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Pseudo-electrochromatography–negative-ion electrospray mass spectrometry of aromatic glucuronides and food colours

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ABSTRACT

Pseudo-electrochromatography is a combination of liquid chromatography and an electromigration technique, especially directed at the separation of ionic compounds prior to mass spectrometric detection with a mobile phase composition compatible with mass spectrometry. The application of pseudo-electrochromatography to the separation of food colours and aromatic glucuronides is described. An example of selectivity tuning by applying voltages of differing polarity during the chromatographic run is given. The coupling of pseudo-electrochromatography with electrospray mass spectrometry is demonstrated. Differences in the effects of the axial potential over the column between silica-based and polymeric packing materials are discussed.

INTRODUCTION

Pseudo-electrochromatography (PEC) [1] was introduced to combine the benefits of conventional liquid chromatography and electromigration methods such as capillary electrophoresis (CE) and electrochromatography (EC). The technique consists of a pressure-driven liquid flow through a packed bed with an axial potential difference. In a recent review on electrochromatography by Tsuda [2] pseudo-electrochromatography was also covered.

In pure electrochromatography as described by Knox and Grant [3], the mobile phase is driven through a packed capillary of 50–300 μm I.D. by applying a potential gradient of 10–50 kV/m. In fused silica the walls bear a fixed negative charge caused by deprotonated silanol and adsorbed hydroxyl groups, whereas the liquid in contact forms a thin layer (*ca.* 10 nm) of positive charge to compensate for the negative charge. This sheath of positive charge encloses the rest of the neutral solvent and takes it along in the direction of the negative electrode as soon as a potential is applied. This so-called electroosmotic flow (EOF) is the only driving force of the mobile phase in EC and has a flow profile approaching a perfect plug [3]. In pressure-driven chromatography the flow has a parabolic profile. This is the reason for the lower efficiency of LC than electromigration methods such as electrochromatography. Neutral compounds are separated in EC by the interaction with the stationary phase whereas the migration of charged compounds is additionally dependent on their electrophoretic mobility, which is a function of the field strength and the size and charge of the ion [4].

Unfortunately, the EOF is very sensitive to pH, electrolyte concentration and organic modifier content of the mobile phase [5]. A serious problem in EC is Joule heating through high currents in the capillary resulting in peak dispersion and bubble formation [6]. Therefore, small column diameters (<300 μm) and low

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buffer concentrations (<20 m*M*) have to be used in EC. The combination of EC and LC by superposing a pressure-driven flow supplied by an HPLC pump was first described by Tsuda [7], originally used to suppress bubble formation. Verheij *et al.* [1] named this pressure-assisted method pseudo-electrochromatography (PEC), to distinguish it from pure electrically driven electrochromatography. The advantages of pseudo-electrochromatography are higher flow-rates, shorter analysis times and the loss of the limitations on pH and buffer composition, because the EOF is no longer important. On the other hand, the flow profile is approaching LC and therefore the efficiency of PEC lies between those of LC and EC, depending on the ratio of pump flow and EOF. This is probably a reason why PEC has not yet become a common analytical technique. The separation of ionic compounds is more readily accomplished with the use of ion pairs and buffer gradients, where the usual HPLC equipment can be used. However, if a mass spectrometer is used as a detector, most ion-pairing agents and many buffers cannot be used, because of the rapid contamination of the ion source.

Experiments have been made to replace non-volatile ion-pair reagents by volatile compounds such as di- or trialkylamines for anions and perfluorinated sulphonic acids for cations [8].

As mentioned before, the retention time of ionic compounds can easily be influenced with the help of a high voltage. Continuing the work of Verheij *et al.* [1], new classes of compounds such as aromatic glucuronides and food colours (sulphonated azo dyes) were investigated. The possibility of changing the polarity of the voltage during the analysis to obtain both acceleration and retardation of compounds in the same run was of special interest. Polymer packing material was used for the first time in PEC and compared with the normally used octadecylsilanized (ODS) stationary phases.

