Kinetic investigation of the transformations of indol-3-ylcarbinol into oligomeric indolyl compounds based on micellar electrokinetic capillary chromatography

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Abstract

Indol-3-ylcarbinol (I3C) and indol-3-ylmethylcarbonium ion are intermediate products formed in aqueous solutions as a result of indol-3-ylmethylglucosinolate (glucobrassicin) hydrolysis, resulting in various physiologically active compounds. A micellar electrokinetic capillary chromatography (MECC) method has been developed for qualitative and quantitative determination of indolyl oligomers. The method, which is based on cholate as the surfactant, allows efficient separations of di(indol-3-yl)methane (DIM), 2,3-bis(indol-3-ylmethyl)indole (TRI), monomeric, and other oligomeric indolyl products. Theoretical plate numbers per meter of capillary of $1-4 \cdot 10^5$ have been found. The resolution between I3C and DIM, DIM and TRI, and between TRI and a possible tetramer, were 30, 34 and 5, respectively. Repeatability and linearity of the method have been determined, giving relative standard deviations for the migration times of between 0.3 and 3% and linear correlation coefficients of around 0.99, respectively. The kinetics (reaction orders, rate constants) of I3C transformation into indolyl oligomerisation products, and of the reaction between I3C and a nucleophile (1-propanol), have been studied. The use of the method for separation of the degradation products of a mixture of glucobrassicin and N-methoxyindol-3-ylmethylglucosinolate (neoglucobrassicin) is shown.

Keywords: Kinetic studies; Indolylcarbinol; Indolylglucosinolates

1. Introduction

Transformation of indol-3-ylcarbinol (I3C) into various types of indolyl compounds seems to occur in aqueous solution via a carbonium ion intermediate and the resulting compounds call for special attention, due to their physiological effects [1]. Oligomerisation of I3C depends on the pH of the solution, and a number of oligomerisation products have been described [2–4]. The chromatographic procedures previously applied for analyses of the indolyl compounds resulting from enzymatic and non-enzymatic degradation of indolylglucosinolates, including as-

corbigens and dimeric, trimeric and tetrameric oligomerisation products of I3C, have most often been based on high-performance liquid chromatography (HPLC) [3–6].

A capillary electrophoresis method, based on micellar electrokinetic capillary chromatography (MECC) with dodecyltrimethylammonium bromide (DTAB) as the surfactant, for determination of indolyl glucosinolates and their degradation products, has been described previously [7]. This DTAB method had high separation capacity for monomeric indolyl compounds, including I3C and ascorbigen. However, the method was not acceptable with respect to the separation of dimeric, trimeric and tetrameric oligomerisation products of I3C. During a

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study of the reaction between I3C and proteins, using a previously described MECC method based on cholate as the surfactant, a good separation of various low-molecular mass oligomerisation products of I3C was observed [8]. The MECC method now described makes use of the differential interaction of cholate micelles with I3C oligomerisation products. The method allows efficient separation of various I3C oligomerisation products, as well as the separation of these and corresponding compounds produced in the enzyme-catalysed degradation of a mixture of glucobrassicin and neoglucobrassicin.

2. Experimental

2.1. Capillary electrophoresis

The apparatus used was an ABI Model 270A (or ABI 270A-HT) capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) with a 76 cm×50 μm I.D. fused-silica capillary. Samples were introduced from the anodic end of the capillary by 1 s and 127 mm Hg vacuum injection. Detection was at a position 53 cm from the injection end as on-column UV detection. Data were processed with a Shimadzu C-R3A Chromatopac (Kyoto, Japan) or a PE Nelson 900 Series Interfase and an IBM compatible 486 DX, 50 MHz personal computer with Turbochrom (4.0) (PE Nelson, Perkin Elmer, Beaconsfield, UK). Before each run, the capillary was washed with 1 M NaOH for 2 min, followed by a wash with buffer for 5 min. The electrophoresis buffer was an aqueous solution containing 15 mM sodium cholate, 100 mM Na$_2$HPO$_4$ and 12% of 1-propanol (the pH of the solution was not adjusted). The conditions of electrophoresis were 20 kV (or 15 kV), 30°C, and detection at 220 or 280 nm, with a detector rise time of 0.5 s. The calculation of normalised peak areas (NA, peak area/migration time), numbers of theoretical plates per meter, resolution, repeatability and linearity of the method was performed as described elsewhere [9]. The determination of the detection limit and linearity was done with the ABI 270A-HT apparatus (Applied Biosystems) and the PE Nelson processing equipment (Perkin-Elmer). The capillary stayed fixed in the

detector fittings during the measurements reported in Table 2.

