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Analysis of dansyl amino acids in feedstuffs and skin by micellar electrokinetic capillary chromatography

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Abstract

Micellar electrokinetic capillary chromatography (MECC) using sodium dodecyl sulphate (SDS) and sodium cholate have been used for analyses of 30 dansylated (Dns) amino acids. The influences of sample preparation, Dns/amino acid ratio, sample solvent composition, and separation conditions including voltage, temperature, pH and buffer composition were investigated. Complete separations of acidic and neutral amino acids were obtained within 45 min in the SDS system. The efficiency expressed as number of theoretical plates for the applied capillary 0.52 m long were between 210 000 and 343 000, and the repeatability was very good with relative standard deviations on relative migration times between 0.09 and 0.70% and on relative normalised peak areas (RNPAs) between 0.85 and 3.41%. The linearity studies gave correlation coefficients between 0.9957 and 0.9993 for RNPAs against concentration. Detection limits were between 3 and 6 fmol or approximately 2 pg of each amino acid. Basic amino acids were separated in a MECC system using sodium cholate. Procedures and problems using Dns derivatisation for amino acids analysed by the MECC methods are described. Finally, examples of analyses of hydrolysates of real complex samples show, that this method can be applied to determine the amino acid composition of proteins in feedstuffs and skin.

1. Introduction

Efficient methods of amino acid analyses are required in connection with determining the nutritional value of proteins in food and feedstuffs including the special non-protein amino acids [1]. Furthermore, studies of the properties of connective tissues such as skin also requires suitable methods of analyses for the amino acids in especially the proteins collagen and elastin. The amino acid composition of such proteins includes Pro, 4-HyPro, 5-HyLys (Hy = hydroxy), desmosine and isodesmosine [2]. Analyses of secondary amines and imino acids like Pro and 4-HyPro demands special derivatisation methods [1,2]. Dansyl (Dns) derivatisation is well suited, as it works on secondary amino groups, and the obtained derivatives are very stable [1].

High-performance capillary electrophoresis (HPCE) methods have gained increasing interest due to their many advantages [3]. Many HPCE papers describing amino acid separations have

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been published. The methods published include the use of Dns-amino acid derivatives [4–8], phenylthiohydantoin (PTH) [9,10], naphthalene dicarboxaldehyde (NDA) [11,12], dabsyl [13,14], fluorescein isothiocyanate (FITC) [15–17], *o*-phthaldialdehyde (OPA) [17,18], fluorescamine [4,17], 9-fluorenylmethylchloroformate (FMOC) [17] and 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA) [19]. The HPCE methods described in these papers include free zone and micellar electrokinetic capillary chromatography (MECC) as well as inclusion chromatography for amino acid enantiomers. The MECC methods show the most promising results. However, most papers are only dealing with few amino acids and with purchased amino acid derivatives of various types. Hereby problems with interfering reagent compounds have not been considered, Furthermore, the performance including the qualitative aspects of the methods have not been reported. Finally, results from analyses of amino acids in hydrolysates of complex samples like feedstuffs and skin have not been reported either.

This paper describes the use of MECC with sodium dodecyl sulphate (SDS) and sodium cholate for analysing Dns-amino acids. The influences of sample preparation, Dns/amino acid ratio, sample solvent composition, and separation conditions including voltage, temperature, pH and buffer composition were investigated. Procedures and problems using Dns derivatisation for amino acids analysed by HPCE are reported. Determination of the performance including linearity, repeatability and response factors were performed. Finally, amino acid analyses of real complex samples are shown. The procedure described gives an efficient and reliable determination of amino acids in complex samples.

2. Experimental

2.1. Apparatus

An ABI Model 270 A-HT capillary electrophoresis system and a Model 600 data analysis system was used (Applied Biosystems, Foster City, CA, USA). The fused silica capillary was 720 mm × 50 μm I.D. (J & W Scientific, Folsom, CA, USA), and the detection point was 520 mm from the injection end of the capillary.

2.2. Materials and reagents

Free amino acids including *cis*-4-Hy-L-Pro (C4Hy-L-Pro), *cis*-4-Hy-D-Pro (C4Hy-D-Pro) and δ-Hy-Lys (HyLys) as well as the derivatives Dns-L-Asp (Asp), Dns-L-Glu (Glu), N-Dns-L-Ser (Ser), Dns-Gly (Gly), N-Dns-L-Thr (Thr), Dns-L-Ala (Ala), O-Dns-L-Tyr (Tyr), N,O-di-Dns-L-Tyr (DiTyr), Dns-L-Val (Val), Dns-L-Met (Met), Dns-L-Phe (Phe), Dns-L-Ile (Ile), Dns-L-Leu (Leu), Dns-L-Pro (Pro), N-Dns-*trans*-4-Hy-L-Pro (T4HyPro), N^{α}-Dns-L-Trp (Trp), N,N-di-Dns-L-cystine (Di2Cys), Dns-L-cysteic acid (cysteic acid), Dns-L-Norvaline (Nor), Dns-L-α-amino-*n*-butyric acid (α-ABA), Dns-γ-amino-*n*-butyric acid (γ-ABA), α-Dns-L-Arg (Arg), N^{ϵ}-Dns-L-Lys (Lys), di-Dns-L-Lys (DiLys), Dns-β-Ala (β-Ala), Dns-tryptamine (Trypt), Dns-cadavarine (Cad), di-Dns-cadavarine (DiCad), Dns-spermidine (Sper), di-Dns-1,4-diaminobutane (Putr) and di-Dns-histamine (Hisn) were obtained from Sigma (St. Louis, MO, USA). Desmosine and isodesmosine were obtained from Elastin Product Co. (Owensville, MO, USA).

