# Micellar electrokinetic capillary chromatography as a fast, cheap, and efficient HPCE method for separation and quantification of intact and desulfo glucosinolates

**Pei Wu LI (1), Søren MICHAELSEN (2), Peter MØLLER (1) and Hilmer SØRENSEN (1)**

(1) Chemistry Department, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Denmark

(2) Department of Research in Small animals, National Institute of Animal Science, P.O. Box 39, DK-8830 Tjele, Denmark.

## ABSTRACT

Advantages and disadvantages of methods of analyses for glucosinolates based on HPLC and high performance capillary electrophoresis (HPCE) are presented and discussed. HPCE methods are in nearly every concern advantageous to HPLC methods. The HPCE method based on micellar electrokinetic capillary chromatography (MECC) using cetyltrimethylammonium bromide (CTAB) (1,2) has been further optimised and applied for the analysis of intact glucosinolates accumulated in seeds and vegetative parts of rapeseed. The optimised separation conditions gave a complete separation of glucosinolates in rapeseed in less than 18 minutes, and with the number of theoretical plates between 240.000 and 566.000 per metre of capillary. Complete separations of glucosinolates could be obtained in less than 8 minutes with only small reductions in resolution. The relative standard deviations were low for both relative migration times ranging from 0.1 to 0.5 percent and for relative normalized peak areas ranging from 0.8 to 2.0 percent. The linearity of the method was good ($0.9823 < r^2 < 0.9992$), and the detection limits were between 15 and 40 fmol. Examples of analyses performed by the HPCE method after applying a new simple filtration step in sample purification are shown.

## INTRODUCTION

Glucosinolates are well-known plant constituents involved in off-flavour, antinutritive and toxic effects to animals if present in too high concentrations (3). In food for humans glucosinolates and degradation products of glucosinolates can also have positive effects as anticarcinogenes and by their special flavour (4,5). In consequence of the large interest in glucosinolates efficient methods of analyses are needed. HPLC methods of intact and desulfo glucosinolates are now recommended as official reference methods in the EU (6). However, new and efficient methods based on capillary electrophoresis has drawn much attention within many areas of analyses. The previously presented HPCE method for analyses of glucosinolates based on hydrophobic interaction and ion-pairing chromatography (1,2) have been further investigated, and the possibilities and advantages using this method are presented here. A fast, simple and efficient procedure have been obtained for the determination of glucosinolates by combining this HPCE method with group separation (7) or filtration.

## MATERIALS AND METHODS

The instruments were ABI Model 270A or 270A-HT capillary electrophoresis systems (Applied Biosystems, USA) with 720 mm x 50 $\mu$m I.D. fused-silica capillaries (J & W Scientific, USA). Detection was performed by measurements of UV absorption at 235 nm at a position 500 mm from the injection end of the capillary.

Glucosinolates (potassium salts) were from the collection in this laboratory (8). The rapeseed used was Danish-grown single and double low spring rape of different varieties. Isolation and purification of intact glucosinolates were performed according to Bjerg and Sørensen (7). The glucosinolates shown in figures here are: **1** sinigrin, **2** gluconapin, **3** glucobrassicanapin, **4** progoitrin, **5** epiprogoitrin, **6** napoleiferin, **7** glucoibervirin, **8** glucoerucin, **10** glucoiberin, **11** glucoraphanin, **12** glucoalyssin, **14** glucocheirolin, **16** glucotropaeolin, **17** gluconasturtiin, **18** glucobarbarin, **22** glucoaubrietin, **23** glucobrassicin, **24** neoglucobrassicin, **26** 4-hydroxyglucobrassicin, **30** epiglucobarbarin, **35** 6'-isoferuloyl-glucosibarin. Disodium hydrogenphosphate, disodium tetraborate, alkyltrimethylammonium bromides, 1-propanol and other chemicals used were of analytical reagent grade.

Buffers were prepared as described by Michaelsen *et al.* (10), and the capillary was washed with 0.1 M NaOH for 2-4 min and with buffer for 5 min before each analysis. Unless otherwise stated the sample was introduced at the cathode by vacuum for 1 second and the separation was performed at - 20 kV (reversed polarity) and 30 °C.

