ELSEVIER

Journal of Chromatography A, 836 (1999) 115–127

JOURNAL OF CHROMATOGRAPHY A

Micellar electrokinetic capillary chromatography of thiocarbamoyl derivatives produced in reactions between isothiocyanates and amino acids

Charlotte Bjergegaard, Peter Møller, Hilmer Sørensen*, Jens Christian Sørensen, Susanne Sørensen

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

Abstract

The reactions of amino acids and ammonia with the isothiocyanate derivatives: allyl-, benzyl-, but-3-enyl-, phenethyl- and phenylisothiocyanate have been investigated by UV spectroscopy, ^{1}H-NMR, and micellar electrokinetic capillary chromatography (MECC). The influence of reaction time, media, temperature, and type of solvent used to dissolve the evaporated reaction mixture were investigated with respect to the formation and stability of substituted thiocarbamoyl (STC) derivatives of amino acids, produced in reactions with allylisothiocyanate. Long reaction time in 50% pyridine at ambient temperature resulted in the production of STC, substituted thiohydantoin derivatives and various oxidation products including dimers with net charge −2. Increased temperature speeded up the reaction, as did increment in pH. Too high pH (>11) resulted in decreased product stability, which however varied considerably both with respect to the individual amino acids used as precursors, and the solvating medium. Modification of the reaction conditions allowed selective production of allylthiocarbamoylated amino acids, neutral as well as acidic, which separated well in the developed MECC system using sodium cholate as surfactant.

Keywords: Derivatization, electrophoresis; Isothiocyanates; Amino acids; Thiocarbamoyl-amino acids

1. Introduction

Isothiocyanates or other glucosinolate derived products are formed in food and feed containing cruciferous plant material owing to enzymatic or non-enzymatic reactions [1,2]. The result is various flavour or bioactive compounds that can give positive or negative physiological effects depending on glucosinolate precursor, -type and reaction conditions [3–7]. The isothiocyanates produced in β-thioglucoside glucohydrolase (E.C. 3.2.3.1.; myrosinase) reactions [5,8] can be transformed into thiourea derivatives by reaction with ammonia [9] and they are as well found to be bound in proteins [10–14]. The reaction between phenylisothiocyanate (PITC) and amino acids in proteins are used in the Edman sequencing procedure, which are also a well-known analytical procedure with high-performance liquid chromatography (HPLC) determinations of the reaction products phenylthiohydantoin (PTH) derivatives [15,16]. However, the potential precursor for PITC, phenylglucosinolate, does not occur as natural product and only limited information is available on the thiocarbamoyl derivatives, thiourea derivatives and other derivatives formed between isothiocyanates produced from glucosinolates and various nucleophiles including functional groups in amino

*Corresponding author. Tel.: +45-35-282-432; fax: +45-35-282-398; e-mail: hils@kvl.dk

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00109-0

acids. There is thus a need for more specific information on the thiocarbamoylderivatives and other products formed in reactions between amino acids, allylisothiocyanate (AITC) produced from the glucosinolate sinigrin and other isothiocyanates produced from other glucosinolates [2]. Methods of analyses for individual thiocarbamoyl derivatives of amino acids, which have an efficient chromophore system [6,17] are thus of interest, both for amino acid analyses and for determination of glucosinolate degradation products in biological materials.

In the present study, the importance of various factors for the formation of allylthiocarbamoylated amino acids and derivatives hereof were studied by means of UV spectroscopy, ^{1}H-nuclear magnetic resonance (NMR) and micellar electrokinetic capillary chromatography (MECC). The MECC procedure, using a bile salt as surfactant, gave efficient separation of the various products formed, which separated in groups corresponding to their net charges, and within these groups in accordance to size or hydrophobic properties. The method gives thus a good tool for qualitative evaluation of these compounds both when present standard solutions are used and in real samples.

2. Experimental

2.1. Samples and reagents

All chemicals were of analytical-reagent grade and the water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Sodium cholate and amino acids were obtained from Sigma (St. Louis, MO, USA), whereas glucosinolates and myrosinase were from the collection of our laboratories [2,8]. Ammonia, disodium hydrogenphosphate, ethyl acetate and pyridine were from Riedel-de Haën (Seelze, Germany). Allyl-, benzyl-, phenethyl- and phenylisothiocyanate were from Aldrich (Steinheim, Germany), and triethylamine was from Fluka (Buchs, Switzerland). Allyl-, but-3-enyl-, benzyl- and phenethylisothiocyanate were in addition produced from the glucosinolates in myrosinase catalysed reactions.

2.2. Preparation of thioureas

Isothiocyanate (allyl-, but-3-enyl, benzyl-, phenethyl or phenylisothiocyanate) (120 μl) was mixed with 3 ml 20% NH_3–96% ethanol (1:4). The samples were sealed and left to react for 2 h at 50°C. The samples were evaporated under air, redissolved in CD_3O^2H and investigated by ^{1}H-NMR. Samples for UV were redissolved in water. Samples for MECC were redissolved in run buffer.

2.3. Preparation of allylthiocarbamoylated amino acids

Three series of experiments were performed:

(a) Amino acids (Gly, Ala, Val, Ile and Leu; 1–3 mg of each) were dissolved in 1 ml 50% pyridine and AITC (50 μl) added. The sample was sealed and allowed to react at ambient temperature. Aliquots (100 μl) of the reaction mixture were taken after incubation for 10, 30, 180, 360 min and overnight, respectively, evaporated under air, redissolved in 100 μl run buffer and analysed by MECC.

(b) Amino acids (Gly, Thr and Ala; 4 mg of each) were dissolved in 2 ml 50% pyridine. This mixture was then divided into 2×1 ml, to which were added 50 μl AITC each. The samples were sealed and left to react at ambient temperature (A) or 50°C (B). Two aliquots (75 μl) of each reaction mixture were taken after 20, 40, 60, 90 and 120 min, respectively. One portion (1) was evaporated under air, redissolved in 75 μl run buffer and exposed directly to MECC, whereas the other portion (2) was added 40 μl 2 *M* NH_3 prior to this treatment.

(c) Final optimisation of the derivatisation procedure included variations of several factors as indicated below. Note that all combinations were not performed. Ca. 2–3 mg of each amino acid (type and number depending on experiment) were dissolved in (1) 500 μl 50% pyridine+500 μl 4.0 *M* NaOH or (2) 500 μl 50% pyridine+500 μl 80 m*M* Li_2CO_3 or (3) 500 μl triethylamine+250 μl water+250 μl 100% pyridine and AITC (50 μl) added. The sample was sealed and allowed to react at ambient temperature. Aliquots (100 μl) of the reaction mixture were taken after 5, 10, 15, 30, 60 and 120 min, respectively, evaporated under air, and redissolved in (1) water, (2) 15% 1-propanol, (3) 15% ethyl acetate,

(4) 15% ethanol, (5) run buffer, (6) 15% acetonitrile, (7) 0.5 *M* acetic acid, or (8) 0.5 *M* sodium hydroxide.