2.3. Procedure

Buffers for HPCE separations were made in pure water, and buffers were filtered through a 0.2-μm filter prior to use. Dns-Amino acids were dissolved in methanol and diluted to 20% methanol with water. Samples were introduced from the anodic end of the capillary by 1-s vacuum injection. Separations were performed under various separation conditions as described in the text. On-column UV detection was at 216 nm unless otherwise stated. The capillary was washed for 2 min with 1 *M* NaOH, 2 min with water and 5 min with run buffer prior to each analysis. Data processing was performed with the data analysis system and according to Michaelsen *et al.* [20].

Proteins in the samples (10–30 mg) were hydrolysed in 6 *M* HCl (10 ml) at 120°C and for 20 h. The hydrolysate was dried with air, redissolved in water and dried again. The sample was then redissolved in 2.0 ml of water and the internal standards Nor (0.010 mmol/sample) and 3,4-dimethoxyphenylammonium chloride (0.005 mmol/sample) were added. For group separation of amino acids 0.5 ml of the redissolved sample was applied to two connected columns set up vertically on top of each other. From top to bottom the columns were: (A) CM-Sephadex C-25 (H^+; 1.0 ml slurry of water and column material, 1:1), (B) Dowex 50W-X8, 200–400 mesh (H^+; 1.0 ml slurry of water and column material, 1:1) [1]. The columns were washed with 9.5 ml water, separated and eluted with 20 ml 2 *M* acetic acid–methanol (1:1) for the A column and with 10 ml 1 *M* pyridine for the B column. The eluates were dried with air and redissolved in 0.5 ml water. The derivatisation was performed in the dark for 2 h with 50 μl sample added to 1.0 ml 25 m*M* Dns-Cl in acetonitrile and 1.0 ml 40 m*M* $LiCO_3$, pH 9.5. The reaction was then stopped by adding 100 μl 4% ethylamine, and the derivatised sample was dried with air. The derivatised sample was redissolved in 20% aqueous methanol, where most amino acid derivatives are dissolved but not all the products of the reagents. The samples were centrifuged at 2000 *g* for 2 min before analysis by HPCE.

3. Results and discussion

The separation mechanisms which are most likely to give acceptable separations of Dns-amino acids are exploitation of hydrophobic interaction of the amino acid side chain and Dns with surfactant monomers and micelles and differences in electrophoretic mobilities of the negatively charged Dns-derivatives at pH values above 4.5. The most promising results published are obtained by use of MECC with SDS in boric acid [8,21]. This MECC method was therefore tested for our purpose.

Dns-Amino acids and amines were dissolved in methanol (4 mg/ml) and diluted ($\times$86) to a final concentration in 20% aqueous methanol. Tyr could only be dissolved in a lower concentration of 2 mg/ml. Trypt, Cad, DiCad and γ-ABA precipitated when diluted to 20% aqueous methanol in the standards. Furthermore, Di2Cys, β-Ala and Putr could not be dissolved to acceptable concentrations in other solvents tested (acetonitrile, 1-propanol solutions, dimethyl sulphoxide, formamide, MECC buffers). All other 28 Dns-amino acids were dissolved and diluted without problems in methanol.

3.1. Separation conditions

The influence of separation conditions on the separations expressed as migration times (t_M), relative migration times (RMT), and the number of theoretical plates per meter of capillary (*N*/m) were investigated. The standard used contained 18 amino acids Asp, Glu, Ser, Gly, Thr, Ala, DiTyr, Val, Met, Phe, Ile, Leu, Pro, T4HyPro, cysteic acid, Trp, Asn and Gln. Not all results are shown here, but the evaluation and determination of the best separation conditions are based on all results. The most important parameters are mentioned here. The initial HPCE conditions were a buffer of 100 m*M* boric acid, 100 m*M* SDS, pH 8.3, a temperature of 27°C and a voltage of 15 kV. Asn and Ser were not completely separated under these conditions, which has also been reported by Miyashita and Terabe [21].