## RESULTS AND DISCUSSION

The previously described MECC method using cetyltrimethylammonium bromide as surfactant (1,2) has been further investigated. The separation is based on hydrophobic and ion-pairing interaction of the negatively charged glucosinolates and the positively charged CTAB micelles and the CTAB-coated capillary wall. This results in differential partitioning of glucosinolates between the CTAB phase and the aqueous phase in a similar way to that described for the reversed phase HPLC methods (6,9).

The influence of separation conditions including buffer composition (electrolyte and concentration), buffer pH, surfactants (dodecyl-, tetradecyl-, cetyl-, and octadecyltrimethyl-ammonium bromide), organic modifier (2-propanol), voltage, temperature, and sample solvent have been investigated. The separations obtained were evaluated on the basis of migration order, migration time and relative migration time, peak area and normalised peak area (peak area/migration time), resolution, and the number of theoretical plates. In total over 25 closely related but structurally different intact glucosinolates were included in this study (2).

The conditions applied had very large influences on the separation parameters, for a detailed description see Michaelsen *et al.* (2). The best set of separation conditions called standard conditions for intact glucosinolates were determined to be a 18 mM disodium tetraborate - 30 mM disodium hydrogenphosphate - 50 mM CTAB, pH 7.0 buffer, a voltage of -20 kV and a temperature of 30 °C. At these standard conditions separations of glucosi-

nolates were obtained within 15 to 18 minutes. An example of a glucosinolate standard analysed by this HPCE method is shown in Figure 1 A. Complete separations with only small reductions in resolutions can be obtained in less than 8 minutes by simple changes in separation conditions. This is illustrated in Figure 2 with rapeseed glucosinolates from a single low variety obtained from QMA ion-exchange purification (8). The short time of analysis greatly increases the possibilities of using HPCE for routine analyses.