For the coupling of PEC and mass spectrometry, continuous-flow fast atom bombardment (FAB) and electrospray or ionspray are most promising, because of the low flow-rate of only a few microlitres per minute used in PEC. Verheij *et al.* [1] showed the possibility of coupling PEC with FAB-MS. In this work the coupling of PEC with electrospray MS is described.

EXPERIMENTAL

Chemicals and solid phases

Methanol, 2-propanol (Baker, Deventer, Netherlands) and acetonitrile (Rathburn, Walkerburn, UK) were of HPLC grade. Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Phenyl-β-D-glucuronide (PG), *o*-aminophenyl-β-D-glucuronide (APG), *p*-nitrophenyl-β-D-glucuronide (NPG) and α-naphthyl-β-D-glucuronide (NAG) were supplied by Sigma (Brussels, Belgium), the food colours (>85% pure) E102 [Colour Index (CI) Food Yellow 4, Acid Yellow 23), E110 (CI Food Yellow 3), E122 (CI Food Red 3, Acid Red 14), E123 (CI Food Red 9, Acid Red 27) and E124 (CI Food Red 7, Acid Red 18) by Morton (Amersfoort, Netherlands) and ammonium acetate (reinst) by Merck (Darmstadt, Germany). The column packings were Nucleosil 100-5C_{18}, Nucleogel RP100-5/150 (Macherey–Nagel, Düren, Germany) and 12–20-μm PRP-1 (Hamilton, Reno, NV, USA).

Instrumentation

The PEC–MS system is outlined in Fig. 1. A Phoenix 20 CU syringe pump (Carlo Erba, Rodano, Italy) was used to deliver the solvent. A Swagelock tee (Crawford Fitting, Solon, OH, USA) with a 40 cm × 50 μm I.D. fused-silica capillary was used to split the mobile phase in a ratio of about 1:50 to 1:100 in order to obtain a flow-rate between 0.5 and 2 μl/min. A 10-mm guard column (Chrompack, Bergen op Zoom, Netherlands) filled with Nucleosil 100-5C_{18} was placed between the pump and the preinjector split to retain small impurities of the solvent. It has been found that the usual solvent filters at the pump inlet were not sufficient for microcolumns. Even when the solvent was filtered before use through 0.2-μm filters, the columns became clogged in less than 24 h. The microcolumns were laboratory prepared and packed (see below) and directly connected to a Valco CI4W internal volume (60/150 nl) micro-

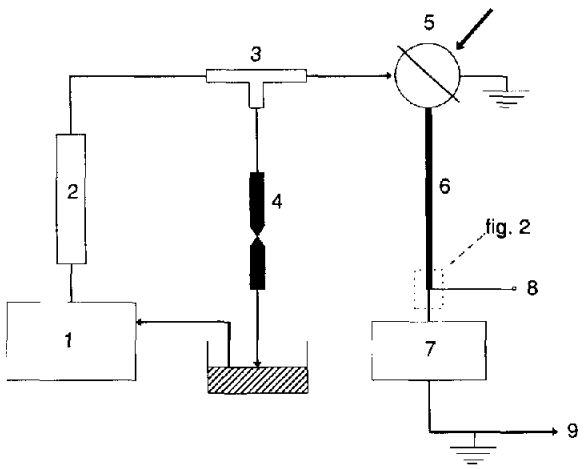

Fig. 1. Scheme of the PEC system. 1 = Syringe pump; 2 = 1-cm guard column filled with 5-μm ODS stationary phase; 3 = preinjector split; 4 = restrictor; 5 = 150-nl microinjector; 6 = microcolumn; 7 = UV detector; 8 = high voltage; 9 = electrospray mass spectrometer.

injector (Valco Instruments, Houston, TX, USA). A window of 10 mm length was burned in the polyimide coating of the 50 μm I.D. fused-silica outlet capillary, which was placed in a modified UV cell of a Spectroflow 757 UV detector (Kratos Analytical, Ramsey, NJ, USA). Signals were registered on a BD41 multi-range recorder (Kipp & Zonen, Delft, Netherlands). The high voltage was delivered from a switchable 0 to ±30 kV Spellman CZE 1000R power supply (Spellman High Voltage Electronics, Plainview, NY, USA) and connected to the Valco union at the end of the column. The injector was set to ground potential.