The migration orders changed for Glu, Asp, Val, Gly, Ala, Gln and cysteic acid with increasing SDS concentration from 100 to 180 m*M* (Fig. 1). Asn and Ser coeluted at concentrations between 100 and 140 m*M* as did Glu and Val at 120 m*M* SDS. At a concentration of 150 m*M* SDS all 18 amino acids could be separated, whereas at concentrations from 160 to 180 m*M* the amino acids Asp and Gly, Gly and cysteic acid as well as Glu and Gln were not completely separated. A concentration of 150 m*M* SDS was therefore chosen, as this also resulted in an acceptable migration time of 44 min for the latest appearing amino acid DiTyr. The observed ef-

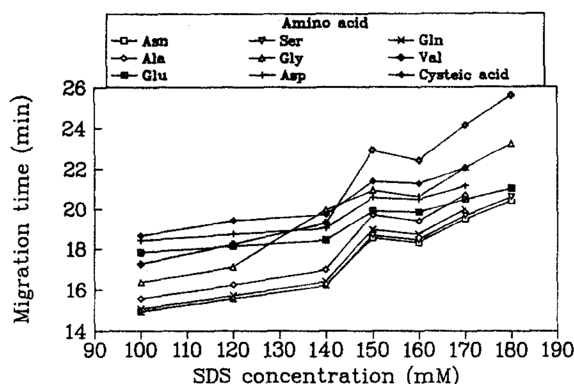

Fig. 1. Influence of SDS concentration on migration times of 9 amino acids. Conditions: 100 m*M* boric acid, pH 8.3, 27°C, 15 kV, 216 nm, vacuum injection 1 s.

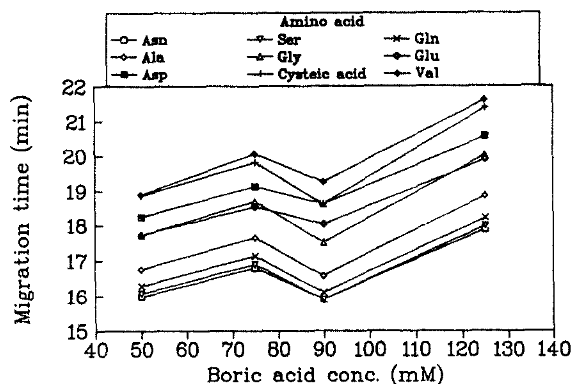

Fig. 2. Influence of boric acid concentration on migration times of 9 amino acids. Conditions: 150 m*M* SDS, other conditions as in Fig. 1.

fects of increasing SDS concentrations are probably a combination of changes in the shape and size of the micelles as well as the surface charge due to the presence of counter ions, increased ratio of the volume of the micellar phase to that of the aqueous phase and decreased electroosmotic flow, due to viscosity changes in the buffer [20]. The effects of SDS in concentrations from 30 to 50 m*M* have been thoroughly described by Ong *et al.* [8]. However, the number of amino acids included in their study was lower and did not include Asn, Gln, T4HyPro, Pro, Ala and Ile. The low concentrations of SDS are not sufficient to separate these amino acids.

Boric acid concentrations of 50, 75, 90, 100 and 125 m*M* gave poor separations of Asn and Ser except at 100 and 125 m*M*. Other amino acids did also coelute at various concentrations except at 100 m*M* (Fig. 2, data for 100 m*M* in Fig. 1). As a consequence hereof a concentration of 100 m*M* boric acid was chosen.

The separations obtained using pH values of 7.5, 8.0, 8.3, 8.5, 8.8, 9.0 and 9.3 were investigated. Increasing pH resulted in changed migration order of Asp, Gly and Glu. The best separations were obtained at pH 7.5 and 8.3. The other pH values of the buffer gave coelution of Gly and Glu or Asn and Ser. A buffer value of pH 8.3 was chosen. A general decrease in migration times with increasing pH was expected due to increased electroosmotic flow [20]. However, this was not seen, and changed properties of amino acids are probably the cause of that [8]. Ong *et al.* [8] also describes the effect of pH, but this is from pH 6.6 to 8.0.

Four voltages of 11, 13, 15 and 17 kV were tested. Good separations were obtained for all amino acids except for Asp and Gly at 17 kV. Furthermore, a small increase in *N* was observed when going from 11 to 15 kV. A voltage of 15 kV was chosen due to these results and because of a decrease in migration time of the latest appearing amino acid DiTyr from 61 to 43 min when going from 11 to 15 kV.

Temperatures of 22.4, 23.7, 25, 26, 27 and 29°C were also tested. The temperature was found to be very important for the separation of especially Thr, Asn and Ser; Ala and Glu as well as Asp and Gly (Fig. 3). The results clearly showed the best separation at the lowest temperature, and even such small changes in temperature of 1°C had a great effect (Fig. 3). Values of *N* were generally highest at the lowest temperatures. Values of *N* obtained with the applied capillary 0.52 m long were for Met, Phe and Trp determined to be 170 000, 210 000 and 190 000 at 22.4°C, 149 000, 168 000 and 165 000 at 25°C, 127 000, 147 000 and 145 000 at 27°C and 123 000, 133 000 and 130 000 at 29°C. Unfortunately, obtaining lower temperatures than 27°C is not possible with the applied ABI instrument, because there is no cooling possibility.

