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Determination of heterocyclic compounds by micellar electrokinetic capillary chromatography

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Abstract

Micellar electrokinetic capillary chromatography (MECC) based on sodium cholate (NaCh) and sodium dodecyl sulphate (SDS) was developed for the determination of aromatic amino acids and heterocyclic legume constituents. The influence of temperature, voltage, micellar system, pH, zwitterion and modifier concentrations in the buffer on migration times, peak areas, resolution and number of theoretical plates was investigated. This MECC method makes possible the sensitive determination of the individual compounds with detection limits in the picomole range. Up to 300 000 theoretical plates per metre of capillary were obtained together with satisfactory linearity and repeatability of the NaCh method. The applicability of MECC to samples prepared from plant material, following a fast and simple technique of isolation, purification and group separation, is illustrated by selected examples.

1. Introduction

Non-protein amino acids with a side-chain containing an aromatic or a heterocyclic ring and heterocyclic compounds without an amino acid group occur in various plants including leguminoses [1–3]. These heterocyclic compounds are structurally different from the purine and pyrimidine bases in nucleic acids. They often have appreciable physiological effects, e.g., favism and influence on growth and egg production caused by the pyrimidines vicine and convicine in *Vicia* [4–6], and effects from isoxazolines in *Lathyrus* and *Pisum* in relation to lathyrism [3,7]. In various legumes, other pyrimidine derivatives occur such as willardine, isowillardine and lathyrine in addition to the pyridine derivatives trigonelline, trigonelline amide and mimosine, which may have coenzyme inhibitory effects [1,4,8]. Moreover, they are important in relation to the natural resistance of plants against pest problems [9,10]. Methods for the determination of the individual heterocyclic compounds are needed for efficient studies of the above problems. Until now, the method of choice has been high-performance liquid chromatography (HPLC) [2,6,11], but high-performance capillary electrophoresis (HPCE) has been shown to display some advantages over HPLC [12,13], making this technique a promising alternative.

Micellar electrokinetic capillary chromatography (MECC), using sodium cholate (NaCh) as the detergent, has recently proved its applicability for the determination of positively charged low-molecular-mass compounds [14]. The purpose of this study was to use this as a basis for developing a MECC method suitable for the separation of the above-mentioned amino acids

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and heterocyclic compounds. The detergents sodium dodecyl sulphate (SDS) and alkyltrimethylammonium bromides (XTAB) with different chain lengths were tested as possible alternatives to NaCh. Two test solutions, comprising selected compounds occurring in *Pisum sativum* L. (test mixture P) and *Vicia faba* L. (test mixture V), were used for the evaluation of the influence of different separation conditions on migration times, peak areas and separation efficiency. Comparison of MECC based on NaCh, SDS and XTAB showed that the best separation for the compounds considered was obtained when NaCh or SDS was used as the detergent. With the combined technique of group separation and MECC now developed, a rapid, sensitive and efficient method for the determination of aromatic amino acids and heterocyclic compounds occurring in legumes is obtained, as illustrated for extracts from *Pisum sativum* L.

2. Experimental

2.1. Apparatus

The apparatus used was an ABI Model 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) with a 760 mm × 0.05 mm I.D. fused-silica capillary tube. Detection was performed by on-column measurements of UV absorption (260 nm) at a position 445 mm from the injection end of the capillary. For data processing, a Shimadzu (Kyoto, Japan) Chromatopac C-R3A integrator was used.

2.2. Samples and reagents

The heterocyclic compounds studied were isolated in our laboratory according to a method described elsewhere [2] or obtained from Sigma (St. Louis, MO, USA). The numbers, names and structures of the heterocyclic compounds in test mixtures P and V are given in Table 1 and Fig. 1. The concentrations of the individual heterocyclic compounds in the test mixtures ranged from $2 \cdot 10^{-3}$ to $3 \cdot 10^{-2}$ *M*.