2.4. High-performance capillary electrophoresis (HPCE)

Analyses were performed using a Hewlett-Packard HP^{3D} CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with diode-array detector and a 645 mm×0.05 mm I.D. fused silica capillary. Detection was performed on-column at 240 nm at a position 560 mm from the inlet. Data processing was carried out by use of a HP Vectra 5/100 mHz Pentium with HP Chemstation V. 6.01. Samples were introduced from the anodic end of the

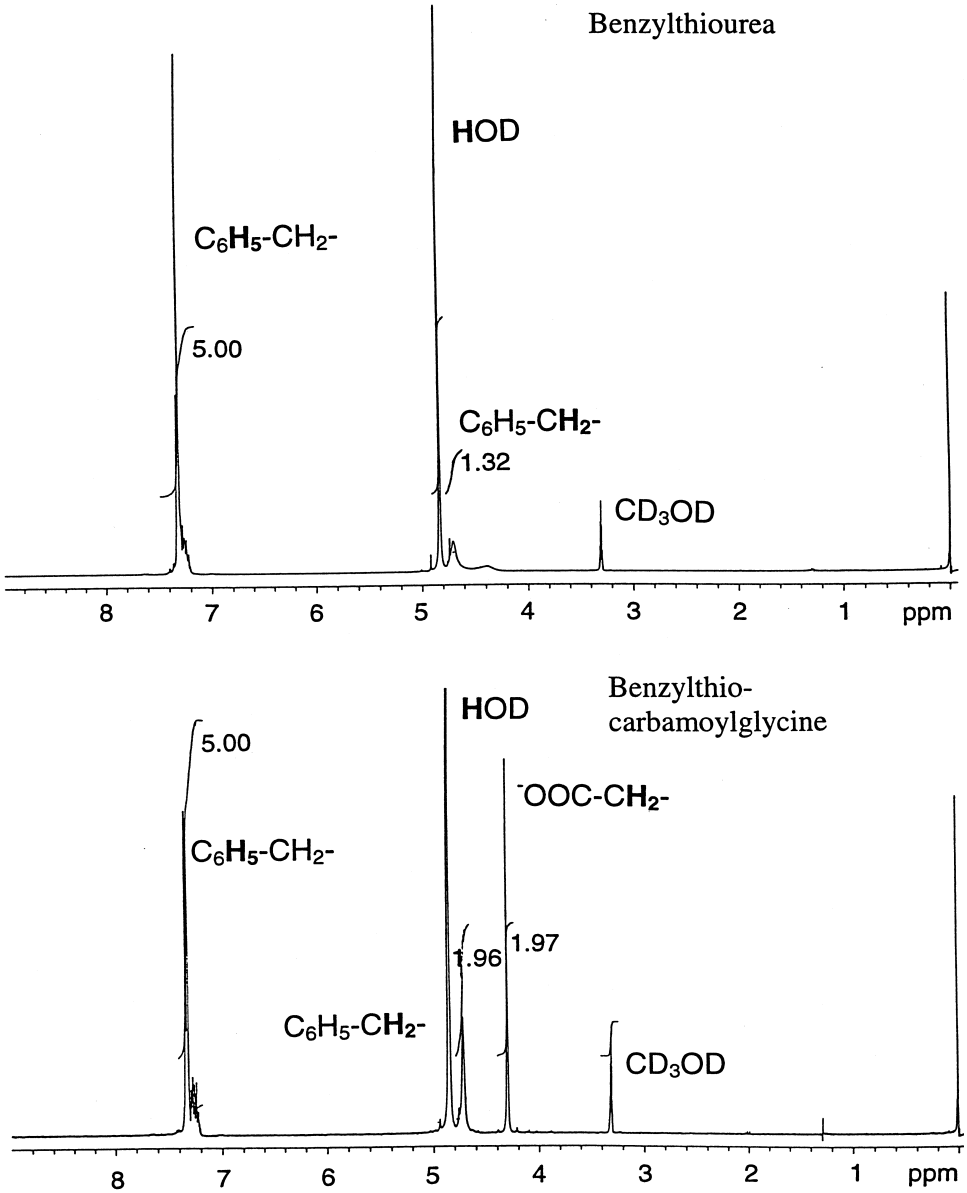

Fig. 1. ^{1}H-NMR spectra of benzylthiourea and benzylthiocarbamoylglycine dissolved in deuteromethanol.

capillary. The specific MECC conditions depended on the AITC-derivatized amino acids to be separated. The run buffer was filtered through a 0.20-μm membrane filter before use. The electrophoreses were run for 60 min and between each run the capillary was washed with 1.0 *M* NaOH for 5 min, water for 2 min and run buffer for 5 min.

3. Results and discussion

The reactivity of isothiocyanates towards nucleophiles is high, and this is utilized in the present study. The thioureas obtained from reaction of ammonia with allyl-, but-3-enyl-, benzyl-, phenethylisothiocyanate (produced from the pure