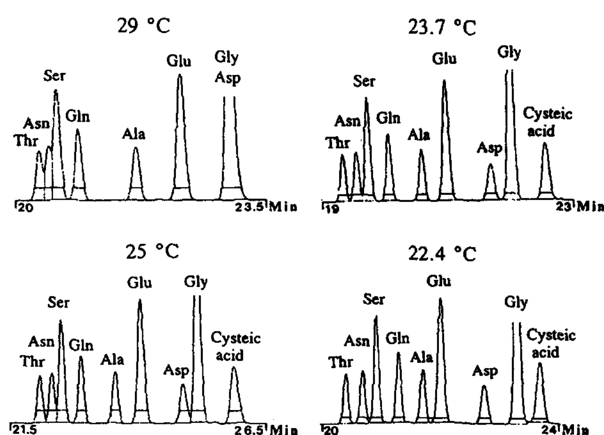

Fig. 3. Influence of temperature on the separation of 9 amino acids. Conditions: 150 m*M* SDS, 13 kV; other conditions as in Fig. 1.

The lowest temperature possible will therefore be determined by the ambient temperature. A temperature of 27°C had to be chosen.

Finally, various concentrations of 1-propanol and tetraethyl ammonium bromide (TEAB) were added to the buffer in order to obtain a higher selectivity of the method, especially for the first appearing amino acids. Addition of 1-propanol had a great influence on migration times, as especially the electroosmotic flow decreased with increasing 1-propanol concentrations. Moreover, changes in migration orders were seen. However, several amino acids co-eluted, and inclusion of 1-propanol can therefore not increase the selectivity. TEAB is supposed to form ion pairs with the amino acid carboxyl group and thereby change selectivity. Furthermore, TEAB may have some influence on the electroosmotic flow, due to interaction with the capillary wall. Addition of 1 to 40 m*M* TEAB to the buffer did also result in changes in migration times and relative migration times, but no general improvement in selectivity was found.

The best detection wavelength was chosen by testing 200, 216 and 245 nm in the optimised method. These values were selected from UV data of the Dns-amino acids. The amino acid peak areas at 216 nm were between 145 and 180% of the areas obtained at 200 nm except for the aromatic amino acids Phe, Trp and DiTyr, which were 0, 138 and 119%, respectively, of the areas at 200 nm. The peak areas at 245 nm were only between 50 and 65% of the areas at 200 nm, except again for the aromatic amino acids. The best sensitivity in UV detection of Dns-amino acids is therefore obtained at 216 nm. However, this wavelength has not been used in any of the previously published papers using UV detection.

The influence of the methanol concentration in Dns-amino acid samples on the separation efficiency was studied. A relatively high methanol concentration in the sample is necessary to dissolve the derivatised amino acids. However, high methanol concentrations in the sample reduced the separation efficiency dramatically (Fig. 4). This may be explained by reduced interaction with SDS or changed stacking conditions in the first minutes after voltage has been applied. The migration time for the tested amino acid Phe slightly decreased from 24.7 to 22.2 min, when the methanol concentration increased from 5 to 50%. This supports the explanation with reduced interaction with SDS. A concentration of 20 to 25% methanol can be used to obtain complete dissolution of Dns-amino acids and only loosing little in separation efficiency. We have used 20% methanol.

The separation obtained by using the optimised conditions on a sample containing 21

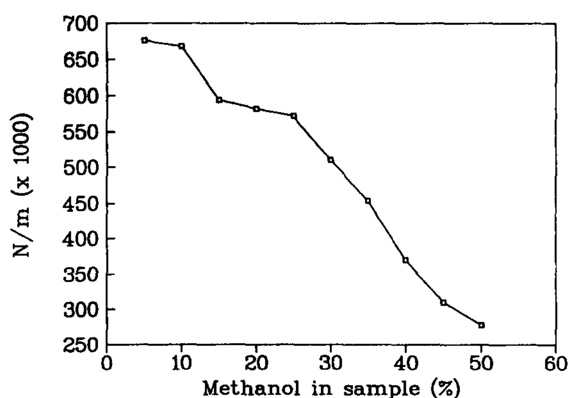

Fig. 4. Influence of the methanol concentration on the separation efficiency (*N*/m) for Phe. Conditions: 150 m*M* SDS, 25°C; other conditions as in Fig. 1.

amino acids is seen in Fig. 5. The migration times were between 17.3 and 44.5 min, and the separation efficiencies were between 403 000 N/m for cysteic acid and 660 000 N/m for Phe, which are considered satisfactory.