2.2. NMR

^{1}H-NMR spectra were measured at 250 MHz on a Bruker AC 250 NMR spectrometer.

2.3. Reference compounds

Reference compounds included potassium thiocyanate, indol-3-ylacetonitrile (IAN) and I3C (Sigma, St. Louis, MO, USA), indol-3-ylaldehyde (I3A) (from Serva, Heidelberg, Germany). 5,11-Dihydroindolo[3,2-b]carbazole (ICZ) was a gift from Dr. Jan Bergman, Department of Organic Chemistry, Royal Institute of Technology, Stockholm, Sweden. Ascorbigen (ASG) was synthesised according to the method described in [10]. Di(indol-3-yl)methane (DIM) slowly precipitated (with 5% TRI) from 6.8 mM I3C dissolved in 50 mM sodium phosphate buffer pH 7.5. 2,3-Bis(indol-3-ylmethyl)indole (TRI) precipitated (in a 1:1 mixture with DIM) from 144 mM sodium acetate, 16 mM acetic acid, 20% acetonitrile (pH ca. 5.6). Indol-3-ylmethylmethyl ether (IME), indol-3-ylmethylethyl ether (IEE), indol-3-ylmethylpropyl ether (IPE) and indol-3-ylmethylbutyl ether (IBE) were formed from I3C dissolved in weakly acidic buffers containing 20% methanol, ethanol, 1-propanol or 1-butanol, respectively. The structures of ICZ, DIM, TRI and ASG were confirmed by ^{1}H-NMR. The NMR spectrum of TRI was only obtained from a 1:1 mixture with DIM {[^{2}H$_6$]dimethyl sulfoxide (DMSO-d$_6$), DMSO=δ 2.49; signals associated with TRI: δ 4.20, s, 2H; δ 4.25, s, 2H; δ 6.8–7.55, m, ca. 16 H; δ 10.75, s, 1 H; δ 10.80, s, 1 H; δ 10.93, s, 1 H}. The structures of the compounds used in this study, together with the abbreviations used in the text and in other figures, are shown in Fig. 1. Peak identification was carried out by a comparison of the migration times and was confirmed by spiking, when relevant.

2.4. Kinetic experiments

Kinetic experiments were carried out as follows: I3C was dissolved in an aqueous solution of 1 mM Na$_2$HPO$_4$, and its concentration, purity and stability
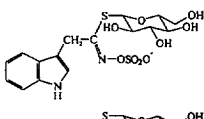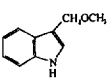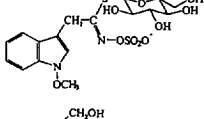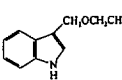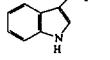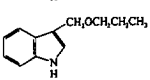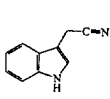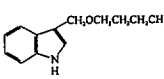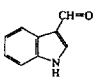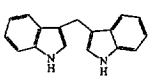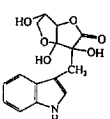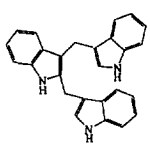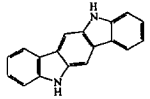
Abbreviation	Structure	Name	Abbreviation	Structure	Name
GB		glucobrassicin (indol-3-ylmethyl-glucosinolate)	IME		indol-3-ylmethylmethyl ether
neoGB		neoglucobrassicin (N-methoxyindol-3-yl-methylglucosinolate)	IEE		indol-3-ylmethylethyl ether
I3C		indol-3-ylcarbinol	IPE		indol-3-ylmethylpropyl ether
IAN		indol-3-ylacetonitrile	IBE		indol-3-ylmethylbutyl ether
I3A		indol-3-ylaldehyde	DIM		di(indol-3-yl)methane
ASG		ascorbigen	TRI		2,3-bis(indol-3-ylmethyl)indole
			ICZ		5,11-dihydroindolo[3,2-b]carbazole

Fig. 1. Names and structures of the indolyl compounds used in the MECC analyses.

were controlled by UV and MECC. A 75-μl volume of the freshly prepared I3C solution was mixed with 125 μl of 1-propanol and 300 μl of 180 mM sodium acetate, 20 mM acetic acid in water. The pH of the final reaction mixture was 6.0 at 30°C. The reaction mixture was immediately placed in the capillary electrophoresis autosampler (30°C) and analysed every 50 min. A representative chromatogram from a kinetic experiment is shown in Fig. 2.

