07	–
	2	0.87	0.73	3.86	2.40	3.80	0.75	9.44	1.04
	18	0.95	0.78	5.51	4.27	3.82	0.80	10.13	2.95
	20	0.98	0.82	6.49	5.30	3.91	0.94	10.01	1.50
	16	1.16	0.98	5.10	3.65	3.87	0.88	9.81	1.56
B	**10**	–	–	–	–	0.22	0.22	–	–
	13	2.72	1.57	3.28	5.53	0.21	0.21	5.10	8.90
	14	2.78	1.61	4.73	6.89	0.21	0.21	6.34	2.68
	21	3.71	2.59	6.30	–	0.30	0.31	7.89	–
	17	4.26	3.18	6.93	5.13	0.42	0.42	5.83	2.52

Numbers as in Fig. 1. Separation conditions as described in the Experimental section.
[a] Relative to the internal standard trigonellinamide.
[b] Relative to sinigrin (**1**) in standard A and relative to glucolimnanthin (**21**) in standard B.

Table 3
Results from linearity studies of desulphoglucosinolates analysed in the MECC cholate system ($n = 6–8$)

Test mixture	Number	50-μm Capillary		75-μm Capillary	
		Concentration range (mM)	r^2	Concentration range (mM)	r^2
A	**1**	0.1338–4.28	0.9998	0.0334–4.28	0.9996
	2	0.1066–3.41	0.9998	0.0266–3.41	0.9995
	18	0.1119–3.58	0.9999	0.0280–3.58	0.9994
	20	0.1553–4.97	0.9999	0.0388–4.97	0.9993
	16	0.1119–3.58	0.9997	0.0280–3.58	0.9994
B	**10**	–	–	0.0950–3.04	0.9866
	13	0.2113–3.38	0.9876	0.2113–3.38	0.9982
	14	0.0853–2.73	0.9889	0.0853–2.73	0.9995
	21	0.1441–4.61	0.9890	0.1441–4.61	0.9988
	17	0.1016–3.25	0.9896	0.1016–3.25	0.9997

Numbers as in Fig. 1. Separation conditions as described in the Experimental section.

Table 4
Results from sensitivity studies of desulphoglucosinolates analysed in the MECC cholate system

Capillary type	NA of number				
	1	**2**	**18**	**20**	**16**
50 μm	147.3	114.8	115.9	309.2	132.9
75 μm	325.5	255.1	256.6	724.6	301.3
75 μm[a]	3466.0	2701.6	2746.0	6721.9	3091.5

Numbers as in Fig. 1. Separation conditions as described in the Experimental section.
[a] High-sensitivity optical cell assembly (Z-cell).

4. Conclusions

In conclusion, MECC based on cholate proved to be a very effective system for separating a wide range of structurally different desulphoglucosinolates. The migration order of the investigated compounds could easily be explained by the structure-property variation among the

Table 5
Separation efficiency (R_s and N/m) obtained for test mixture A analysed in the MECC cholate system

Capillary type	N/m of number					R_s of numbers			
	1	**2**	**18**	**20**	**16**	**1–2**	**2–18**	**18–20**	**20–16**
50 μm	267 843	251 464	259 904	274 482	266 189	5.6	6.1	2.8	4.0
75 μm	216 979	188 445	200 201	209 167	185 463	4.9	5.1	2.0	3.7
75 μm[a]	218 971	211 959	241 549	218 967	214 513	5.0	–	2.2	3.5

Numbers as in Fig. 1. Separation conditions as described in the Experimental section.
[a] High-sensitivity optical cell assembly (Z-cell).

analytes, including hydrophobicity and size of the R-group, but stereoisomers were separated as well. Differences in mass/charge ratio and ion-pairing with the micelles, as in the case of the intact glucosinolates, were, on the other hand, without importance, as the desulphoglucosinolates are uncharged compounds, which explains the advantage of the cholate micelles compared with the system used for the negatively charged intact glucosinolates.