PEC–electrospray coupling

The mass spectrometer used was a Finnigan MAT (San Jose, CA, USA) TSQ 70 triply quadrupole instrument equipped with a Finnigan MAT electrospray interface. The system was operated in the negative-ion mode. A sheath flow consisting of 10% water in 2-propanol (1–2 μl/min) was delivered by a Model 2400 syringe pump (Harvard Apparatus, Edenbridge, UK). A 40 μm I.D. fused-silica capillary was inserted in the sheath-flow needle of the electrospray interface. The capillary was connected to the outlet of the UV detector by a Valco zero dead volume union, which lies at earth potential. If no UV detector is used, the capillary is directly connected to the column outlet.

Column preparation

The capillary columns were made of 220 μm I.D. fused-silica capillary tubing (SGE, Melbourne, Australia) or 250 μm I.D., 1/16 in. O.D. (1 in. = 2.54 cm) polyether ether ketone (PEEK) tubing (Jour Research, Onsala, Sweden). To make the connection of the fused-silica capillary easier, they were glued in a 0.5 mm I.D., 1/16 in. O.D. PEEK tube with a two-component epoxy glue (Torr Seal, Varian, Lexington, MA, USA), so that the fused-silica capillary protruded a few centimeters. The space between the fused-silica and PEEK tubing has to be carefully filled to avoid dead volumes. After the glue had hardened the protruding ends were cut with a sharp knife and the remaining glue removed from the PEEK capillary. In this way fused-silica columns could be connected with the usual 1/16 in. Valco stainless-steel ferrules or PEEK fingertight fittings. To keep the packing inside the column, 1/16 in., 2 μm metal screen filters (Valco) or 3 μm Fluoropore filters, cut with a hand-made 1/16 in. disc cutter from commercial Fluoropore filters (Millipore), were placed on both ends of the column. This is much simpler than making sintered frits [9] or using a plug of quartz-wool [1,10]. Further, the filters always have the same thickness and pore size, which is very important when making microcolumns of reproducible quality. The column end with the filter was connected to a Valco zero dead volume union, which also served as electric contact for the high voltage (Fig. 2). Although the bore of the zero dead volume union is 250 μm, the separation is not improved by using a through union.

Different packing procedures have been described [11]; acetonitrile is the most common solvent for the packing of ODS stationary phases. According to Cappiello *et al.* [12], other conditions are necessary to pack a PEEK column, because of repulsive forces between the particles themselves and the internal column walls. They developed a method called soap packing, with a solution of 1% aqueous sodium

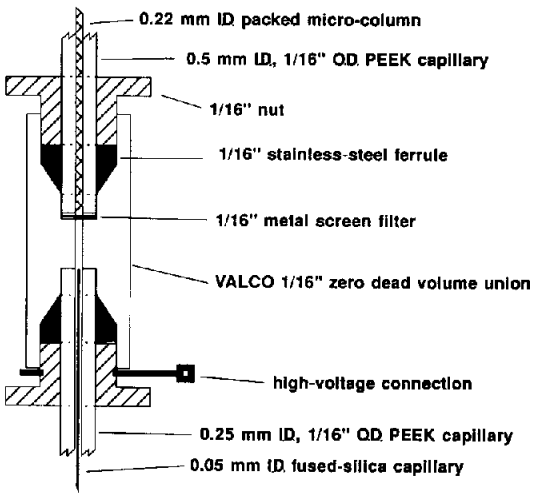

Fig. 2. Connection between microcolumn and detector. " = Inch.

laurylsulphate as packing solvent. However, this method gave poor results with our packing apparatus, which was different from that described in their paper. The same packing conditions as for the fused-silica columns were used for the preparation of the PEEK columns. The efficiency of PEEK columns was *ca.* 30% lower than that of silica columns, but can probably be optimized. For polymer packing material (Nucleogel RP100-5/150 and Hamilton PRP-1) methanol–water (6:4) proved to be superior to acetonitrile.