The initial concentration of I3C in the reaction mixture was either 1.7 or 0.4 mM. In some experiments DIM was added (dissolved in 1-propanol) to 0.5 or 0.3 mM. Quantification of I3C, IPE, DIM and TRI was performed by analysing external standards of I3C (ϵ_{277}=5630 M^{-1} cm^{-1} [7]) and DIM (ϵ_{280}= 1.21·10^4 M^{-1} cm^{-1} [4]). The response factor obtained for I3C was also used for IPE. The relative molar response factor for TRI compared to DIM was determined experimentally from a comparison of ^{1}H-NMR and MECC of the mixture of DIM and TRI (Fig. 3), and calculated as [NA(TRI)/NA(DIM)]· ([DIM]/[TRI]). In the kinetic experiments, the molar
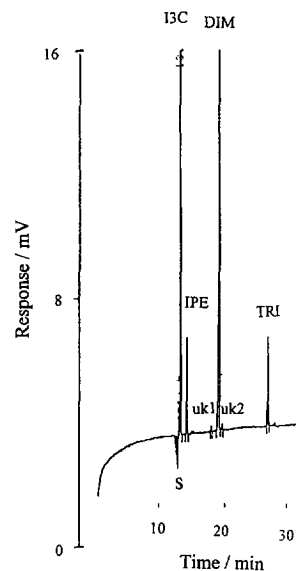
Fig. 2. MECC chromatogram of the mixture of compounds resulting from I3C oligomerisation in the presence of DIM and 1-propanol (Experiment 2, Table 3, 123 min). MECC conditions: 15 mM sodium cholate, 100 mM Na$_2$HPO$_4$, 12% 1-propanol; 20 kV; 30°C; 220 nm. In this and other chromatograms, S is the solvent band.
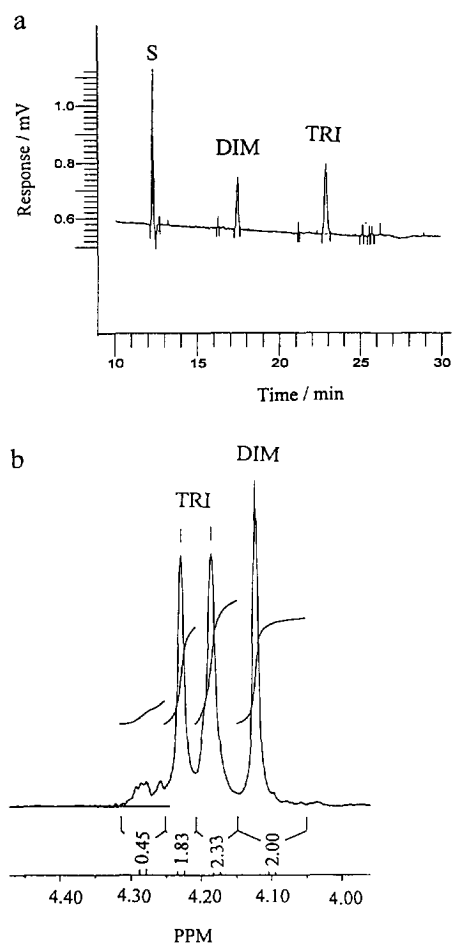
Fig. 3. MECC chromatogram (a) and ^{1}H-NMR (b) of the same mixture of DIM and TRI, for calculation of the relative molar response factor. MECC conditions are the same as in Fig. 2. 250 MHz ^{1}H-NMR in DMSO-d_6, internal standard, tetramethylsilane (0 ppm). Only the region of the NMR spectrum representing the CH_2 groups is shown.

response factor for TRI was calculated as the molar response factor for DIM multiplied by the relative molar response factor 1.5.

Reaction orders were determined from plots of concentrations of reactants versus formation rates of IPE and TRI. Determination of rate constants was performed by computer simulation of the course of individual reactions, either using a simple linear approximation for each 1 min interval, or an improved approximation, based on the Runge-Kutta method of numerical integration [11].