Disodium hydrogenphosphate, disodium tetraborate, taurine, NaCh, SDS and alkyltrimethylammonium bromides (XTABs: C-12, C-14, C-16) were obtained from Sigma and 1-propanol from Merck (Darmstadt, Germany).

2.3. Procedure

The different separation buffers tested in the NaCh system were prepared with variations in concentration of 1-propanol (0–20%), taurine (0–600 m*M*) and NaCh (15–50 m*M*). The applied voltage and temperature ranged from 10 to 25 kV and from 27 to 60°C, respectively. The electrolyte concentration at 100 m*M* Na_2HPO_4 remained fixed, and the pH of the buffer was 7.3, except when testing the pH dependence (pH 7.3–8.5).

The separation buffers in the SDS and XTAB systems were prepared with 50 m*M* detergent, 30

Table 1
Names and numbering of heterocyclic compounds in test mixtures V and P used in MECC analyses

Test mixture V		Test mixture P	
No.	Name	No.	Name
1	Trigonelline amide	**1**	Trigonelline amide
2	Trigonelline	**2**	Trigonelline
3	Vicine	**8**	Tryptophan
4	Tyrosine	**4**	Tyrosine
5	Dopa-glucoside	**9**	Willardine
6	Dopa	**10**	Isoxazoline
7	Convicine		[3-(Isoxazolin-5-one-2-yl)alanine]

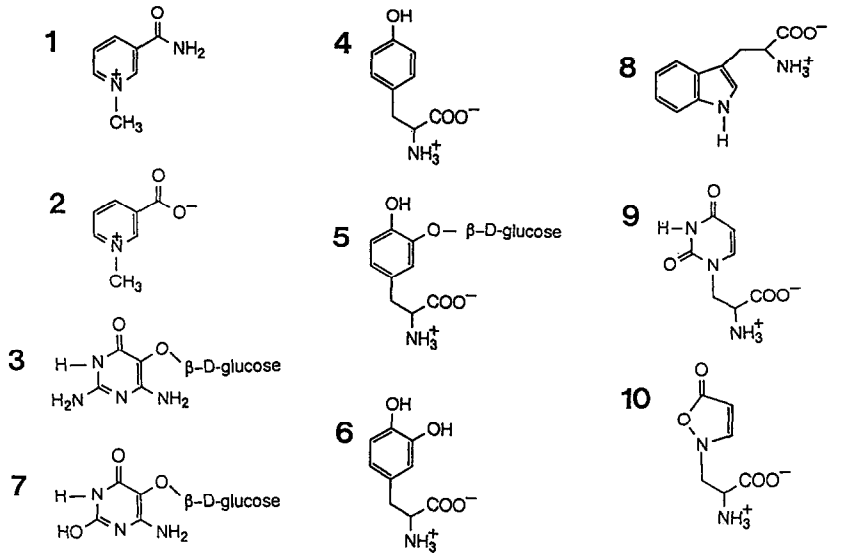

Fig. 1. Structures of some aromatic amino acids and heterocyclic compounds naturally occurring in *Pisum sativum* L. and *Vicia faba* L. and selected for the test mixtures in this study. The numbers refer to Table 1.

m*M* disodium hydrogenphosphate, 18 m*M* disodium tetraborate and 5% 1-propanol. The pH was adjusted to 7.0 in all systems.

Common to the different systems was filtration of buffers through a 0.45-μm membrane filter prior to use. Washing of the capillary was performed with 1.0 *M* NaOH for 2 min and with buffer for 5 min before each analysis. When the buffer composition was changed, the washing procedure was extended to 15 min with the new buffer. In the optimization study, the buffer was changed manually after about ten analyses, whereas the linearity and repeatability studies included a buffer change after each analysis. In the NaCh and SDS systems, the samples were introduced from the positive end of the capillary and in the XTAB system from the negative end by vacuum for 1 s. Unless stated otherwise, the separations were performed at 50°C and 20 kV (NaCh) or 40°C and 18 kV (SDS and XTAB)

Calculations of relative migration times (*RMT*), normalized peak areas (*NA*), number of theoretical plates per metre of capillary (*N*/m) and resolution (R_s) were performed as described previously [15]. Repeatabilities were estimated from the means and relative standard deviations (R.S.D.). The linearity of the NaCh method was determined from linear regression analysis based on least-squares estimates. The detection limits for the compounds investigated were determined from the linearity analysis and a signal-to-noise ratio of 2:1.