3.2. Migration order

The migration order and RMT values relative to mesityl oxide included as a neutral marker are shown for 30 amino acids in Table 1. Most of the amino acids can be separated by the HPCE method at the optimised conditions. However, Glu and α-ABA, Val and γ-ABA, as well as DiLys, Tyr, Trypt, HyLys and Cad are not separated well enough. Tyr and Lys with only one Dns group will not be present, when real samples are analysed, and a relatively large surplus of Dns-Cl is used. This surplus should be 10 times or more of Dns-Cl compared to amino acid [22].

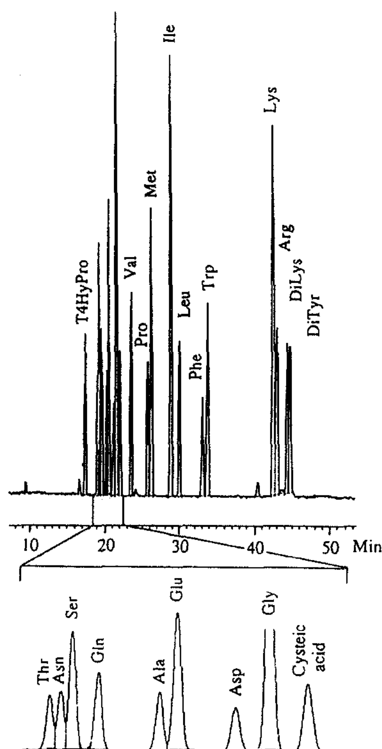

Fig. 5. Separation of 21 amino acids in the optimised method. Conditions as in Fig. 1 except 150 mM SDS.

Table 1
Migration order and RMT values (relative to mesityl oxide) for amino acids

Amino acid	RMT	Amino Acid	RMT
T4HyPro	0.824	Nor	1.206
Thr	0.900	Pro	1.215
Asn	0.909	Met	1.236
Ser	0.913	Ile	1.362
Gln	0.930	Leu	1.416
Ala	0.960	Phe	1.571
Glu	0.982	Trp	1.604
α-ABA	0.989	Lys	2.055
Mesityl oxide	1.000	Arg	2.076
Asp	1.015	DiLys	2.138
Gly	1.027	Tyr	2.145
Cysteic acid	1.055	Trypt	2.156
Val	1.104	DiHyLys	2.160
γ-ABA	1.105	Cad	2.176
C4Hy-D-Pro	1.142	DiTyr	2.191
C4Hy-L-Pro	1.144		

Conditions as in Fig. 5. except a temperature of 25°C.

The separation mechanisms involved are differences in electrophoretic mobility of the Dns-amino acids due to variations in the side chains, hydrophobic interaction of the Dns groups and SDS and the amino acid side chains and SDS in combination with ion repulsion of the negatively charged amino acid carboxyl groups and the negatively charged SDS. The electroosmotic flow will move the amino acids towards the cathode, and the interaction with SDS and the electrophoretic mobility of the amino acids will have a retarding effect on the compounds.

The migration order of *e.g.* Ala, Val and Leu as well as Asn and Gln illustrates the increased hydrophobic interaction with SDS, as the side chain becomes more hydrophobic. The electrophoretic mobilities of the amino acids with the longer side chains are lower, but the increased hydrophobicity has a larger effect. Nor shows a further increased interaction with SDS compared to Val due to the linear side chain of Nor. The appearance of Gly between Ala and Val is probably due to a higher electrophoretic mobility of Gly than of Ala in combination with the hydrophobic interaction. Glu and Asp appears quite early in the electropherograms even though

their electrophoretic mobility is high because of two negative charges. This is probably due to the hydrophilic character of the side chains in combination with increased ion repulsion with SDS. The migration order of α-ABA and γ-ABA is likely to be explained by the increased hydrophobic interaction of γ-ABA compared to α-ABA. This is due to less ion repulsion with SDS of the carboxyl group in γ-ABA than in α-ABA, caused by the position of the carboxyl group compared to the Dns group in the two compounds. T4HyPro migrates faster than C4Hy-L-Pro and C4Hy-D-Pro, and this is likely to be caused by facilitated hydrophobic interaction of HyPro and SDS, when the carboxy and the hydroxy groups of HyPro are on the same side of the cyclic ring of Pro as in the *cis* forms. Pro shows a further increased interaction with SDS, because it lacks the hydroxy group. Met, Leu and Ile have relatively non-polar side chains and appear therefore in the middle of the electropherograms. The aromatic amino acids Phe, Trp and Tyr will interact relatively strongly with SDS, and therefore these compounds appear late in the electropherogram. This is especially seen for DiTyr. Lys has a large and relatively non-polar side chain as well as a net zero charge which both results in strong interaction with SDS. DiLys shows a further increased interaction with SDS, and therefore appears after Lys. Ong *et al.* [8] also describe the migration order in MECC using SDS, but not all amino acids included in this study were included in their study. Moreover, lower concentrations of SDS were used.