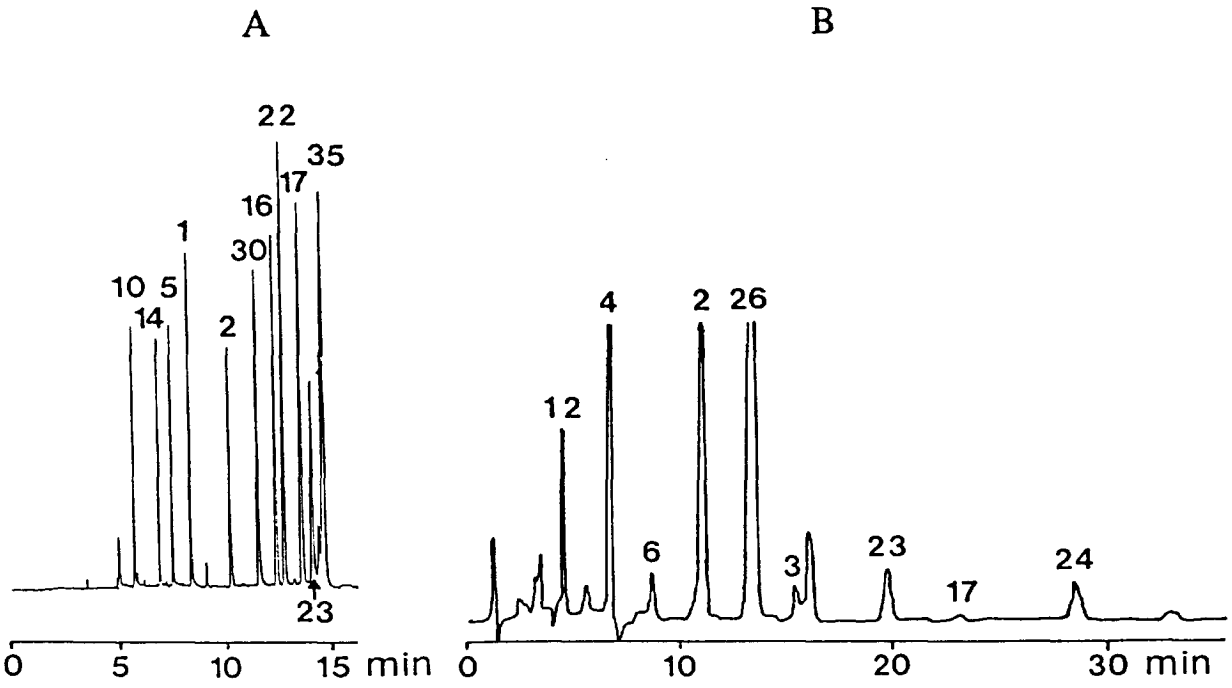


Figure 1. Comparison of analyses performed by HPCE and reversed phase HPLC on intact glucosinolates. A) HPCE analysis of glucosinolate mixture performed at standard conditions (see text), B) Reversed phase HPLC analysis of rapeseed glucosinolates using a 10 mM phosphate, pH 7.0, 5 mM tetraheptylammonium bromide, 30 % acetonitrile buffer, 1.0 ml/min, Spherisorb S3ODS2, 150 x 4.6 mm, 70 °C, 235 nm.

The performance of the method is shown in Table I. The number of theoretical plates per metre of capillary (N) was very high, up to over half a million. Repeatabilities expressed as relative standard deviations (RSD) were also very good with respect to relative migration times, normalized peak areas and relative normalized peak areas. Relative migration times gave better RSD values due to elimination of small changes in migration times, when many samples are analysed in series. Relative migration times are therefore used for identification purposes. Normalised peak areas are used in HPCE due to the influence of analyte velocity on peak area (2). Finally, the linearity of the method is also satisfactory. The results obtained with three different capillaries (different manufacturers) were also compared. The migration times and the repeatability of relative migration times depended on which capillary was used. The other separation parameters showed nonsystematic variations. Therefore, it is advisable to use capillaries from only one manufacturer. Negative sample solvent effects were seen, if the samples contained over 12.5 percent acetonitrile or over 12 - 17 percent methanol. This

is due to the negative effect of these organic sample solvents on the CTAB micelles and the disturbances of the hydrophobic and ion-pairing chromatography principles responsible for the separations.

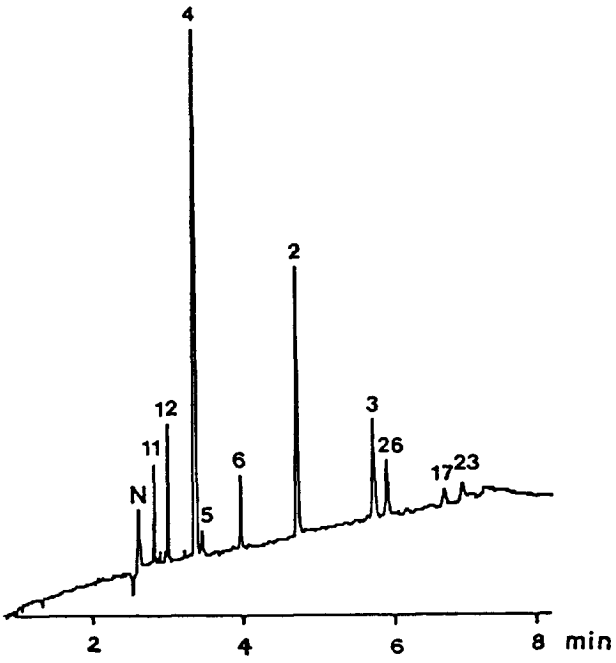


Figure 2. Fast HPCE analysis of rapeseed glucosinolates from a single low rapeseed variety obtained after QMA ion-exchange, evaporated and redissolved in water. Standard separation conditions (see text) except 30 kV and 50 °C.