2.5. Glucosinolate isolation and degradation

Myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) was isolated from seeds of *Sinapis alba* L. cv. "Albatros", as described previously [12]. Intact glucosinolates were extracted from freeze-dried broccoli (*Brassica oleraceae* L. var. *italica* cv. "Emperor") and were isolated on an analytical scale as strongly acidic compounds, using QMA columns and principles described elsewhere [13]. Intact glucosinolates were determined by MECC using cetyltrimethylammonium bromide (CTAB) as the surfactant [9]. Myrosinase-catalysed glucosinolate degradation was carried out by dissolving the isolated intact glucosinolates in 20 mM acetic acid, 180 mM sodium acetate (pH 5.6). To 30 μl of the glucosinolate solution (3.0 mM glucobrassicin, 2.7 mM neoglucobrassicin and small amounts of other glucosinolates) was added 1 μl of myrosinase (5 nmol/min μl) dissolved in 0.5 M NaCl, 20 mM Tris–HCl (pH 7.4), 0.25 mM methyl-α-D-mannopyranoside. After overnight incubation at 25°C, 1-propanol was added to 25% and the glucosinolate degradation products were analysed by MECC using the method based on cholate described in Section 2.1, as well as the method based on CTAB for determination of intact glucosinolates [9]. A control incubation of glucosinolates without myrosinase was analysed in a parallel experiment. Peaks representing DIM and TRI were identified both by spiking and by comparison of migration times.

3. Results

3.1. Chromatography of reference compounds

The migration times of various indolyl compounds were tested (Fig. 2). I3C migrated just after the solvent band, with baseline separation between I3C and IAN/I3A (Table 1). IME, IEE, IPE and IBE migrated a little slower than I3C, appearing in the above-mentioned order. Baseline separation of I3C and IME was not obtained, but was obtained for I3C and the higher order alkyl ether homologues. The migration times of the monomers mentioned were in the range of 0–2 min after the solvent band. The

Table 1

Migration times $(t_m)\pm$relative standard deviations (R.S.D.), numbers of theoretical plates per meter of capillary (N/m) and resolution (R_s) of selected compounds in the MECC analyses. MECC conditions as in Fig. 2. TET is a peak that might represent a tetrameric indolyl compound

Analyte	t_m (min)	R.S.D. (%)	N/m ($\times 10^5$ m^{-1})	R_s
I3C	12.86	–	1.8	
				6
IAN	13.28	–	1.5	
				–
I3A	13.44	–	1.5	
I3C	13	0.3	3.2	
				6
IPE	14	0.2	3.6	
				27
DIM	18	2.3	3.8	
				34
TRI	23	2.7	4.4	
				5
TET	26	–	1.4	

selectivity of the method for the monomers mentioned was not impressive.

In contrast, the migration times of DIM, TRI, the unknown I3C product uk1, ASG, glucobrassicin, neoglucobrassicin and several putative indolyl tetramers were much longer than the migration time of the solvent band (Figs. 2, 4 and 7a). ASG migrated between DIM and TRI. For unknown reasons we could not detect ICZ at either 215 or at 335 nm. The numbers of theoretical plates per meter of capillary for I3C and its oligomerisation products were 1–4· 10^5, and the resolution of these compounds was good (Table 1).

Samples were normally dissolved in an aqueous solution containing 15 mM sodium cholate, 50 mM Na$_2$HPO$_4$, with 25% 1-propanol or 20% acetonitrile added. In this solvent, I3C is fairly stable and the oligomeric indolyl compounds have sufficient solubility. A large systematic increase in the migration times during a series of analyses occurred occasionally. The phenomenon seemed to be related to precipitation of analytes on the inner surface of the capillary and could be partly counteracted by increasing the voltage. Similarly, TRI in some instances seemed to partially precipitate in either the samples or during the electrophoresis, resulting in
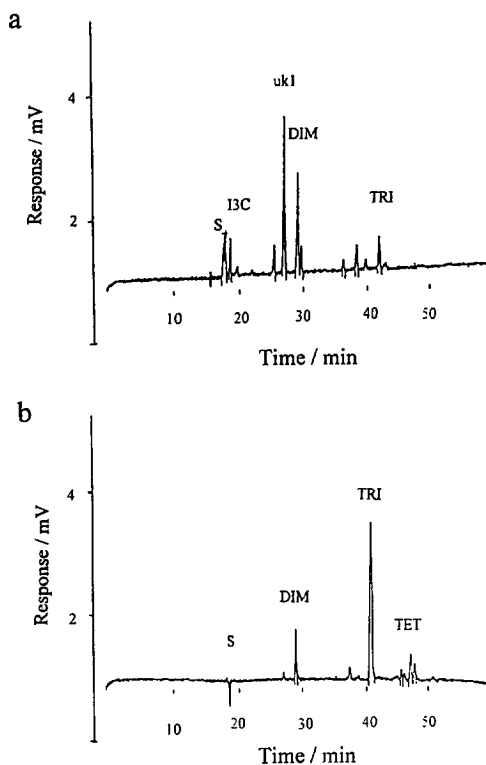