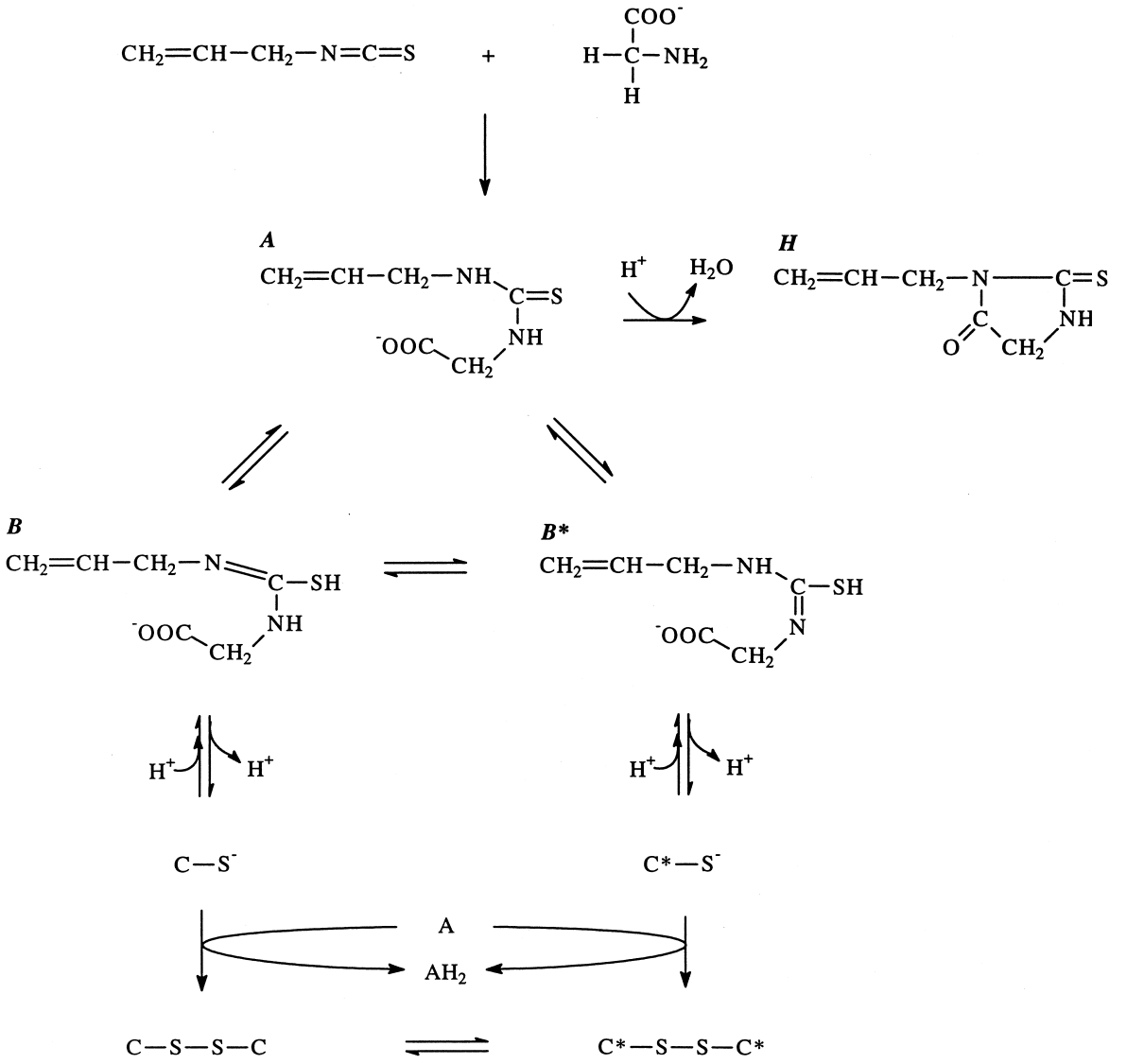

Fig. 2. The reaction of allylisothiocyanate with Gly. The various products (described by letters) are commented on in the text.

glucosinolates by myrosinase catalysis or from pure isothiocyanates) and from pure phenylisothiocyanate were easily separated by MECC, with allylthiourea having the shortest migration time (t_m) and phenethylthiourea the longest t_m, as also expected from hydrophobicity of these isothiocyanates and their thiourea derivatives. This migration pattern was also consistent for thiocarbamoylated amino acids obtained from reaction with the different isothiocyanate derivatives, but for these negatively charged compounds, t_m values were in the reversed order (see below).

Considering the great number of glucosinolate producing isothiocyanates [2,5] and also the great number of amino acids [18] and other nucleophiles, it will, however, not be possible to have all compounds of these types separated in one system. It is therefore important to have specific knowledge to the method of analysis including factors decisive for separation of the various types of products, based on well defined starting points with respect to isothiocyanates and amino acids. In this paper, the main focus has been placed on AITC-derived products as model compounds used for method development.

Reaction of isothiocyanates with ammonia in ethanol resulted in thiourea derivatives as confirmed by ^{1}H-NMR (Fig. 1) confirming the high reactivity of the amino group in ammonia compared to the hydroxy group in ethanol. This was also found for amino acids, as amino acids having hydroxy groups in their side chain (e.g., serine, threonine) only produce thiocarbamoylated amino acids by reaction between the free α-amino group and isothiocyanate. Lysine, having an ϵ-amino group reacts at both amino groups when dissolved in solvents for the amino groups with pH higher than pK_a values [17].

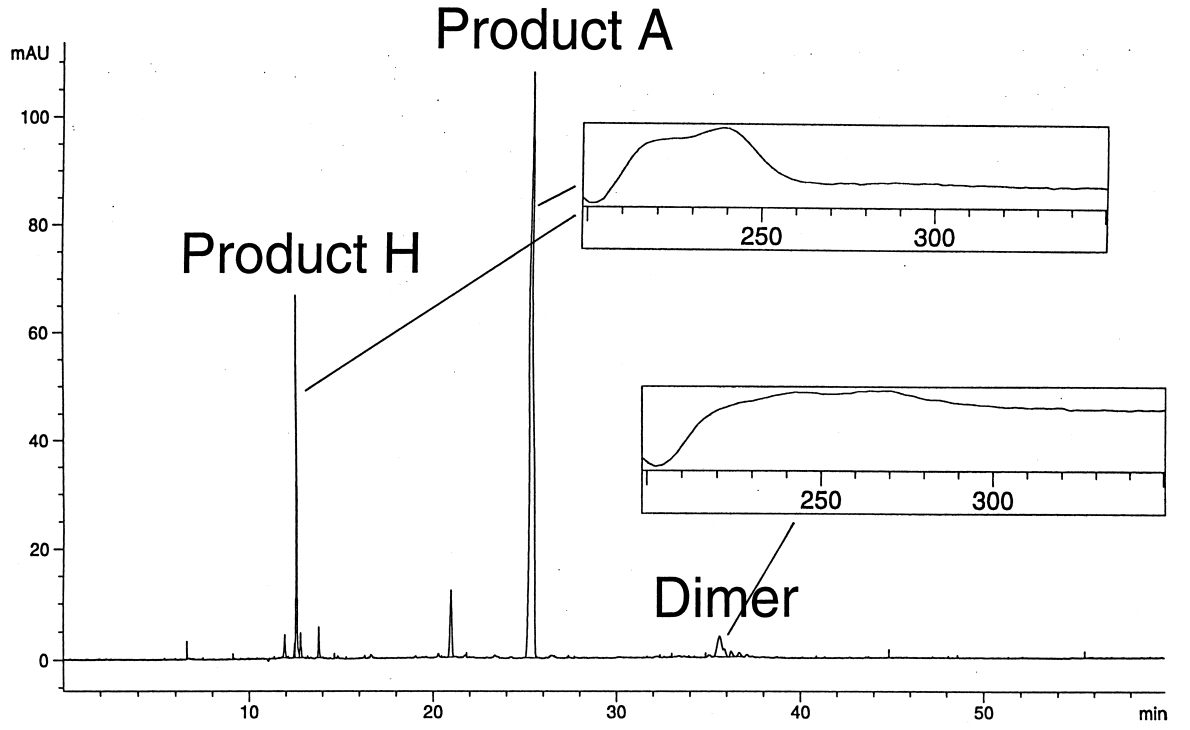

Fig. 3. Electropherogram of compounds produced after 24 h of reaction between AITC and Gly. Separation conditions: temperature, 20°C; voltage, 15 kV; buffer composition: 80 m*M* sodium cholate and 50 m*M* disodium hydrogenphosphate, adjusted to pH 8.0 by 4 *M* phosphoric acid. UV detection at 240 nm. The UV spectra characteristic for the products A and H are nearly identical whereas the derivatives B, B*, C and C* (Fig. 2) have slightly changed conjugated systems as reflected in their UV spectra.

In the studies of the reactions between allylisothiocyanate and amino acids, allylisothiocyanate was added in excess (ca. 20–60 times), as preliminary results showed highest reaction velocity at these conditions. The proposed reaction scheme for allylisothiocyanate and the amino acid Gly is outlined in Fig. 2.