3.3. Detection limits

Detection limits have been determined from a signal-to-noise ratio of 2 using dilutions of a sample containing 17 amino acids. The optimised separation conditions were used with vacuum injection for 1 s. The detection limits were found to be between 0.7 and 1.3 μM, except for the aromatic amino acids Trp at 0.5 μM and DiTyr at 0.1 μM. The injected volume is approximately 4.5 nl with the applied temperature of 27°C and assuming a viscosity in the buffer and the sample identical to water [23]. The detection limits are therefore between 3 and 6 fmol or approximately 2 pg of each amino acid. Use of instruments with fluorescence detection will reduce detection limits considerably. The possibility of analysing samples having a lower sample concentration than 0.7 to 1.3 μM by increasing the capillary diameter was investigated. Increasing the diameter increases the injected sample volume and the length of the light pathway in the capillary, and thereby increased peak areas are obtained. Increasing the diameter from 50 to 75 μm resulted in increased normalised peak areas (NPAs) of approximately a factor 5.5 and decreased migration times of a factor 0.7 for Ala and Phe. However, the separation efficiency expressed as N/m decreased a factor 3.4. Increased capillary diameter can therefore only be recommended, when the obtained separation efficiency is not critical.

3.4. Repeatability and linearity

The repeatabilities of the method expressed as relative standard deviation (R.S.D.) of RMT, NPA and relative NPA (RNPA) values are shown in Table 2. R.S.D.s of RMT values were very low lying between 0.09 and 0.70%. This is in the same level as reported for other MECC methods [20,24]. R.S.D.s of NPA values were quite high especially for Glu, Asp and DiTyr. Integration of DiTyr is difficult, and this results in the high R.S.D. Problems with Glu and Asp will be explained elsewhere. Finally, R.S.D.s of RNPA values were much improved compared to the NPA values lying between 0.85 and 3.41% except for Glu, Asp and DiTyr. This improvement compared to NPA values is caused by elimination of variations in injection volumes. Altogether, both qualitative and quantitative analyses can be performed satisfactory in regard to repeatability.

The linearity of the method was determined by using a standard containing 14 amino acids at 6 concentration levels and with 5 repetitions. Linear regression analyses of NPA values against concentrations gave correlation coefficients between 0.9824 and 0.9912 except for DiTyr at

Table 2
Repeatability as relative standard deviation of RMT, NPA and RNPA values for amino acids

Amino acid	R.S.D. (%)		
	RMT	NPA	RNPA
T4HyPro	0.29	6.36	2.32
Thr	0.22	7.26	2.31
Asn	0.24	6.99	2.89
Ser	0.24	6.47	3.41
Gln	0.23	6.48	2.32
Ala	0.16	6.25	1.70
Glu	0.26	8.47	5.85
Asp	0.24	7.53	4.37
Gly	0.14	6.03	1.95
Val[a]		5.62	
Pro	0.09	5.97	0.85
Met	0.12	5.64	1.21
Ile	0.19	6.12	1.22
Leu	0.23	5.93	1.42
Phe	0.31	6.16	1.70
Trp	0.32	5.78	1.71
DiTyr	0.70	14.45	9.92

Conditions as in Fig. 5, 14 repetitions.
[a] Other amino acids relative to Val.

0.9222. Correlation coefficients between 0.9957 and 0.9993 were obtained by using RNPA values. Hereby variations in injection volumes are eliminated, and these variations have nothing to do with linearity. The most correct evaluation of linearity is therefore based on RNPA values, and the linearity of this method is good.

3.5. Relative response factors

Relative response factors (RRF) for 15 amino acids were calculated (Table 3). The RRF values were calculated from response factors (RF) as $RRF = RF_x/RF_{Leu}$, where RF = NPA/concentration in sample. The RRF values were from 0.6881 to 1.5466 and depended strongly on the amino acid, although most of the amino acids have one identical Dns group. However, the amino acids Tyr and Trp have a considerable UV absorption at this wavelength. This accounts for the higher RRF values of these two amino acids [2]. R.S.D.s of RRF values were between 1.8 and 4.8%, with the highest value for Glu. These results show that either complete standards must be included in each series of analyses, or the RRF values for all amino acids under the actual conditions must be determined.

3.6. Derivatisation and examples of analyses

Samples of skin from mink and various feedstuffs were hydrolysed and derivatised (see Experimental). The necessary amount of ethylamine to stop the reaction was determined in a series of derivatisations. Unfortunately, HPCE analyses directly of the reaction mixture after derivatisation gave very bad results. This is due to the high amount of acetonitrile in the sample. Dissolution in methanol–water gives much better results. The amount of 20% methanol in water to dissolve the derivatised amino acids was also

Table 3
Relative response factors (RRF) for amino acids

Amino acid	RRF	R.S.D. (%)	Amino acid	RRF	R.S.D. (%)
T4HyPro	0.730	3.1	Pro	0.743	2.0
Thr	0.834	2.6	Met	0.688	4.6
Asn	0.762	2.2	Ile	1.093	2.6
Ser	1.007	1.9	Leu	1	
Gln	0.779	2.5	Phe	0.784	4.5
Ala	0.801	3.0	Trp	1.547	1.8
Glu	0.911	4.8	DiTyr	1.409	15.9
Val	0.970	1.9			