Fig. 4. MECC chromatogram of products from overnight oligomerisation of I3C in 20 mM sodium acetate buffer, 20% acetonitrile, pH 4.8. (a) Solvent phase analysed directly. (b) The formed precipitate redissolved in aqueous 25% 1-propanol, 15 mM sodium cholate, 50 mM Na$_2$HPO$_4$. A group of possible tetrameric indolyls is marked "TET". MECC conditions: 15 kV, otherwise as in Fig. 2.

distorted peaks. When sufficiently diluted samples were used, the problem of precipitation of TRI did not occur and the reproducibility of the migration times was excellent (Table 1). Concentrations of DIM and TRI of below 0.25–0.5 mM in samples is therefore recommended.

3.2. Quantitative aspects

Standard curves of I3C, DIM and TRI were made (Table 2). The detection wavelength, 220 nm, was a compromise between the absorption maxima of I3C (217 nm) and DIM (223 nm). The detector response at 220 nm for DIM and TRI was ca. five-times higher than the response at 280 nm. The detection limits (220 nm) of DIM and TRI were near 1 μM

Table 2
Linear regression data for standard curves of I3C, DIM and TRI. Detection wavelength, λ, was 220 or 280 nm, as indicated. MECC conditions otherwise were as in Fig. 2

Analyte	λ (nm)	Range (μM)	n	$\alpha \pm SD$ (μV s/min)	$\beta \pm SD$ [μV s/(min μM)]	r
I3C	220	26–519	8	-7 ± 15	0.67 ± 0.05	0.9865
I3C	280	26–519	7	1.2 ± 1.6	0.264 ± 0.005	0.9990
DIM	220	1–27	6	-0.51 ± 0.8	2.76 ± 0.06	0.9990
DIM	220	27–550	13	18 ± 46	2.42 ± 0.15	0.9883
DIM	280	5–550	9	-0.9 ± 6	0.56 ± 0.02	0.9947
TRI	220	1–67	11		$k \cdot 4.5$	0.9914
TRI	280	8–67	4		k	0.9699

N = number of observations.
α = intercept. β = slope. SD = standard deviation.
r is the observed correlation coefficient

(signal-to-noise ratio = 6). Repeated determinations of DIM and TRI concentrations gave standard deviations of between 2 and 6%. The standard curve of TRI was linear, but the slope of the curve could not be determined because of a lack of sufficiently pure TRI.

The molar response factor for TRI relative to DIM was therefore determined by comparing normalised areas in MECC chromatograms with ^{1}H-NMR data of the same mixture of DIM and TRI. Because of some minor impurities in the sample, the molar ratio of DIM to TRI in the NMR sample could not be determined exactly. Based on the NMR signals of the CH_2 groups, the molar ratio between DIM and TRI was determined to be 1.3; due to the presence of minor impurities, peak heights rather than peak areas were used. Based on peak areas the molar ratio was 2.00/1.83 = 1.1 (Fig. 3). The ratio NA(TRI)/NA(DIM) from MECC chromatograms of the mixture was 1.46 ± 0.10 at 220 nm ($n = 17$) and 1.53 ± 0.23 at 280 nm ($n = 10$). These ratios of normalised areas were determined from different dilutions of the NMR sample in different solvents and there were no signs of precipitation of TRI. The molar response factor for TRI relative to DIM at 220 nm seemed therefore to be $1.3 \cdot 1.46 = 1.9$, based on NMR peak heights, or $1.1 \cdot 1.46 = 1.6$, based on NMR peak areas.