The allylthiocarbamoylated Gly produced may exist in tautomeric equilibriums with the reaction products B, B* or the corresponding thiolate ions (C, C*), which can be formed in reactions resulting from dissociation or release of H^+. Corresponding reactions occur as well with other amino acids and isothiocyanates as also revealed from the ^{1}H-NMR of benzylthiocarbamoylglycine (Fig. 1). The tautomerisations and reaction products are the reasons for the occasional presence in MECC of one to two minor peaks in addition to the initial main product A. Adjustments in the reaction procedure (see below) may reduce this phenomenon. The substituted thiohydantoin derivatives (product H; uncharged) are expected at acidic conditions, but appears also to be formed at the present conditions. As the case is for the thiocarbamoylderivatives, thiohydantoins may also give the corresponding tautomeric equilibriums and reactions. In addition, hydrolytic desulphuration may moreover transform the compounds into their corresponding oxygen analogues, and oxidation may lead to production of dimers (net charge −2) by formation of a disulfide bridge. In MECC, using sodium cholate as surfactant, the uncharged thiohydantoins appear just after the solvent front (including pyridine), whereas the dimers have higher t_m values than the thiocarbamoyl amino acids (A) due to a net charge of −2. Product A (−1) migrates thus at intermediate t_m (Fig. 3). As seen from the figure, the allylthiocarbamoylated products are easily characterised by their UV absorption pattern.

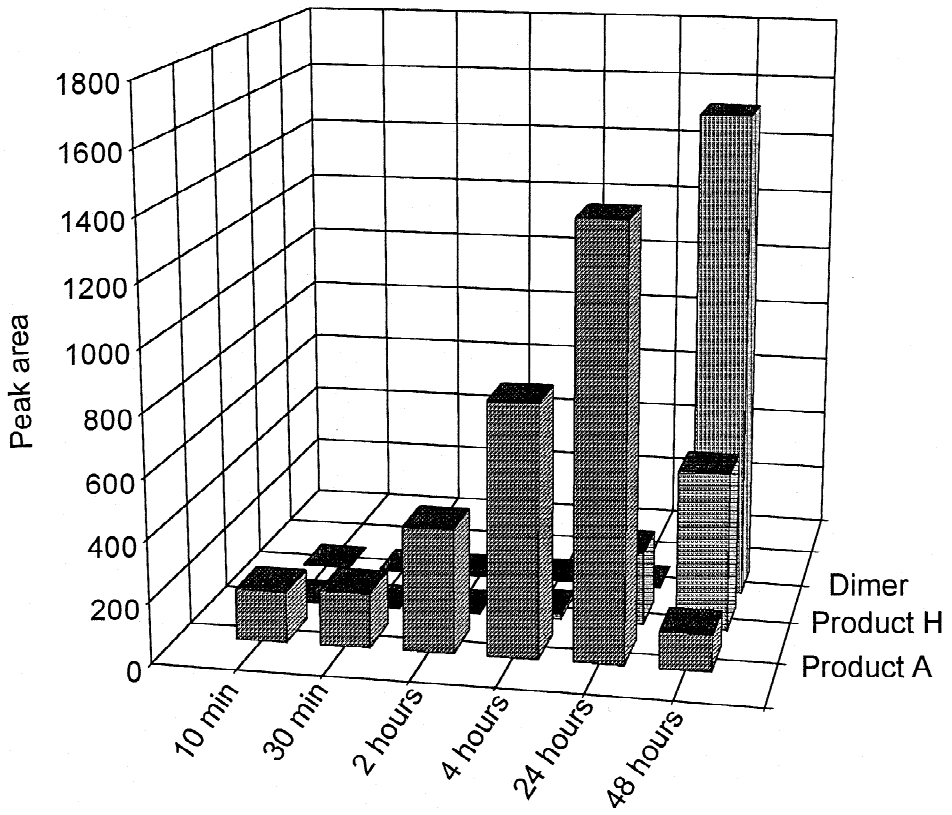

Fig. 4. Formation of the products A and H and dimers (C and C*, Fig. 2), as a function of time of reaction between AITC and Gly. Peak areas of individual compounds were determined as normalised areas in MECC (Fig. 3).

Results from study a indicated that time is an important factor in determining the amount and type of reaction products formed. Increased reaction time leads to increased production of product A, but only up to a certain limit, after which the thiohydantoins and, for some of the amino acid tested, the dimers (Figs. 2 and 3) were produced at the expense of product A (Fig. 4).

Study b showed, that an increment in temperature increased the reaction rate considerably (Fig. 5). The addition of NH_3 prior to air evaporation was performed in order to consume unreacted AITC by formation of allylthiourea. The appearance of this compound was verified by the peak in MECC and by UV spectroscopy. The thiourea derivatives migrated in this system with t_m close to peaks for thiohydantoins and the peak representing the electroosmotic flow (EOF). These peaks were found close to the peak for the solvent pyridine and, therefore, these peaks did not interfere with the product peaks (compare to Fig. 3).

Termination of the reaction with excess of NH_3 also resulted in a considerable increase in the formation of product A compared to the amount of product formed without use of NH_3 as terminating step. This indicates, that a stronger base than pyridine is needed for sufficient deprotonisation of the amino group in the amino acids, as an amino group with a free electron pair increases the reactivity or is necessary for an appropriate reactivity. These results were verified in an experiment testing the product formation by reaction of six amino acids with AITC at pH 9, 10 and 11. Slow reactions were observed at pH 9, whereas pH 10 resulted in fast reactions and relatively good stability of the products. At pH 11, fast degradations of the products were observed. The importance of pH has also been studied by Cejpek et al. [19], who found a higher

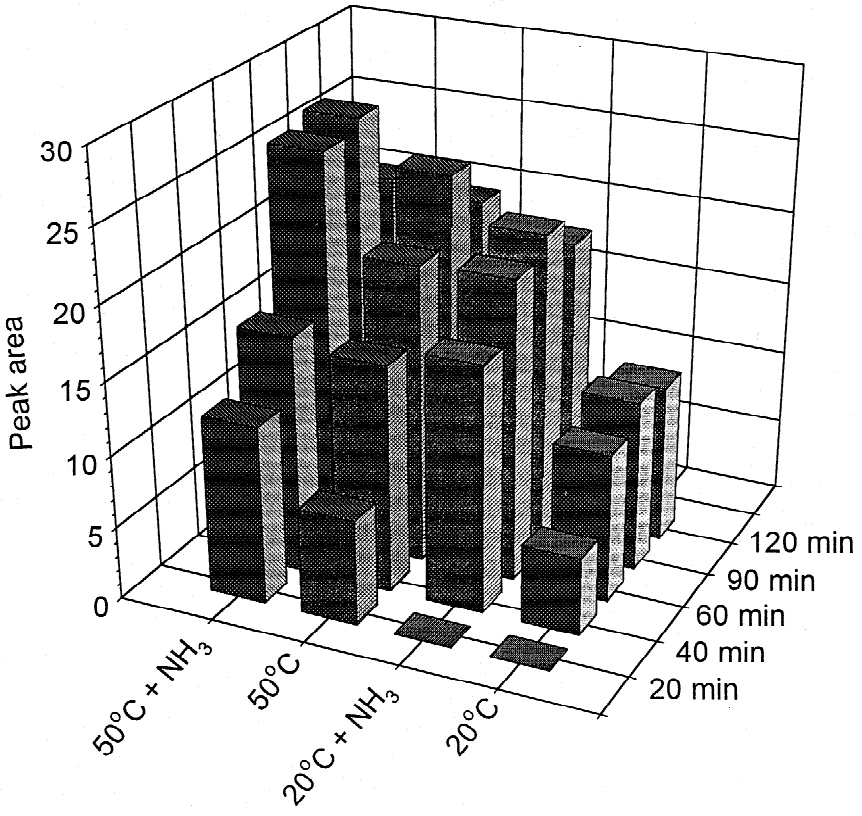

Fig. 5. Temperature and pH dependence (NH_3 addition) for formation of product A (allylthiocarbamoylated amino acid), obtained from reaction between Gly and AITC.

stability of the allylthiocarbamoylated amino acids compared to the corresponding thiohydantoins at pH 10.