3. Results and discussion

3.1. Migration order

The selective retention of analytes in a MECC system is obtained due to differential partitioning between the aqueous buffer and the micellar phase. The interactions of analytes with the micellar phase consist of both hydrophobic and ionic interactions, the extent depending on the structure and possible charge of the sample molecules. The aromatic amino acids and heterocyclic compounds included in this study have a zero net charge at pH values of about 4–8, except for trigonelline amide (**1**), which is positively charged. Owing to the electrophoretic mobility towards the cathode, trigonelline amide (**1**) is thus the fastest migrating analyte in MECC systems with negative detergents (NaCh, SDS), whereas the migration order for the other com-

pounds depends on the detergent used. For NaCh, the migration order was **1**, **2**, **8**, **4**, **9**, **10** for test mixture P and **1**, **2**, **3**, **4**, **5**, **6**, **7** for test mixture V, whereas use of SDS changed the migration order to **1**, **2**, **4**, **9**, **10**, **8** for test mixture P and **1**, **2**, **3**, **5**, **4**, **7**, **6** for test mixture V (Fig. 2).

In test mixture P, tryptophan (**8**), with the indolyl structure, apparently interacts more strongly with the SDS micelles than with the NaCh micelles, shifting it from a position next to tyrosine in the middle of the electropherogram in the NaCh system to a position as the last-eluted compound in the SDS system. Different types of indolylic compounds including **8** can be efficiently separated in XTAB systems [16]. The other compounds in test mixture P were eluted in identical order in the NaCh and SDS systems. *MT* varied considerably, however, especially for the last-eluted analytes. As described previously [14,17,18], NaCh and SDS result in micelles of different structure, SDS micelles having the charged portions pointing outward whereas NaCh micelles are hydrophobic at the surface, hiding the polar part in the centre of the micelle. The generally slower migration of analytes in the NaCh system compared with the SDS system, using a lower detergent concentration, higher voltage and higher temperature, indicates a stronger interaction of the analytes with NaCh micelles than with the SDS micelles. The hydrophobic properties of the aromatic structures of the analytes can give a likely explanation for this. The critical micellar concentrations (CMC) for SDS and NaCh are 8.2 and 13.0 m*M*, respectively [19].