3.3. Separation of unknown oligomerisation products

Incubation of I3C in aqueous buffer of pH 4–7 resulted in the formation of DIM, TRI and trace amounts of other products. Some experiments with I3C oligomerisation in more hydrophobic reaction mixtures were carried out by including 20% acetonitrile in the reaction mixtures. Under these conditions, several additional reaction products were seen (Fig. 4). From their migration times and poor solubility, we suggest that the slowly migrating peaks in Fig. 4b are various tetrameric indolyl compounds.

3.4. Kinetic experiments

Kinetic experiments: The kinetic experiments showed that the rate of formation of IPE was first order in I3C, at a constant concentration of 1-propanol. The data were consistent with the assumption that the rate of formation of TRI was first order in both I3C and DIM. In the kinetic modelling, it was further assumed that the formation of DIM was second order in I3C, i.e.

$$d[IPE]/dt = k_1[I3C]$$

$$d[DIM]/dt = k_2[I3C]^2 - d[TRI]/dt$$

$$d[TRI]/dt = k_3[I3C][DIM]$$

$$d[I3C]/dt = -d[IPE]/dt - 2k_2[I3C]^2 - d[TRI]/dt$$

With the exception of the very minor reaction products uk1 and uk2 (Fig. 2), only IPE, DIM and TRI were formed in the pH 6.0 reaction mixture. The formation of uk1 and uk2 was neglected in the kinetic modelling. By adjusting k_1, k_2 and k_3 manually, the theoretical curves were fitted to the ob-
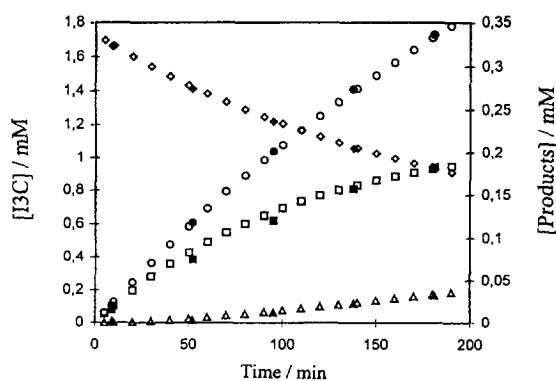
Fig. 5. Comparisons of observed and predicted concentrations of reactant and product as a function of time in an I3C oligomerisation reaction (experiment 1, Table 3). Filled symbols, observed concentrations; open symbols, predicted concentrations. ◆=I3C, ●=IPE, ■=DIM, ▲=TRI.

served concentrations (Fig. 5). There was no significant difference between the rate constants obtained by the two kinetic models. Rate constants determined from three experiments with different starting conditions are reported in Table 3. The rate constants should be considered as preliminary results.

3.5. Degradation products of indolyl glucosinolates

The glucosinolates isolated from broccoli were dominated by glucobrassicin and neoglucobrassicin. Ascorbic acid is not to be expected among the strongly acidic compounds, and the chromatograms of the isolated glucosinolates showed no significant amount of ascorbic acid. Incubation of the glucosinolates with myrosinase resulted in their complete degradation to various compounds, notably thiocyanate and some compounds that interacted strongly with both CTAB and cholate micelles. The latter compounds were only poorly resolved with the MECC method for intact glucosinolates (Fig. 6), but were well resolved by the MECC method described
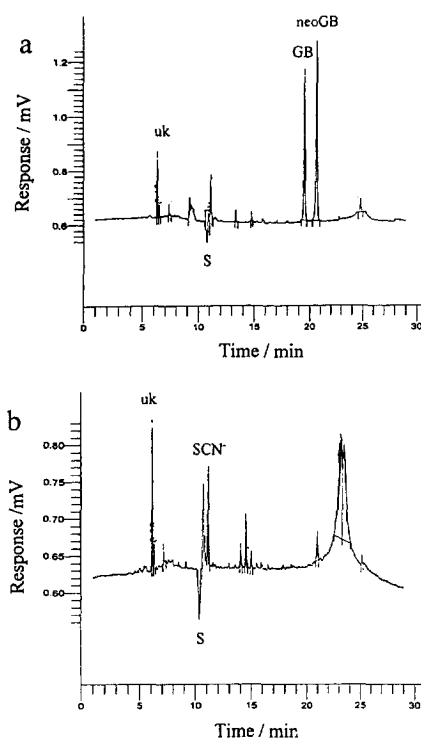