The separation capacity of the MECC system was high, with N/m around 200 000–300 000, and R_s ranging from 2.2 to 6.1. The repeatability and linearity of the method were in general satisfactory; however, some improvements are still needed for acceptable quantitative determination of the actual compounds. The experience using the high-sensitivity optical cell assembly was very good, improving the sensitivity of the system considerably without losing any separation efficiency. It thus provides an evident alternative to the standard unbowed capillary for low-concentration samples, as may be the case with some plant extracts.

Acknowledgements

The authors gratefully acknowledge support from the Danish Agricultural and Veterinary Council and from the Danish Agricultural Ministry.

References

[1] H. Sørensen, in F. Shahidi (Editor), Rapeseed/Canola: Production, Chemistry, Nutrition and Processing Technology, Van Nostrand Reinhold, New York, 1990, Ch. 9, p. 149.
[2] C. Bjergegaard, P.W. Li, S. Michaelsen, P. Møller, J. Otte and H. Sørensen, Bioactive Subst. Food Plant Orig., 1 (1994) 1.
[3] N. Bille, B.O. Eggum, I. Jacobsen, O. Olsen and H. Sørensen, Z. Tierphysiol. Tierernähr. Futtermittelkd., 49 (1983) 195.
[4] R. McDanell, A.E.M. McLean, A.B. Hanley, R.K. Heaney and G.R. Fenwick, Food Chem. Toxicol., 26 (1988) 59.
[5] B. Bjerg, B.O. Eggum, I. Jacobsen, J. Otte and H. Sørensen, Z. Tierphysiol. Tierernähr. Futtermittelkd., 61 (1989) 227.
[6] S. Loft, J. Otte, H.E. Poulsen and H. Sørensen, Food Chem. Toxicol., 30 (1992) 927.
[7] S.R.R. Musk and I.T. Johnson, Carcinogenesis, 14 (1993) 2079.
[8] L.M. Larsen, J.K. Nielsen and H. Sørensen, Entomol. Exp. Appl., 64 (1992) 49.
[9] S. Michaelsen, J. Otte, L.-O. Simonsen and H. Sørensen, Acta Agric. Scand., 44 (1994) 25.
[10] P. Helboe, O. Olsen and H. Sørensen, J. Chromatogr., 197 (1980) 199.
[11] O. Olsen and H. Sørensen, J. Am. Oil Chem. Soc., 58 (1981) 857.
[12] B. Bjerg and H. Sørensen, in J.P. Wathelet (Editor), World Crops: Production, Utilization, Description: Glucosinolates in Rapeseeds: Analytical Aspects, Vol. 13, Martinus Nijhoff, Dordrecht, 1987, p. 59.
[13] J. European Communities (1990) No. L 170, 03.07.27-34.
[14] C. Bjergegaard, S. Michaelsen, P. Møller and H. Sørensen, GCIRC-Congress, Saskatoon, Canada, III (1991) 822.
[15] S. Michaelsen, P. Møller and H. Sørensen, J. Chromatogr., 608 (1992) 363.
[16] S. Michaelsen and H. Sørensen, Pol. J. Food Nutrition Sci., 3/44, No. 1 (1994) 5.
[17] C. Feldl, P. Møller, J. Otte and H. Sørensen, Anal. Biochem., 217 (1994) 62.
[18] B. Bjerg and H. Sørensen, in J.P. Wathelet (Editor), World Crops: Production, Utilization, Description: Glucosinolates in Rapeseeds: Analytical Aspects, Vol. 13, Martinus Nijhoff, Dordrecht, 1987, p. 125.
[19] P. Møller, O. Olsen, A. Plöger, K.W. Rasmussen and H. Sørensen, in H. Sørensen (Editor), Advances in the Production and Utilization of Cruciferous Crops, Vol. 11, Martinus Nijhoff/Dr. W. Junk Publ., Dordrecht, 1985, p. 111.