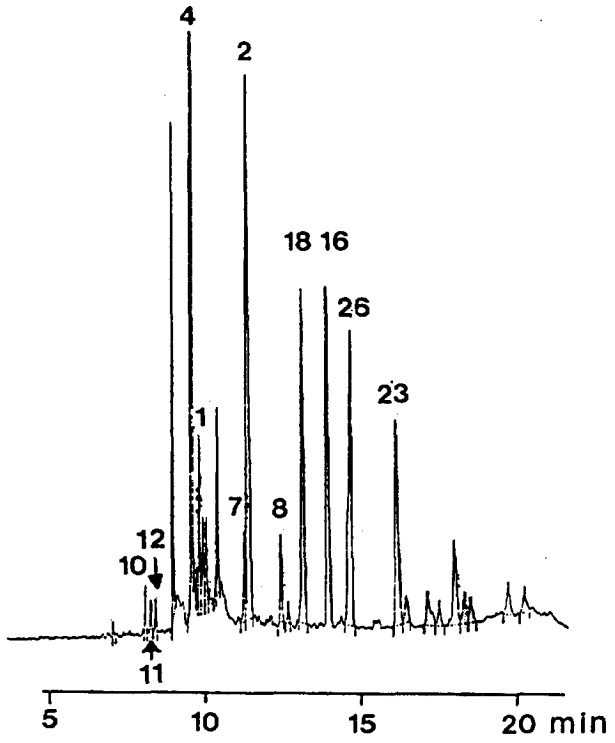


Figure 3. HPCE analysis of rapeseed glucosinolates (breeding program) obtained after filtration of crude extract through a Dowex 50 x 8 ($H^+$) column, evaporated and redissolved in water. Standard separation conditions (see text) except 10 percent 1-propanol included.

Table I. Performance of the HPCE method for intact glucosinolates. Several series performed in repeatability and linearity tests.

| | | |
|---|---|---|
| Efficiency | N/meter of capillary | 240.000 - 566.000 |
| Repeatability | Migration times | 0.4 - 2.5 |
| (RSD,%, n = 7 - 15) | Relative migration times | 0.1 - 0.5 |
| | Normalized peak areas | 0.8 - 2.6 |
| | Relative normalized peak areas | 0.8 - 2.0 |
| Linearity | Concentration range, mM | 0.04 - 1.12 |
| (5 - 10 conc. levels) | Correlation coefficients | 0.9723 - 0.9992 |
| Detection limit | Concentration, $\mu$M | 3 - 8 |
| (at applied conditions) | Amount, fmol | 15 - 40 |

The detection limit with regard to concentration is not very low in HPCE due to the short light pass in the capillary, whereas with regard to absolute amount it is very low (Table I). Different approaches can be made to improve detectability of glucosinolates in a specific sample if necessary. Redissolving of the samples in a smaller volume of water is the first choice, but at very low concentrations of glucosinolates this can give another problem as too high ionic strength may be the result. In HPCE it is necessary with a relatively low ionic strength in the sample to obtain sample stacking and thereby sharp peaks. However, this is usually no problem in concern with the glucosinolate samples. The injection time may also be increased to improve detectability. This leads to further bandbroadening and resolution loss at long injection times. However, injection times up to 6 seconds can be applied without loosing to much in resolution. This will improve detectability in terms of concentration 6 times compared to the results shown in Table I. Finally, the capillary diameter may also be increased from 50 to 75 $\mu$m I.D.. This also results in further bandbroadening and resolution loss, but may in many connections give acceptable separations. Migration times are generally reduced by 25 percent giving faster analyses at equally applied conditions. The detection limits are improved by a factor 3. Both increased injection time and increased capillary diameter can therefore often be used to increase detectability of analyses by HPCE. However, the obtained detection limits in HPCE are usually sufficient for analyses of glucosinolates.

Inclusion of 10 percent 1-propanol improved the separation of glucosinolates appearing late in the electropherogram such as the glucosinolates with aromatic, especially indolyl sidechains. Furthermore, using 50 mM dodecyltrimethylammonium bromide instead of CTAB and including 10 percent 1-propanol or 2-propanol gave improved separation of the aromatic glucosinolates but a slightly reduced separation of the glucosinolates with aliphatic sidechains. Therefore, the buffer of choice depends on the glucosinolate composition in the actual plant material to be analysed.

Desulfo glucosinolates can also be analysed efficiently by HPCE. However, the desulfatation step is timeconsuming and gives problems with correct conditions to obtain complete desulfatation without destroying any of the glucosinolates (7,11). Therefore, HPCE of intact glucosinolates is preferred.