Fig. 6. MECC chromatograms of isolated broccoli glucosinolates incubated in sodium acetate buffer, pH 5.6, (a) without myrosinase and (b) with myrosinase. (The particular chromatograms in Fig. 5a–b are from a degradation experiment with three times more diluted glucosinolates than described in Section 2. The chromatograms of undiluted glucosinolates and their degradation products were similar to the ones shown). MECC conditions: 18 mM $Na_2B_4O_7$, 30 mM Na_2HPO_4, 50 mM CTAB, in H_2O, pH 7, 10% 1-propanol; 20 kV; 30°C; 235 nm; sample injection from the cathodic end of the capillary [9].

here (Fig. 7). Only trace amounts of DIM and TRI could be detected among the degradation products of the mixture of glucosinolates isolated from broccoli. Instead, two partly overlapping peaks that might represent mono- and di-N-methoxylated forms of DIM, and several peaks that might represent different mono-, di- and tri-N-methoxylated forms of TRI

Table 3
Rate constants (as defined in the text) for the I3C oligomerisation reaction and for the reaction with 25% 1-propanol, determined in three experiments with different initial concentrations of I3C and DIM

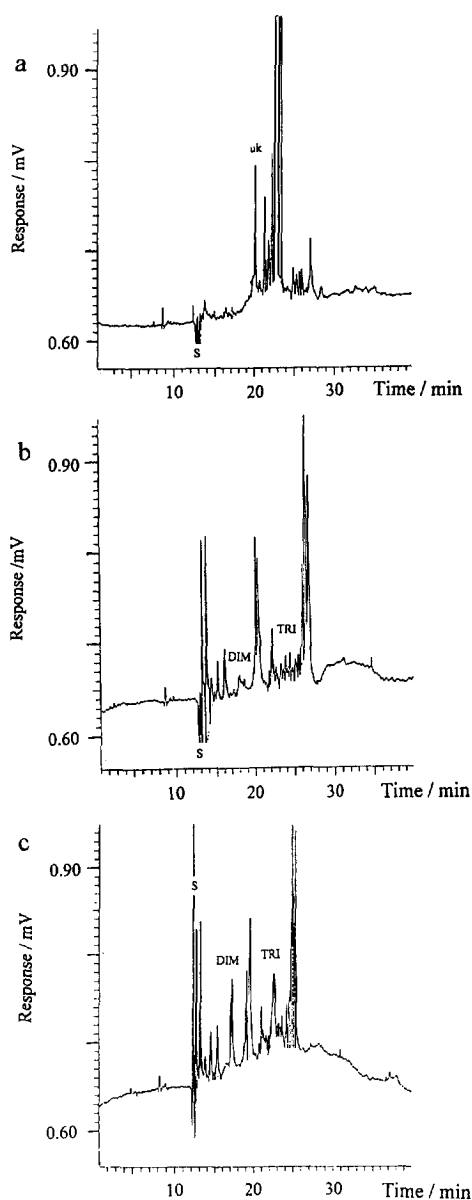| Experiment	[I3C] (mM)	[DIM] (mM)	k_1 (10^{-3} min^{-1})	k_2 (10^{-3} mM^{-1} min^{-1})	k_3 (10^{-3} mM min^{-1})
1	1.7	0	1.45	0.71	1.4
2	1.7	0.5	1.25	0.7	0.8
3	0.5	0.3	1.17	0.9	1.17

Fig. 7. MECC chromatograms of isolated broccoli glucosinolates incubated in sodium acetate buffer, pH 5.6, (a) without myrosinase and (b) with myrosinase. (c) Spiking of the glucosinolate degradation products in (b) with DIM and TRI. MECC conditions as in Fig. 2.

were seen. Ascorbigen was not present among the glucosinolate degradation products, as shown by MECC using the method based on DTAB [7]. Using the molar response factor obtained for DIM, the

degradation products detected accounted for around 40 nmol of indolyl groups from degradation of 170 nmol of glucobrassicin and neoglucobrassicin.

4. Discussion

The suitability of cholate as a surfactant in the MECC of hydrophobic compounds is well known [14,15], and is believed to be caused by the reversed micelles formed by cholate and by the stability of these micelles, even at quite high concentrations of organic modifier [14]. The migration order of the homologous series of indol-3-ylmethylalkyl ethers and of the mono-, di- and trimeric indolyloligomers show the importance of hydrophobicity for the migration times. However, the high migration time of the hydrophilic molecule, ASG, points to the importance of interactions other than hydrophobic interactions between analytes and cholate micelles. The migration time of the negatively charged indolylglucosinolates was clearly influenced by their negative electrical charge.