Conditions as in Fig. 5, 6 concentration levels and 5 repetitions, RRF values calculated relative to Leu.

determined in a series of experiments. The volume had to be at least 800 μl at the applied conditions of derivatisation (see Experimental). A residue is present in all samples, but the residue does not contain amino acids, as the amino acid peak areas do not increase further by increasing the volume of methanol–water. An example of an analysis of acidic and neutral amino acids in a hydrolysed skin sample is shown in Fig. 6. At least three reagent peaks were seen in all derivatised samples. However, amino acids could be separated by the applied method. The amino acids can be quantified in the samples by using RRF values and an internal standard. Nor was used here as internal standard, but Nor and Pro were not completely separated, and therefore another internal standard may be used. Cys can also be determined by the method after oxidation to cysteic acid (Fig. 5). Analyses of acidic and neutral amino acids in samples of feedstuffs as well as the samples of skin have been performed and works well. Furthermore, analyses of desmosine and isodesmosine, which are two special amino acids present only in elastin and responsible for the special properties of elastin, have been tested in the SDS system. Desmosine and isodesmosine consists each of one lysine and three allysine (lysine with an aldehyde group in the sidechain instead of an amino group) units forming a pyridine ring. It was impossible to dissolve sufficient amounts of the Dns derivatives of these amino acids in various solvents, probably due to the presence of at least four Dns groups on each amino acid. However, analysing the underivatised amino acids was possible in the SDS system (Fig. 7). Desmosine has a molecular extinction coefficient of 4750 M^{-1} cm^{-1} at 234 nm and of 3200 M^{-1} cm^{-1} at 268 nm and isodesmosine has a molecular extinction coefficient of 6450 M^{-1} cm^{-1} at 278 nm and a relatively small absorbance at 234 nm. These properties can be used to identify the amino acids, as seen in Fig. 7.

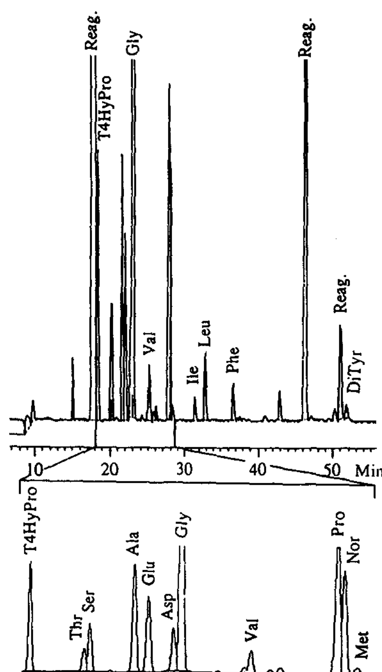

Fig. 6. Separation of Dns-derivatised acidic and neutral amino acids in a hydrolysate of skin from mink. Conditions as in Fig. 1 except 150 mM SDS.

Derivatisation of different amounts of the same sample did not give the expected peak areas for Glu and Asp after HPCE analysis. Furthermore, the larger R.S.D.s of both NPA and RNPA values seen (Table 2) suggests problems in quantifying these amino acids after Dns

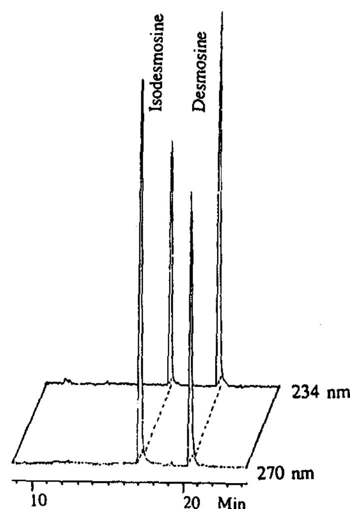

Fig. 7. Separation of underivatised desmosine and isodesmosine in the SDS system. Conditions as in Fig. 1 except 150 mM SDS and the detection wavelength.

Table 4
Peak areas in percent of expected peak areas at decreasing Dns/amino acid ratios in the derivatisation

Dns/amino acid ratio	Area in percent of expected				
	50	20	10	5	2.5
Asp	100	91	86	75	56
Glu	100	94	87	82	70
Ala	100	84	86	90	81
Leu	100	88	99	120	134

Conditons as in Fig. 1 except 150 m*M* SDS. Expected peak areas calculated from the peak areas obtained at the highest Dns/amino acid ratio.

derivatisation. A series of derivatisations with decreasing Dns/amino acid ratio was performed with Asp and Glu, and the results were compared to results obtained with Ala and Leu (Table 4). The expected peak area was calculated from the peak area obtained at the highest Dns/amino acid ratio. The amount of amino acid to be derivatised was increased and the Dns-Cl amount was held constant. The peak areas were much lower than expected for Asp and Glu at low Dns/amino acid ratios, and this was not seen for Ala and Leu. A possible explanation could be the ability of the carboxyl group of especially Glu to form a peptide bond with the amino group of Glu as in pyroglutamic acid, and thereby release Dns. The presence of pyroglutamic acid in the sample prior to derivatisation cannot give these results. However, a lower stability of Dns derivatives of Asp and Glu may be explained by the release of Dns. Furthermore, an additional peak was seen in increasing amounts appearing after Asp and Glu when the Dns/amino acid ratio decreased. This could be the transformation product.