Reactions at pH values below 9 were found to give negligible product formation, except for cysteine, which reacted well even down to pH 6. This observation may be explained by the presence of a free electron pair at the thiol group, making the SH group (but not the NH_3^+ group) in cysteine reactive towards AITC at this pH. The product obtained was shown to have a considerably higher ϵ value than the corresponding products formed between isothiocyanates and amino groups, and this selective reaction of the thiol group may be utilised in specific determination of sulfur in proteins.

Study c was initiated to find a reaction media alternative to pyridine, which is normally used for production of thiohydantoins [20] due to the good solvent properties for the products formed but suffering from low basicity as described above. The best reaction medium was found to be 500 μl triethylamine+250 μl water+250 μl 100% pyridine, as both NaOH and Li_2CO_3 resulted in too much salt in the samples. Reactions performed at room temperature in this medium were completed within 15 min without dimer formation. Evaporation under air was performed to remove excess AITC. Ethyl acetate (15%) proved to be the best solvent for the evaporated samples prior to MECC, giving the electropherogram with fewest interfering peaks.

The stabilities of the allylthiocarbamoylated amino acids (Gly, Thr and Ala) in 15% ethyl acetate were tested by repeated injections of the derivatised samples in MECC (see separation conditions below). The stabilities of the tested amino acid derivatives varied considerably, with the glycine derivative being the most stable (Fig. 6).

Based on the above mentioned results, the final derivatisation procedure selected was as follows: Dissolve amino acid or other nucleophilic compound in a mixture of triethylamine–water–100% pyridine (2:1:1), and add AITC in excess (20–30 times). Seal the sample and react 15 min at ambient temperature. Evaporate the sample under air and redissolve in

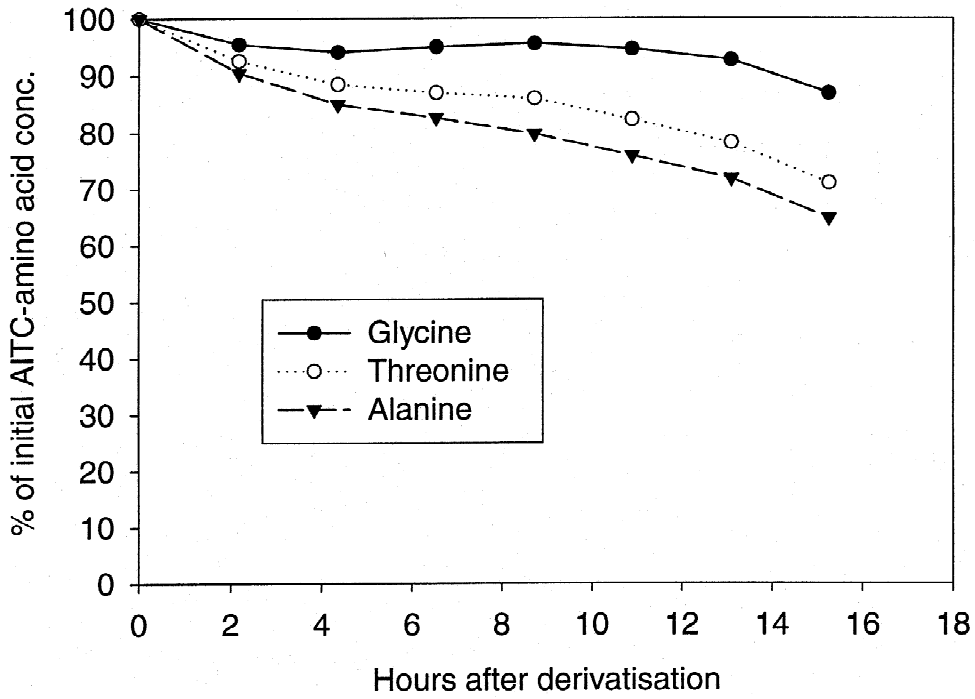

Fig. 6. Product stability of thiocarbamoylated Gly, Thr and Ala measured as in Fig. 3. Stabilities are given as a function of time after derivatisation with allylisothiocyanate.

15% ethyl acetate prior to MECC. As indicated in Fig. 6, quantitative analysis requires simultaneous derivatisation and analysis or alternatively use of individual correction factors for each amino acid. The relatively poor stability over time at the alkaline conditions is however a minor problem with respect to the qualitative aspect of the analysis. Problems with the stability of isothiocyanate derivatised amino acids has also been observed by Puig-Deu and Buxaderas [21], using phenyl isothiocyanate as derivatisation agent.

The buffer composition for MECC separation of allylthiocarbamoylated amino acids was optimised with respect to concentration of surfactant (sodium cholate; 20–300 m*M*), buffer salt (Na_2HPO_4; 10–300 m*M*), and modifier (1-propanol and acetonitrile; 0–20%). The resolution for Gly, Ala and Thr at varying cholate concentration is shown in Fig. 7.

The number of theoretical plates was highest at 100–130 m*M* sodium cholate, but declined at higher concentrations. Corresponding curves for phosphate and modifier concentration showed promising separation conditions at 40 m*M* disodium hydrogenphosphate and 10% acetonitrile using 100 m*M* sodium cholate. This system was directed primarily at separation of thiocarbamoylated neutral amino acids, which mainly migrated in accordance to their molecular mass (low mass – high t_m) (Fig. 8). At the applied buffer pH (pH 8.0) these derivatized amino acids have one negative charge, and compounds with the lowest molecular mass have the highest mobility towards the anode (injection), and thereby the highest t_m. This migration against the EOF gives as expected high t_m for small molecules, but t_m will in addition be affected by binding of analytes to the cholate micelles, which also have migration toward EOF, owing to their negative net charge. This means that thiocarbamoylated neutral amino acids with equal molecular mass can have different t_m values as seen from Fig. 8. Thiocarbamoylated amino acids produced in reactions between the amino acids and but-3-enyl, benzyl, phenethyl- and phenylisothiocyanate instead of allylisothiocyanate behave in the same way. With uncharged thiourea derivatives,