The following packing procedure was used for a 20 cm × 220 μm I.D. fused-silica capillary column. A Brownlee micro gradient system (Brownlee Labs., Santa Clara, CA, USA) dual-syringe pump was used to pack the columns, which were connected to a 1.5-ml laboratory-made high-pressure mixing chamber, equipped with a magnetic stirrer. A slurry of 8 mg of Nucleosil 100-5C_{18} in 1 ml of acetonitrile was homogenized for 5 min in an ultrasonic bath and then transferred with a syringe into the mixing chamber. The microcolumn was directly connected to the mixing chamber with the end of the column pointing upwards. The magnetic stirrer was activated and the programme of the pump (initial pressure 30 bar, maximum pressure 300 bar, flow-rate 100 μl/min) was started. When all the air had been flushed out of the column, the whole installation was turned through 180°, so that the end of the column was pointing downwards. The maximum pressure was reached in about 15 min and maintained for about 1 h. Thereafter, the flow was stopped and the column was removed after the depressurization was complete (1–2 h). For the polymer packings Nucleogel and PRP-1 the slurry concentration was 4 mg/ml in methanol–water (4:6) and a maximum pressure of 200 bar was used. In addition, the column was rinsed afterwards with water for at least 20 h, giving better baseline stability, perhaps owing to impurities in the polymer packing. The column performance was tested with a set of three parabenes [methanol–water (4:6) with silica-based and acetonitrile–water (1:1) with polymer stationary phases].

RESULTS AND DISCUSSION

Separation of food colours

Five water-soluble food colours (E102, E110, E122, E123 and E124) were chosen as model compounds for multiply charged ions to investigate the influence of the high voltage on retention time and peak shape. The dyestuffs, which have two or three sulphonic acid groups and, for E102, also a carboxylic acid group, were separated on a capillary column filled with 5-μm ODS particles with 10 m*M* ammonium acetate–methanol (8:2) as mobile phase.

At high pH the acidic groups are fully deprotonated, forming doubly and triply negatively charged ions. The triply charged dyes (E102, E123, E124) are eluted first, followed by the doubly charged E110 and E122 (Fig. 3a). Without a modifier gradient the analysis time of E122 is very long (240 min, extrapolated from Fig. 3b) and the peak is too broad for detection. Applying a positive voltage over the column shortens the retention time dramatically (Fig. 3). For example, the retention time of E122 is a few hours without a voltage and 33 min at 2 kV, but at 8 kV it is less than 5 min. At the same time, the peak becomes narrower and higher, resulting in improved detection limits.

However, at high voltage joule heating becomes a severe problem, leading to gas bubble formation, which can irreversibly destroy the