These findings of a dependence of amino acid peak areas on the Dns/amino acid ratio are in contradiction with the results described by Tapuhi *et al.* [22]. They stated that such problems were not seen, when derivatisations with corresponding reaction mixtures were performed. However, using other derivatisation conditions Seiler [25] and Neadle and Pollitt [26] reported a dependence of the yield of the derivatisation on the relative amount of Dns-Cl present. The influence of Dns/amino acid ratio on peak areas of Asp and Glu can be minimised by using high Dns/amino acid ratios and by adjusting the amino acid concentrations in samples to a well defined level.

3.7. Cholate system

Other MECC systems than SDS were investigated due to the fact, that reaction products from the Dns derivatisation interfered with the basic amino acids Lys, DiLys, HyLys and Arg as well as Tyr (Fig. 5 and 6). Furthermore, these amino acids had long migration times. The best results were obtained in buffers containing 50 m*M* disodium hydrogenphosphate and 50 or 80 m*M* sodium cholate at pH 8.0 and analysed at 30°C and 20 kV (Fig. 8). The efficiency was between 228 000 and 428 000 *N*/m for Arg, Lys, Tyr and DiLys at these conditions. Furthermore, HyLys (mixed DL and DL *allo* form) gave two well separated peaks appearing between Tyr and DiLys (Fig. 8B). In electropherograms of hydrolysed protein, with the hydrolysate group separated to obtain the basic amino acids and de-

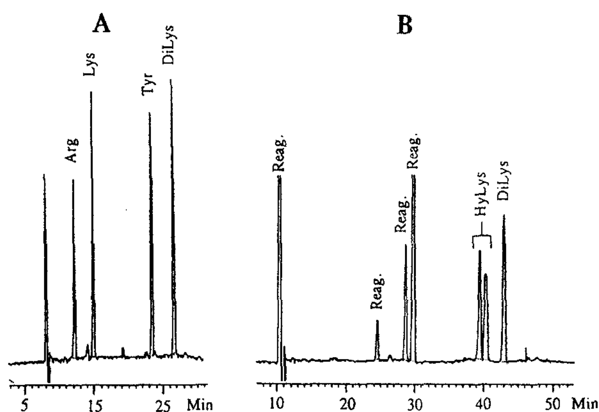

Fig. 8. Separation of amino acids in the cholate system. (A) Basic amino acids and Tyr, 50 m*M* disodium hydrogenphosphate, 50 m*M* sodium cholate, pH 8.0, 30°C, 20 kV, 216 nm, 1 s vacuum injection. (B) DiLys and HyLys forms (derivatised mixed DL and DL *allo* forms), conditions as in (A) except 80 m*M* sodium cholate, 27°C and 18 kV.

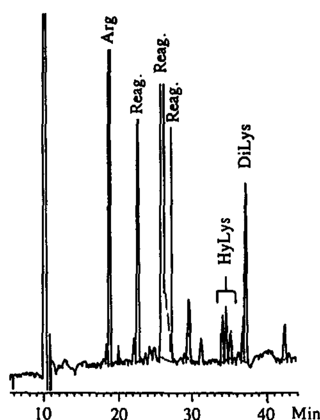

Fig. 9. Separation of derivatised basic amino acids in a hydrolysate of skin from mink. Conditions as in Fig. 8B.

rivatisation of the amino acids these basic amino acids could be seen and their identity tested by spiking (Fig. 9). Further optimisation of parameters and determination of the performance of this method have to be performed, but it seems promising for analyses of the basic amino acids. Apart from Terabe *et al.* [7], who used taurodeoxycholate for chiral separation of six amino acids, no other reports on MECC with cholate for Dns-amino acids have been reported. Analyses of acidic and neutral amino acids were also tested in the cholate system, but many amino acids coeluted. The developed method with SDS is recommended for analyses of these amino acids.

4. Conclusions

The described MECC methods with SDS and cholate can be used to analyse amino acids released from proteins in feedstuffs and skin. The derivatisation step with Dns-Cl is relatively simple and fast. Most amino acids can be dissolved in 20% methanol in water. The optimised SDS method gives complete separations of acidic and neutral amino acids within 45 min, and the efficiency is high with up to 660 000 N/m. Furthermore, the repeatability and linearity are good. The detection limits are in the low femtomole range. Finally, basic amino acids can be separated by the cholate method.

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