Samples were prepared by traditional extraction of glucosinolates from the plant material and a fast and simple purification step based on ion-exchange on QMA columns (8). Alternatively, a fast and simple filtration through a Dowex 50 x 8 ($H^+$) column was applied as the only purification step (Figure 3). This method gave the possibility of concentrating the sample without disturbing the separation in HPCE, because the ionic strength is very low in the sample. For screening purposes by plant breeders this filtration method in combination with HPCE can give possibilities of very fast and cheap analyses.

Comparisons between the HPLC methods now in use for glucosinolate analysis and this HPCE method favours the HPCE method in nearly all points. Figure 1 A and B shows the electropherogram and chromatogram, respectively, of the HPCE method and a typical HPLC method for intact glucosinolates. The only disadvantage of HPCE compared to HPLC may be the relatively high concentration detection limit. However as discussed this can be

overcome by either redissolving in smaller amount of solvent, increase injection time, or increase diameter of capillary, if it is a problem. The advantages of HPCE compared to HPLC methods for the analysis of intact glucosinolates are numerous:

- More simple equipment; the same to 50 percent more expensive than HPLC instruments
- Faster changes of separation conditions when changing method or during method development
- Very cheap capillaries; typically 2-3 percent of the price for HPLC columns
- Long lifetimes of capillaries compared to columns due to very low sensitivity to sample impurities
- Less purified samples can be analysed reducing sample preparation costs
- Inexpensive and unharmful reagents in buffers used in very small amounts (few microlitres/hour)
- Faster analyses; typically 5-15 minutes compared to 20-35 minutes for HPLC
- Very high separation efficiency, 240.000-566.000 N compared to 10.000-30.000 N for HPLC
- Good resolution of compounds
- Very small amounts of sample necessary due to low mass detection limit (picograms or femtomol)
- Repeatbility and linearity comparable to HPLC

Alltogether this results in cheap, fast and reliable analyses using HPCE. Because of these advantages of HPCE compared to HPLC this HPCE method is now beeing used for routine analyses in our laboratory.

## REFERENCES


1. Michaelsen, S., Møller, P., and Sørensen, H. (1991). *GCIRC Bulletin* **No.7**, 97-106.
2. Michaelsen, S., Møller, P., and Sørensen, H. (1992). *J. Chromatogr.* **608**, 363-374.
3. Bjerg, B., Eggum, B.O., Jacobsen, I., Otte, J., and Sørensen, H. (1989). *Zeitschr. Tierphysiol., Tierernährung u. Futtermittel-kd.* **61**, 227-244.
4. Yano, M., Itoh, H., and Saijo, R. (1987). *J. of the Japanese Soc. of Food Sci. and Technol.* **34**, 9, 508-511.
5. Wattenberg, L.W., Hanley, A.B., Barany, G., Sparnins, V.L., Lam, L.K.T., and Fenwick, G.R. (1986). In: Diet, nutrition and cancer (Hayashi *et al.*, Ed's). Japan Sci. Soc. Press, Tokyo/VNU Sci. Press, Utrecht, 193-203.
6. Verordnung (EWG) Nr. 1864/90 vom 29. juni 1990 zur Änderung der Verordnung (EWG) 1470/68. Amtsblatt der Europäischen Gemeinschaften Nr. L 170/27 vom 3.7.1990.
7. Bjerg, B. and Sørensen, H. (1987). In: World Crops: Production; Utilization, Description: Glucosinolates in Rapeseeds: Analytical Aspects (Wathelet, J.-P., Ed.), Martinus Nijhof, Dordrecht, 59-75.
8. Sørensen, H. (1990). In: Rapeseed/Canola: Production, Chemistry, Nutrition and Processing Technology (Shahidi, F., Ed.), Van Nostrand Reinhold, New York, 149-172.
9. Bjerg, B. and Sørensen, H. (1987). In: World Crops: Production; Utilization, Description: Glucosinolates in Rapeseeds: Analytical Aspects (Wathelet, J.-P., Ed.), Martinus Nijhof, Dordrecht, 125-150.
10. Quinsac, A. and Ribaillier, D. (1987). *ibid.* 90-108.
11. Fiebig, H.-J. (1991). *Fat Sci. Technol.* 7, 264-267.