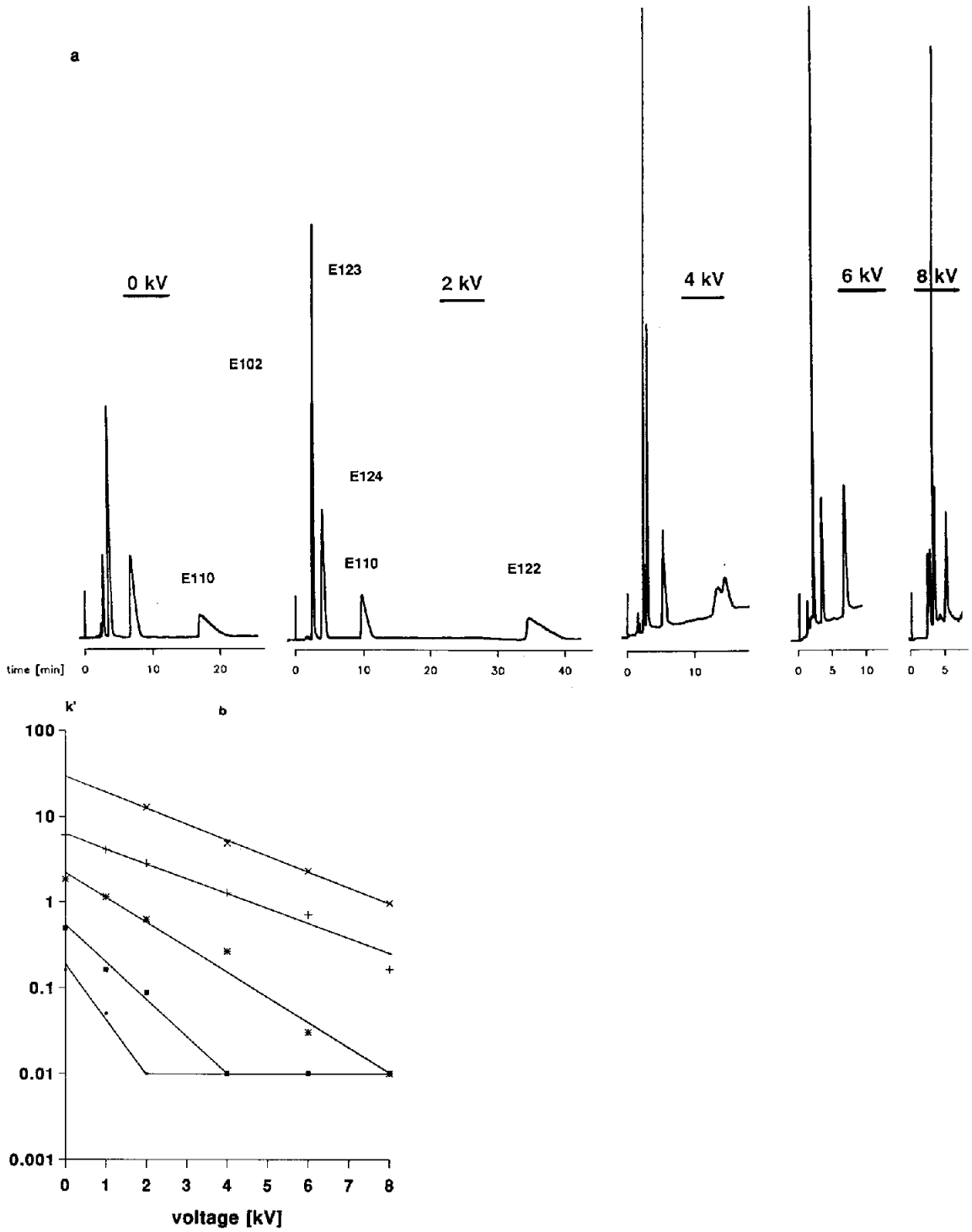

Fig. 3. Separation of food colours. Column, 20 cm × 250 μm I.D. PEEK column packed with Nucleosil 100-5C_{18}; mobile phase, 10 m*M* ammonium acetate (pH 8.5)–methanol (8:2); sample concentration, 0.5 mg/ml each in water. (a) Chromatograms with UV detection at 220 nm with different voltages; (b) plot of k' *vs.* voltage.

packed bed of the column. It is interesting that bubbles are formed more easily in fused-silica than in PEEK capillaries, perhaps because of sharper edges at the column end. Decreasing the buffer concentration reduces the current and therefore also the joule heating in the capillary, but the stability of the system also decreases, leading to less reproducible results. In addition, at lower ammonium acetate concentrations the retention time is also reduced because ammonium acts with anions as an ion-pair reagent.