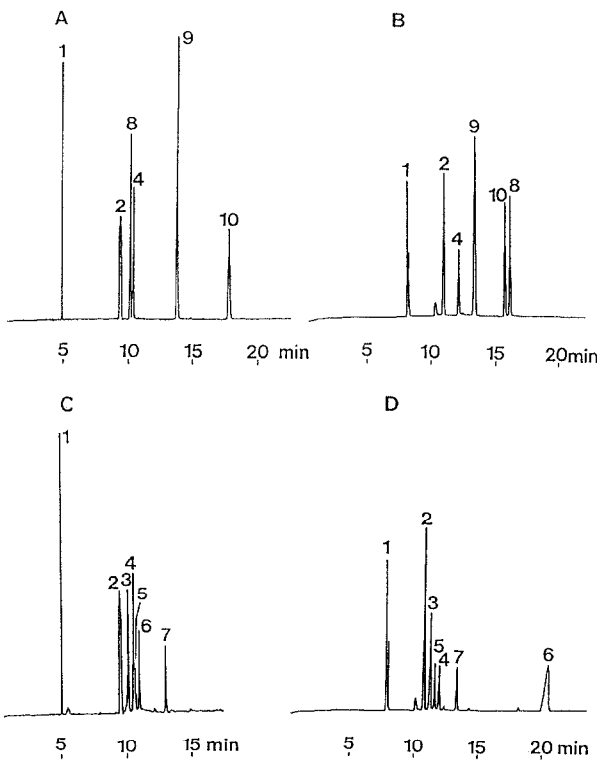

Fig. 2. Separation of aromatic amino acids and heterocyclic compounds by MECC using test mixture P [(A) NaCh, (B) SDS] and test mixture V [(C) NaCh, (D) SDS]. The conditions used for the NaCh system [temperature, 50°C; voltage, 20 kV; buffer, 100 m*M* Na_2HPO_4–15 m*M* NaCh–10% 1-propanol (pH 7.3)] were established from systematic changes of various separation parameters. The conditions used for the SDS system are given under Experimental. Detection in both systems, UV at 260 nm, 64 mV full-scale.

In test mixture V, a change of detergent type from NaCh to SDS affected the migration order of the analytes much more than with test mixture P (see above). Dopa-glucoside (**5**) changed to a position in front of tyrosine (**4**), whereas convicine (**7**) was eluted before dopa (**6**). This shift in migration order shows that the glucosylation leading to high hydrophilicity is of greater importance in the SDS system than in the NaCh system. For example, the difference in *MT* for dopa (**6**) and dopa-glucoside (**5**) is less than 1 min in the NaCh system, whereas the corresponding interval is about 8 min in the SDS system. It should be noted that the larger difference in the SDS system is a result of faster elution of dopa-glucoside (**5**) and the slower elution of dopa (**6**).

An advantage of the SDS over the NaCh system is that the peak of trigonelline (**2**) is moved away from the solvent front. The split peak observed for trigonelline amide (**1**) is, however, a problem that needs to be overcome. Otherwise, an alternative internal standard should preferably be chosen.

The XTAB systems failed to give acceptable separations of the heterocyclic compounds in the test mixtures. XTAB form positively charged

micelles with a structure similar to that of the SDS micelles, with the charges on the surfaces of the micelles.

3.2. Separation parameters

The influence of changed separation conditions on *MT*, *RMT*, *NA* and separation efficiency (N/m and R_s) were evaluated for test mixtures V and P in the NaCh system. No marked differences were observed for the two test mixtures, and the results will be commented upon without distinguishing one from the other.

Variation of the temperature (30, 40, 50 and 60°C) resulted in a non-linear decrease in *MT* (Fig. 3), the decrease being less pronounced at high temperature, as also found previously for flavonoids and aromatic choline esters [14,20]. The same pattern was observed on increasing the voltage (10, 15, 18, 20 and 25 kV), indicating a temperature-related effect on the partitioning of analytes between micelles and the aqueous buffer phase. The decrease in *MT* with increasing temperature results from a decreased viscosity and a change in the diffusion double layer at the inner capillary wall with increased zeta potential as a result [13]. As a compromise, a temperature of 50°C was found to be suitable for the separation of the compounds occurring in *Vicia* and *Pisum*.

Changes of pH (7.3, 8.0 and 8.5) had, as expected, a limited effect on the migration of analytes, as the tested range was relatively narrow. pH values below 7.0 lead to precipitation of NaCh owing to its pK_a value of about 6.4, and such pH values should therefore be avoided in the NaCh system [14]. More alkaline pH values are not recommended owing to the instability of dopa (**6**) and some heteroaromatics under these conditions. Therefore, pH 7.3 was adopted for further experiments. Possibly, a change of electrolyte from phosphate to borate would affect the migration of glucosylated analytes [e.g., vicine (**3**), convicine (**7**) and dopa-glucoside (**5**)] at pH values above 8, owing to complex formation between borate and the sugar part, as demonstrated by the free zone capillary electrophoresis of oligosaccharides [21,22]. The effect of cholate, taurine and 1-propanol concentrations on *MT* was as described previously for the NaCh system [14]. The *RMT* remained relatively constant, although exceptions occurred. A constant *RMT* is crucial for the correct identification of analytes.

Variations in *NA* under different separation conditions were a general observation, and could not be explained by evaporation of the solvent, as illustrated in Fig. 4.