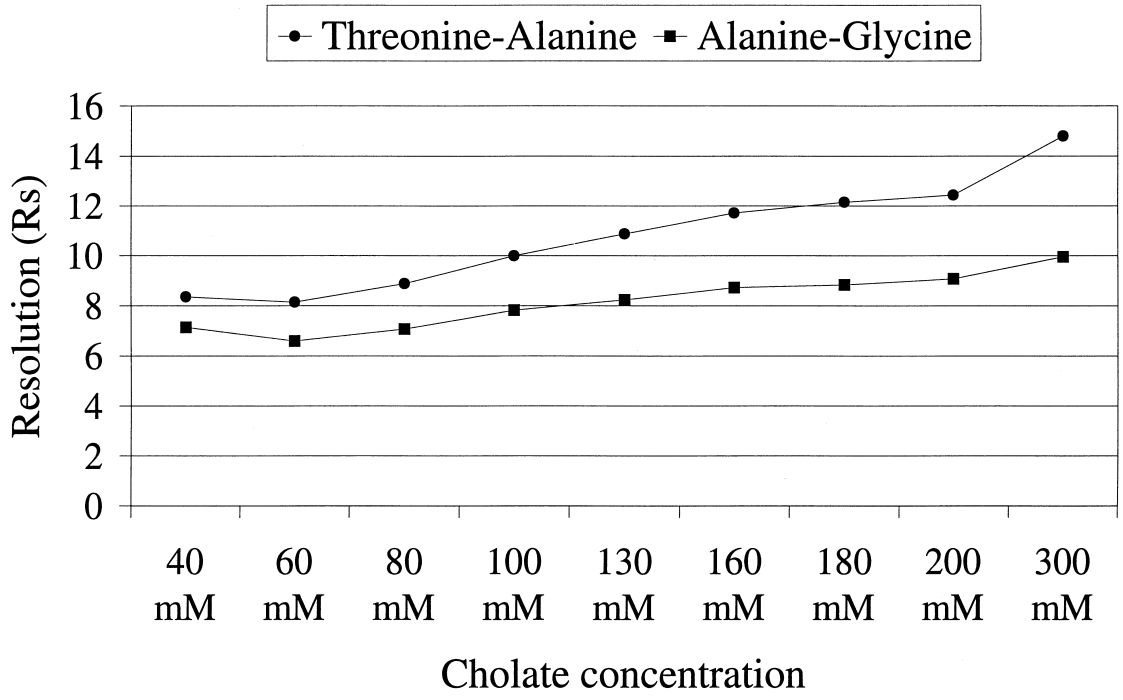

Fig. 7. Resolution for Gly–Ala and Ala–Thr at increasing sodium cholate concentration. Separation conditions: temperature, 20°C; voltage, 15 kV; buffer composition: sodium cholate (varying concentration) and 50 m*M* disodium hydrogenphosphate, adjusted to pH 8.0 by 4 *M* phosphoric acid. UV detection at 240 nm.

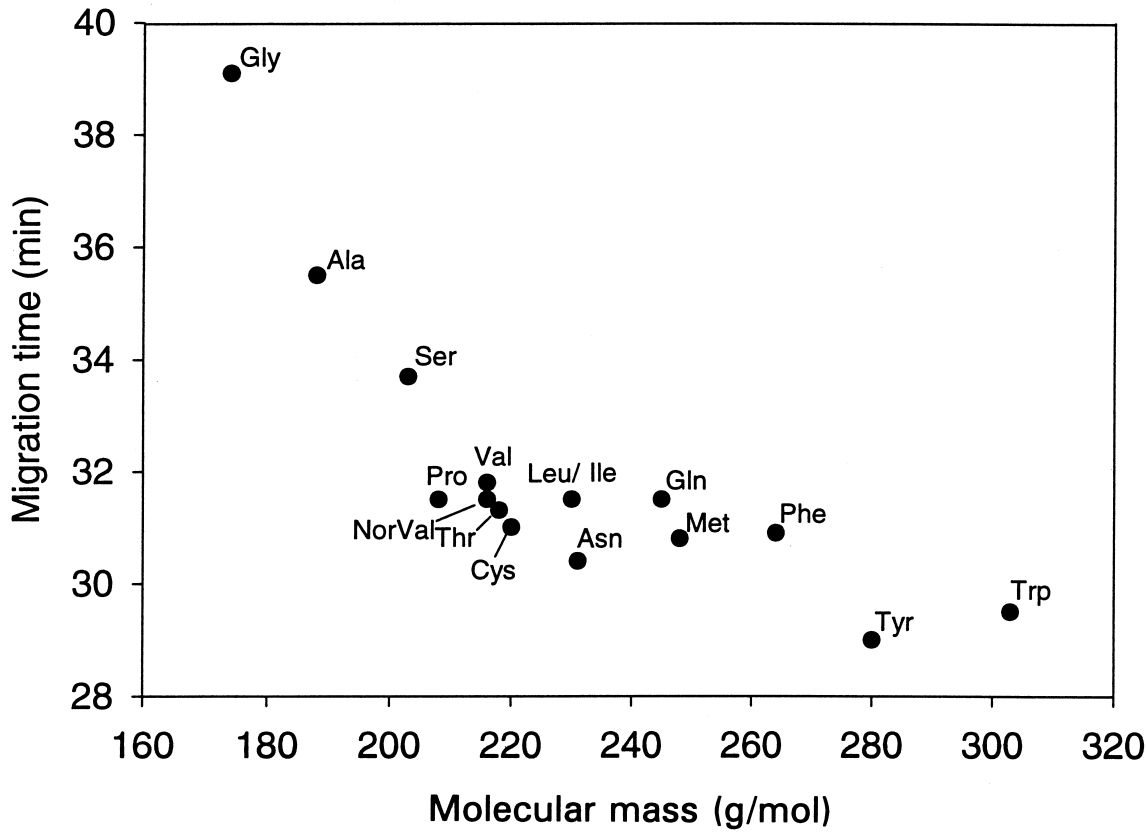

Fig. 8. Migration time for selected neutral amino acids in MECC. Separation conditions: temperature, 20°C; voltage, 15 kV; buffer composition: 100 m*M* sodium cholate, 40 m*M* disodium hydrogenphosphate, 10% acetonitrile, pH adjusted to 8.0 by 4 *M* phosphoric acid. UV detection at 240 nm.

produced in reactions with these isothiocyanates and ammonia, t_m values are determined by EOF and the interaction between analytes and the micelles. This means, that analytes with the strongest association to the micelles will have the highest t_m, and this is often the compounds with the highest molecular mass; t_m values for the thiourea derivatives of allyl-, phenyl- and benzylisothiocyanate were thus 23, 27 and 31 min, respectively.

Thiocarbamoylated acidic amino acids have net charge −2, and appropriate separation of these compounds required slightly changed MECC conditions to obtain acceptable t_m separations and peak shape. These changes comprised a decrease in sodium cholate concentration and an increase in disodium hydrogenphosphate concentration. Moreover, the organic modifier acetonitrile was replaced by 1-propanol and a zwitterion, taurine, was included in the buffer (Fig. 9).

Analysis of acidic amino acids and neutral amino acids present in inflorescences of *Reseda lutea* L. was performed on samples isolated, purified and group separated using the procedure described elsewhere [17,22]. MECC was performed at conditions described in Fig. 9, resulting in electropherograms shown in Figs. 10A and B for non-derivatised and AITC-derivatised samples, respectively.