The method presented here should be useful in the analysis of glucosinolate degradation products in plant material and in the characterisation of myrosinase-catalysed degradation of glucobrassicin and its substituted analogues. By having good selectivity for oligomeric indolyl compounds, it supplements the previously described method based on DTAB, which has good selectivity for monomeric indolyl compounds, notably I3C, but poor selectivity towards the more hydrophobic oligomeric degradation products. Although the structures of the putative tetramers formed by oligomerisation in the presence of 20% acetonitrile (Fig. 4) have not been confirmed, the experiment clearly shows that TRI does not migrate in the end of the migration-time window and therefore can be separated from more hydrophobic compounds.

It has previously been shown that the response factors of hydrophobic glucosinolates in MECC varies significantly with electrophoresis voltage, concentration of surfactant in the electrophoresis buffer and with other factors [9], and therefore relative response factors in MECC do not necessarily equal the relative molar absorptivities of the ana-

lytes. Normalised peak areas must be used in quantification because of the use of on-column detection, to correct for differences in migration rates. We are not sure whether the approximately determined molar response factor of TRI relative to DIM is significantly different from the factor 1.5 that should be expected from the number of identical chromophores in the two molecules. In the calculations of rate constants, the factor 1.5 was therefore used. In the case of I3C and DIM, the ratio of the molar response factors at 280 nm was found to reflect the relative molar absorptivities at the detection wavelength (ϵ_{280}) of the molecules (Table 2).

As examples of the use of the MECC method, we present a kinetic investigation of the I3C oligomerisation, as well as a chromatographic analysis of the degradation products of a mixture of indolylglucosinolates that are representative for broccoli. The method is well suited for both kinds of experiments.

The reaction orders of the I3C oligomerisation reaction obtained in this work are in accordance with the theory that the dominant reaction mechanism for formation of TRI at near neutral pH has DIM as an intermediate [4]. As the rate of formation of TRI was influenced equally by DIM formed in situ or by authentic DIM added to the reaction mixture, a mechanism involving the hypothetical dimeric intermediate 2-(indol-3-ylmethyl)indol-3-ylcarbinol, followed by a nucleophilic attack of this intermediate on the C3 of another molecule of I3C was not significant at pH 6 (route A–B–D–C in scheme II in [4]). The latter reaction mechanism may be relevant at lower pH, resulting in the formation of several indolyl trimers and tetramers. For kinetic experiments at pH values lower than 6, a faster detection method must be used. One possibility in this connection would be to use the present system with a higher voltage or a shorter capillary, as this will shorten the time of analysis.

The formation of the indol-3-ylmethylalkyl ether from I3C and alcohols present in the reaction medium has been reported previously [2,16]. The reaction is probably first order in the alcohol as well as in I3C, and if so, the true second order rate constant is very low. The reaction might still have significance in connection with glucosinolate degradation in cruciferous plant material, as the total concentration of alcohol groups on proteins and polysaccharides in such materials is very high.

The glucosinolate-degradation experiment showed that DIM and TRI were present only in trace amounts after the enzymatic degradation of a mixture of glucobrassicin and neoglucobrassicin in weakly acidic buffer. In contrast to this result, DIM and TRI are the dominating products of I3C oligomerisation under the same conditions. The several dominating degradation products that were seen might be the mixed oligomers that should be expected from reaction between I3C and N-methoxyindol-3-ylcarbinol in the mixture. As ascorbic acid from the freeze-dried broccoli is not present in the same fraction as the isolated glucosinolates, and as ascorbigen was not detected among the degradation products, the observed degradation products were not of the ascorbigen type. The calculated yield of glucosinolate degradation products is not necessarily true, as their response factors are unknown. The solubilities of DIM and TRI were not exceeded, so precipitation of these compounds was not the reason for the very low amounts of these compounds that were found.

It is not known whether the experiment described has any relevance to glucosinolate degradation in broccoli, where the presence of ascorbic acid will influence the kinds of degradation products [1]. The experiment shows, however, that the degradation products of a substituted indolylglucosinolate can be separated from DIM and TRI using the method described.

Acknowledgments

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