The change in *NA* is probably due to an altered interaction of the analytes with the micellar phase, the effect depending on the actual

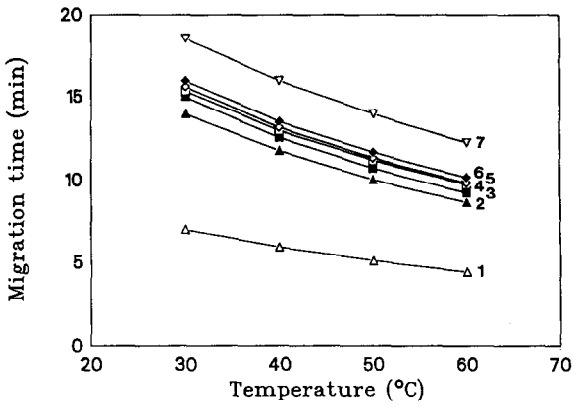

Fig. 3. Example of change in *MT* for test mixture V with variation of temperature in the NaCh system. Numbers as in Table 1. Separation conditions as in Fig. 2.

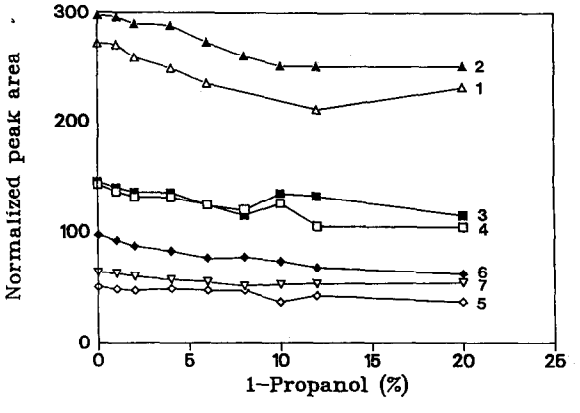

Fig. 4. Example of change in *NA* for test mixture V with variation of concentration of 1-propanol in the NaCh system. Numbers as in Table 1. Separation conditions as in Fig. 2, except for temperature (30°C).

separation conditions. Quantitative determination will require a knowledge of, among other things, the molar absorptivities under the actual separation parameters. The observed tendency of *NA* to decrease as a function of the concentration of 1-propanol in the electrolyte (Fig. 4) was probably due to the higher viscosity of 1-propanol compared with water. As the concentration of 1-propanol increased, the viscosity increased and the peak area decreased.

Changes in the various separation parameters had little influence on R_s, whereas N/m varied considerably. The separation efficiency for the SDS system and the optimized NaCh system was in general high, as shown in Table 2.

The low N/m for trigonelline (**2**) in the NaCh system is caused by the migration close to the solvent front, which leads to interference from the solvent peak (Fig. 2). Using SDS, N/m for trigonelline (**2**) was raised about 2.5 times, whereas N/m for the other compounds in the test mixtures in total was higher in the NaCh than the SDS system. The separations of tyrosine (**4**) and dopa-glucoside (**5**), which are relatively poor in the NaCh system ($R_s < 1$), were considerably improved in the SDS system ($R_s = 2.2$). The R_s values for the other compounds in the test mixtures were generally higher in the NaCh system, although the level was also satisfactory using SDS as the detergent. It is possible that a systematic change of the separation parameters in the SDS system, as performed for NaCh, may result in conditions that improve the separation capacity of this system further.

3.3. Linearity and repeatability

Good linearity and repeatability are essential for quantitative analysis. Correlation coefficients (r^2) and slopes from linear regression analysis by the least-squares method for *NA* and various concentrations of heterocyclic compounds determined in the NaCh system are given in Table 3. The approximate detection limits, corresponding to the optimized conditions applied as described in the Experimental section and in Fig. 2, are also presented. The injection volume was calculated to be 4.28 nl, assuming a viscosity in the buffer and sample identical with that of water.