The results obtained with non-derivatised *R. lutea* amino acids (Fig. 10A) reveal the presence of the 3-carboxy-substituted aromatic amino acids, together with some few of other analytes in the group III area (net charge −1), and Phe and Tyr occur in the group II area (net charge 0). The occurrence of some other compounds with absorption at 214 nm are in agree-

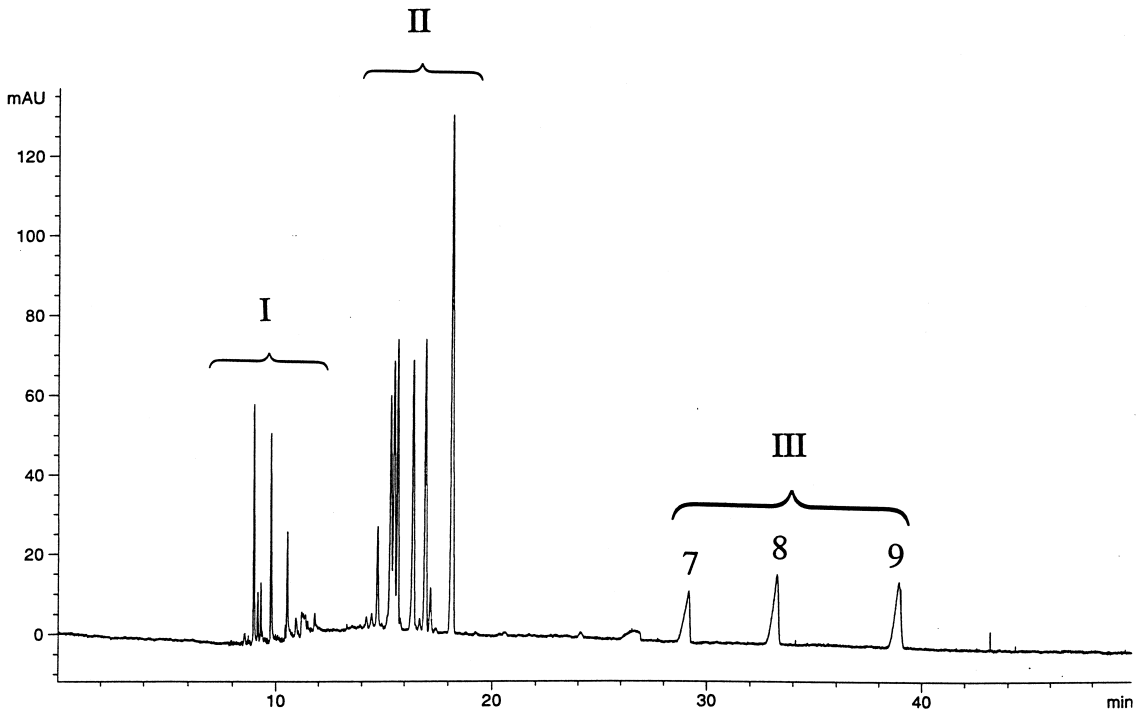

Fig. 9. Electropherogram of AITC-derivatized neutral and acidic amino acids. Separation conditions: temperature, 30°C; voltage, 18 kV; buffer composition: 75 m*M* sodium cholate and 75 m*M* disodium hydrogenphosphate, 4% 1-propanol, 250 m*M* taurine, pH unadjusted. UV detection at 240 nm. I=Reagent peaks, II=neutral amino acids (Tyr, Phe, Asn, Leu, Val, Ser, Ala and Gly) and III=acidic amino acids (7=α-aminoadipic acid, 8=Glu, 9=Asp).

ment with corresponding analyses of samples from the same type of plant material investigated by free zone (FZ) CE [23]. Transformation of *R. lutea* amino acids into allylthiocarbamoyl derivatives (detection at 240 nm; Fig. 10B) shows the presence of quantitatively dominating amounts of neutral amino acids in group II (net charge −1), and Asp and Glu in amounts equal to the above mentioned aromatic amino acids in group III (net charge −2). The occurrence of other aliphatic acidic amino acids in extracts from *R. lutea* inflorescences is as expected from previously described investigations of acidic amino acids in Reseda species [24,25].

The quantitatively dominating amount of aromatic amino acids in electropherograms of non-derivatised samples from *R. lutea* (Fig. 10A) is in accordance with the relatively strong absorbance for such compounds at 214 nm. Aliphatic amino acids have on the other hand only very weak UV absorption at this wavelength [17,18]. When the amino acids are derivatised with AITC or other isothiocyanates, a relatively efficient chromophore system is obtained with extinction coefficients at about 240 nm in the size of $1.2 \cdot 10^4$ M^{-1} cm^{-1}[26]. With absorption maximum at 240 nm this give nearly an equal response for all amino acids, as only few amino acids have chromophore systems, which give absorption in this UV area [17,18].

The MECC systems and procedures for derivatisation of isothiocyanates with ammonia and amino acids now described give a valuable tool for studies both of isothiocyanates formed from glucosinolates and for qualitative amino acid analysis. However, the limited stability of thiocarbamoylated amino acids over time call for attention before it can be a recommendable quantitative method of analyses for amino acids, and such problems needs also to be considered when thiohydantoin derivatives are used for quantitative amino acid analyses [16]. The conditions, especially pH, found to be required for