In general, the repeatability of *MT*, *RMT*, *NA*

Table 2
Separation efficiency (N/m and R_s) for heterocyclic compounds analyzed by MECC (NaCh and SDS)

Test mixture	Detergent	Parameter	Value						
V	NaCh	No.	**1–2**	**2–3**	**3–4**	**4–5**	**5–6**	**6–7**	
		R_s	30.9	3.5	3.6	0.8	2.4	15.5	
		No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
		N/m	255 000	53 000	212 000	179 000	220 000	195 000	286 000
	SDS	No.	**1–2**	**2–3**	**3–5**	**5–4**	**4–7**	**7–6**	
		R_s	18.6	2.6	1.9	2.2	6.9	19.0	
		No.	**1**	**2**	**3**	**5**	**4**	**7**	**6**
		N/m	166 000	139 000	137 000	212 000	187 000	191 000	51 000
P	NaCh	No.	**1–2**	**2–8**	**8–4**	**4–9**	**9–10**		
		R_s	29.4	4.6	2.1	25.2	20.4		
		No.	**1**	**2**	**8**	**4**	**9**	**10**	
		N/m	238 000	47 000	220 000	214 000	202 000	139 000	
	SDS	No.	**1–2**	**2–8**	**8–4**	**4–9**	**9–10**		
		R_s	19.5	6.2	5.6	10.4	2.3		
		No.	**1**	**2**	**8**	**4**	**9**	**10**	
		N/m	184 000	123 000	155 000	98 000	208 000	296 000	

Numbers as in Table 1. Separation conditions as in Fig. 2.

Table 3
Results from linearity studies of heterocyclic compounds analysed by MECC (NaCh)

Test mixture	No.	Regression coefficient (r^2)	Slope	Concentration range (m*M*)	Detection limit (pmol)
V	**1**	0.9989	141.85	0.19–3.79	0.81
	3	0.9989	76.52	0.17–3.29	1.41
	4	0.9906	32.91	1.38–27.6	23.6
	5	0.9971	25.50	0.70–13.9	11.9
	6	0.9998	42.94	1.27–25.4	10.9
	7	0.9957	32.67	0.08–1.64	0.70
P	**1**	0.9981	163.17	0.19–3.79	0.81
	8	0.9930	132.83	0.25–4.90	1.05
	4	0.9943	66.22	1.38–27.6	5.91
	9	0.9974	180.53	0.25–5.02	1.07
	10	0.9883	60.27	0.29–5.81	24.9

Numbers as in Table 1. Separation conditions as in Fig. 2.

and *RNA* ($n = 10$) was satisfactory. For test mixture P, the R.S.D.s were in the ranges 0.82–4.15% (*MT*), 0.88–3.67% (*RMT*), 5.18–9.66% (*NA*) and 2.02–4.66% (*RNA*), and for test mixture V, R.S.D.s were 1.98–2.21% (*MT*), 0.19–0.24% (*RMT*), 3.21–9.22% (*NA*) and 1.07–6.09% (*RNA*). As expected, the use of relative values (*RMT*, *RNA*) resulted in lower R.S.D.s, but some improvements are needed, especially for the reproducibility of peak areas.

3.4. MECC of samples from plant material

Examples of analyses of naturally occurring mixtures of aromatic amino acids and heterocyclic compounds, isolated and purified by group separation of plant extracts, are shown in Fig. 5. These results also illustrate the change in analyte concentration during germination of seeds from *Pisum sativum* L. The ion-exchange technique used for the purification [2] yield a sample of neutral compounds, excluding trigonelline amide from the mixture. The evaporated eluate dissolved in water was used directly for the MECC analyses with NaCh as the detergent.

The content of heterocyclic compounds in the seed extract changed during germination, with trigonelline (**2**) dominating in the 2-day samples, while isoxazoline (**10**) occurred in highest amounts after 8 days of germination. The isoxazoline peak probably cover two or three structural closely related compounds, as splitting of the peak was observed with certain separation parameters. The protein amino acids tryptophan (**8**) and tyrosine (**4**) occurred in low concentration. A possible candidate for the unidentified peak marked X is 4-chlorotryptophan, which has been demonstrated previously to be a constituent of peas [23], but final identification needs further investigation.

The composition of the compounds considered in roots from germinated seeds was investigated, using both NaCh and SDS as detergents (Fig. 6).

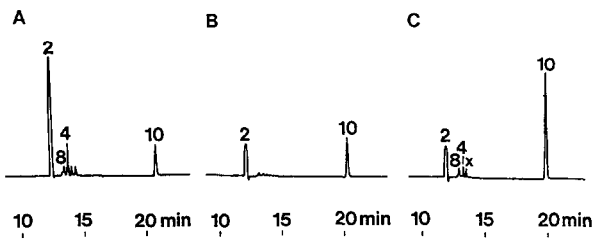

Fig. 5. Aromatic amino acids and heterocyclic compounds in seeds from *Pisum sativum* L. (cv. WSB), (A) 2, (B) 4 and (C) 8 days after start of germination, analysed by MECC (NaCh). Separation conditions as in Fig. 2. Detection, UV at 260 nm, 64 mV full-scale. Peaks were identified by spiking and knowledge of *RMT* of reference compounds.

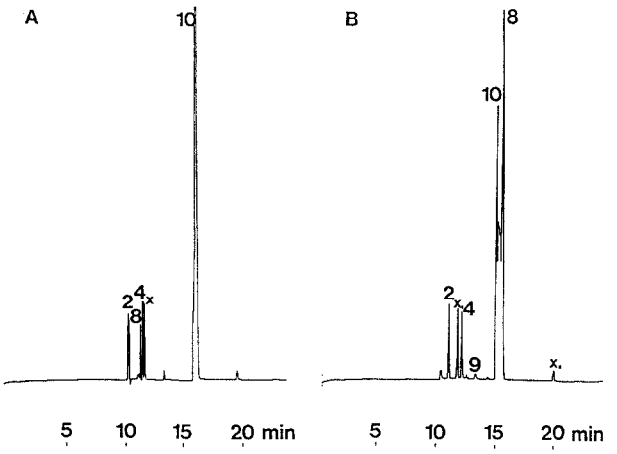

Fig. 6. Aromatic amino acids and heterocyclic compounds in roots from *Pisum sativum* L. (cv. Kelwo) 8 days after start of germination with (A) NaCh and (B) SDS as the detergent. Separation conditions as in Fig. 2. Detection, UV at 260 nm, 64 mV full-scale. Peaks were identified by spiking and knowledge of *RMT* of reference compounds.

The presence of structurally closely related isoxazolines was most clearly demonstrated in the NaCh system, whereas co-elution of the quantitatively dominant isozaxolines with tryptophan occurred in the SDS system. The unidentified peak (X_1) appearing between trigonelline (**2**) and tyrosine (**4**) in the SDS system may represent a glucosylated isoxazoline [1,3]. As for dopa-glucoside (**5**), glucosylation increases the migration rate in the SDS system. 4-Chlorotryptophan is a possible candidate for the peak marked X_2. Comparisons of the peak areas obtained in the NaCh and SDS systems indicated about a twofold higher sensitivity of the NaCh system.

4. Conclusions

MECC with NaCh and SDS as the detergents proved to be successful for the analysis of standard mixtures and group-separated plant extracts containing aromatic amino acids and heterocyclic compounds from leguminous seeds. The migration order for the compounds in the two systems differed, and this may be turned to account for identification purposes. The separation capacity in both systems was high, with $N/m \approx 100\,000$–$300\,000$ and $R_s = 0.8$–30.9. The NaCh system had a sensitivity about two times higher than that of the SDS system. In the NaCh system, *RMT* of the compounds considered had a satisfactory repeatability (mean R.S.D. below 1%), and this technique may therefore be used for identifying aromatic amino acids and heterocyclic compounds in mixtures of unknown composition, although preferably in combination with spiking. However, some improvements concerning quantitative determination still need to be made, including the determination of relative response factors under the optimized separation conditions.

Acknowledgement

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