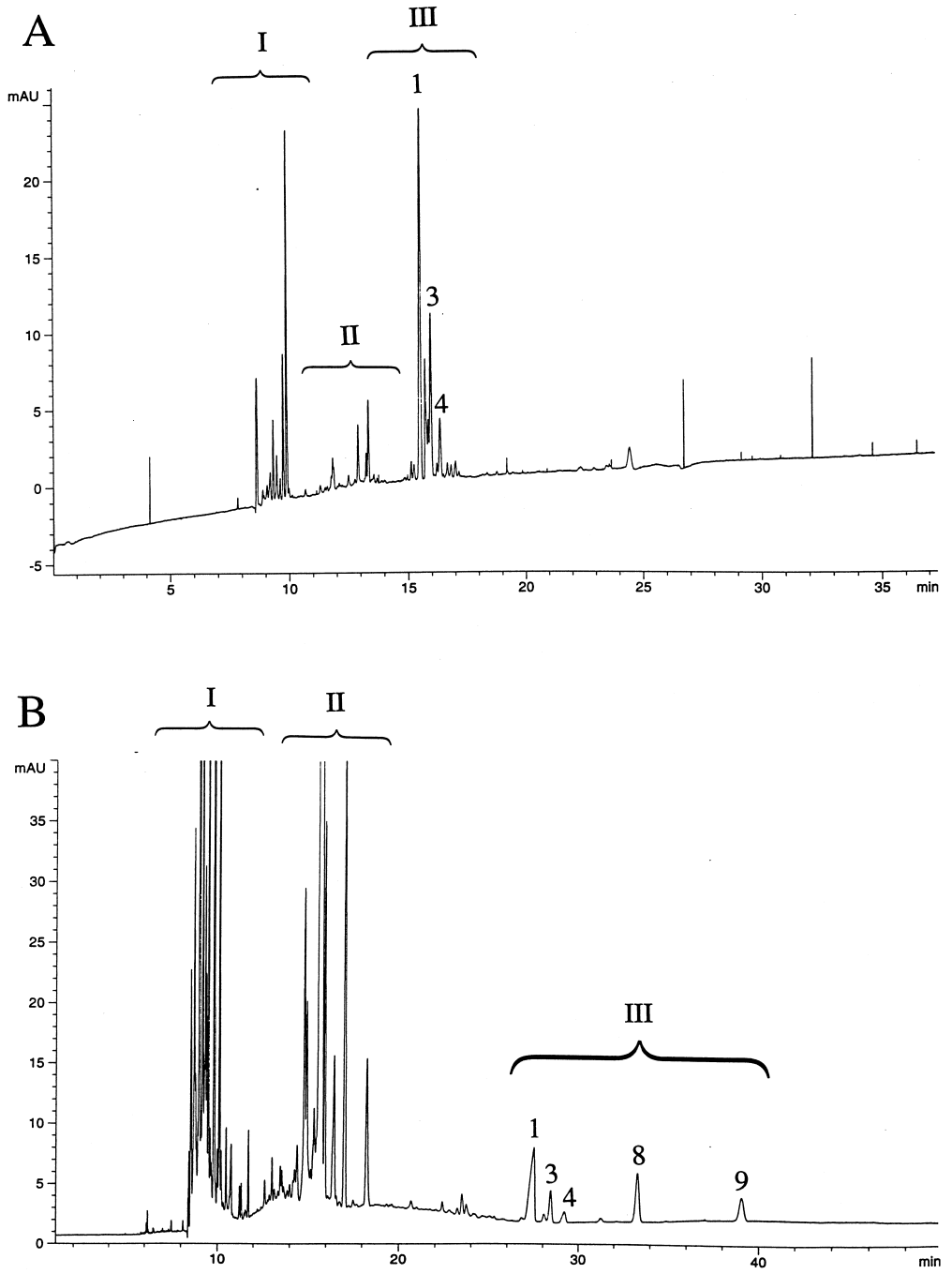

Fig. 10. Electropherograms of samples including neutral and acidic amino acids isolated from inflorescence of *Reseda lutea* L. (A) Non-derivatized sample (214 nm) and (B) AITC-derivatized sample (240 nm). Separation conditions as in Fig. 9, except for the detection wavelength in (A). I=Reagent peaks, II=neutral amino acids, III=acidic amino acids 1=3-(3-carboxyphenyl)alanin, 3=3-(3-carboxy-4-hydroxyphenyl)alanine, 4=3-carboxy-4-hydroxy-phenylglycine, 8=Glu and 9=Asp.

thiocarbamoylation of amino- and thiol-groups are as well important to consider in relation to discussion of interaction between proteins and isothiocyanates produced from glucosinolates [10–14]. Thiocarbamoylation of amino groups demands thus a free amino group corresponding to a pH above, whereas corresponding derivatisation of isothiocyanates occurs even with protonated thiolgroups or pH in weakly acid to neutral solutions.

Acknowledgements

This work was financial supported by the Commission of European Union (Contract FAIR CT 95-0260 and FAIR CT 98-3778).

References

[1] O. Olsen, H. Sørensen, J. Am. Oil Chem. Soc. 58 (1981) 857.
[2] H. Sørensen, Rapeseed/Canola: Production, Chemistry, Nutrition and Processing Technology, Van Nostrand Reinhold, 1990, Ch. 9, p. 149.
[3] N. Bille, B.O. Eggum, I. Jacobsen, O. Olsen, H. Sørensen, Z. Tierphysiol., Tierernähr. Futtermittelkd. 49 (1983) 195.
[4] B. Bjerg, B.O. Eggum, I. Jacobsen, J. Otte, H. Sørensen, Z. Tierphysiol., Tierernähr. Futterrmittelkd. 61 (1989) 227.
[5] C. Bjergegaard, P.W. Li, S. Michaelsen, P. Møller, J. Otte, H. Sørensen, in: H. Kozlowska, J. Fornal, Z. Zdunczyk (Eds.), Proceedings of Euro Food Tox IV, Centrum Agrotechnologii i Weterynarii Polska Akademia Nauk, Olztyn, Poland, 1994, p. 1.
[6] N. Agerbirk, C. Bjergegaard, C.E. Olsen, H. Sørensen, J. Chromatogr. A 745 (1996) 239.
[7] N. Agerbirk, C.E. Olsen, H. Sørensen, J. Agric. Food Chem. 46 (1998) 1563.
[8] S. Palmieri, P. Rollin, H. Sørensen, S. Sørensen, Agro. Food Ind. High Tech. 9 (1998) 24.
[9] L.R. Wetter, C.G. Young, J. Am. Oil Chem. Soc. 53 (1976) 162.
[10] R. Björkman, Phytochemistry 12 (1973) 1585.
[11] S. Kawakishi, T. Kaneko, J. Agric. Food Chem. 35 (1987) 85.
[12] J. Kroll, H. Rawel, R. Kröck, J. Proll, W. Schnaak, Nahrung 38 (1994) 53.
[13] H. Rawel, J. Kroll, Nahrung 39 (1995) 465.
[14] J. Kroll, H. Rawel, J. Sci. Food Agric 72 (1996) 376.
[15] T. Iida, H. Matsunaga, T. Fukushima, T. Santa, H. Homma, K. Imai, Anal. Chem. 69 (1997) 4463.
[16] T. Iida, H. Matsunaga, T. Santa, T. Fukushima, H. Homma, K. Imai, J. Chromatogr. A 813 (1998) 267.
[17] H. Sørensen, S. Sørensen, C. Bjergegaard, S. Michaelsen, Chromatography and Capillary Electrophoresis in Food Analysis, The Royal Society of Chemistry, Cambridge, 1999, 470 pp.
[18] B.O. Eggum, H. Sørensen, in: M. Friedman (Ed.), Absorption and Utilization of Amino Acids, CRC Press, Boca Raton, FL, 1989, p. 265, Ch. 17.
[19] K. Cejpek, J. Valusek, J. Velisek, in: Proceedings of Euro Food Chem IX, 24–26 September 1997, Interlaken.
[20] H. Matsunaga, T. Santa, T. Iada, T. Fukushima, H. Homma, K. Imai, Anal. Chem. 68 (1996) 2850.
[21] M. Puig-Deu, S. Buxaderas, J. Chromatogr. A 685 (1994) 21.
[22] B. Bjerg, O. Olsen, K.W. Rasmussen, H. Sørensen, J. Liq. Chromatogr. 7 (1984) 691.
[23] C. Bjergegaard, L.P. Hansen, P. Møller, H. Sørensen, S. Sørensen, J. Chromatogr. A 836 (1999) 137.
[24] L.K. Meier, O. Olsen, H. Sørensen, Phytochemistry 18 (1979) 1505.
[25] E.P. Kristensen, L.M. Larsen, O. Olsen, H. Sørensen, Acta Chem. Scand. B B34 (1980) 497.
[26] J. Barrett, F.S. Deghaidy, Spectrochim. Acta 31A (1975) 707.