The structure of E123 and the corresponding mass spectrum in the negative-ion mode are shown in Fig. 4. All sodium ions are exchanged by protons already in solution. Therefore, only signals of the free sulphonic acid (= M) are present. The base peak is the triply charged ion at m/z 178, followed by the doubly charged ion at m/z 268. The single charged $[M - H]^-$ ion can hardly be distinguished from the background. The ion at m/z 597 is probably an adduct with $[M + OAc]^-$. The electrospray (ESP) mass spectra of these azo dyes are almost identical with the ionspray spectra obtained by Edlund *et al.* [13], who also did not observe fragmentation. In negative-ion thermospray (TSP) the doubly charged ion was the base peak in neutral solution [14], whereas in positive-ion TSP (repeller on) the loss of $NASO_3$ has been reported [15].

Separation of aromatic glucuronides

Glucuronides are important metabolities of many biologically active compounds found in urine. The applicability of PEC–ESP-MS to some aromatic glucuronides as model compounds was investigated. Stefansson and Westerlund [16] studied the influence of the stationary phases, pH and counter ions in the separation of these aromatic glucuronides by ion-pair chromatography. The best results were obtained with a mobile phase consisting of 20 m*M* dodecylamine and a zwitterionic 2-(N-morpholino)ethanesulphonic acid (MES) buffer. These mobile phase conditions are clearly not compatible with MS conditions.

The chromatogram of the four glucuronides without the use of an ion pair is shown in Fig. 5a. The three structurally similar phenyl glucuronides are eluted first, NAG coming much later as a broad peak. At 0 kV PG and APG

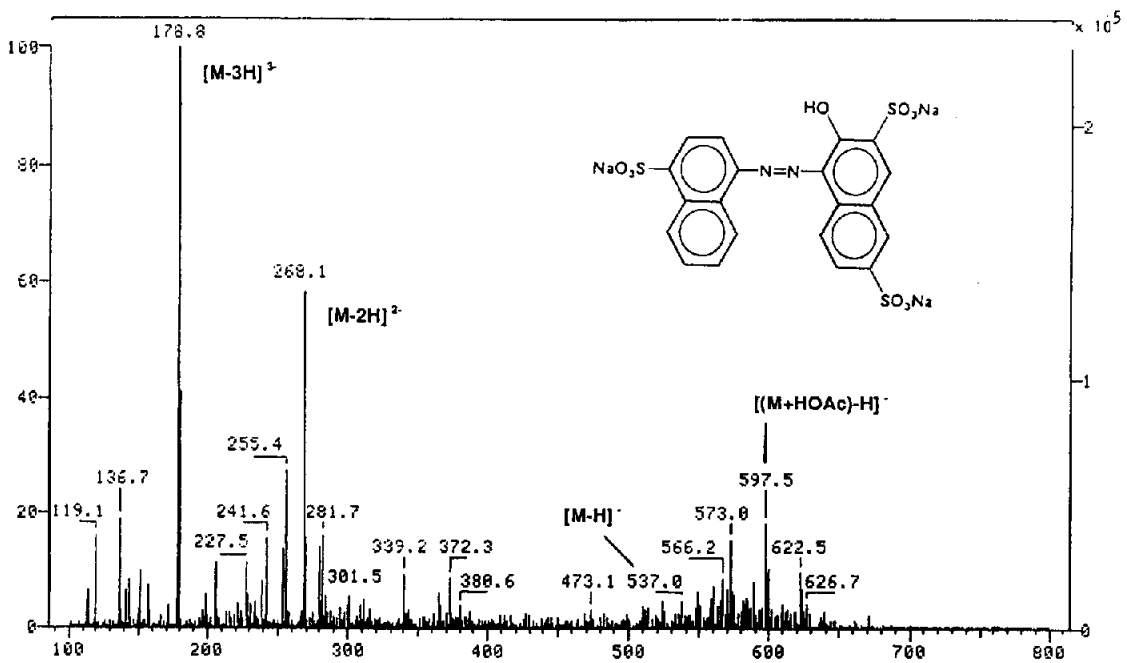

Fig. 4. Mass spectrum of food colour E123 in the negative-ion mode (M is the free acid). Constant infusion (1 μl/min) of 100 μg/ml in 2 m*M* ammonium acetate; sheath flow 2 μl/min of 2-propanol–2 m*M* ammonium acetate (9:1).

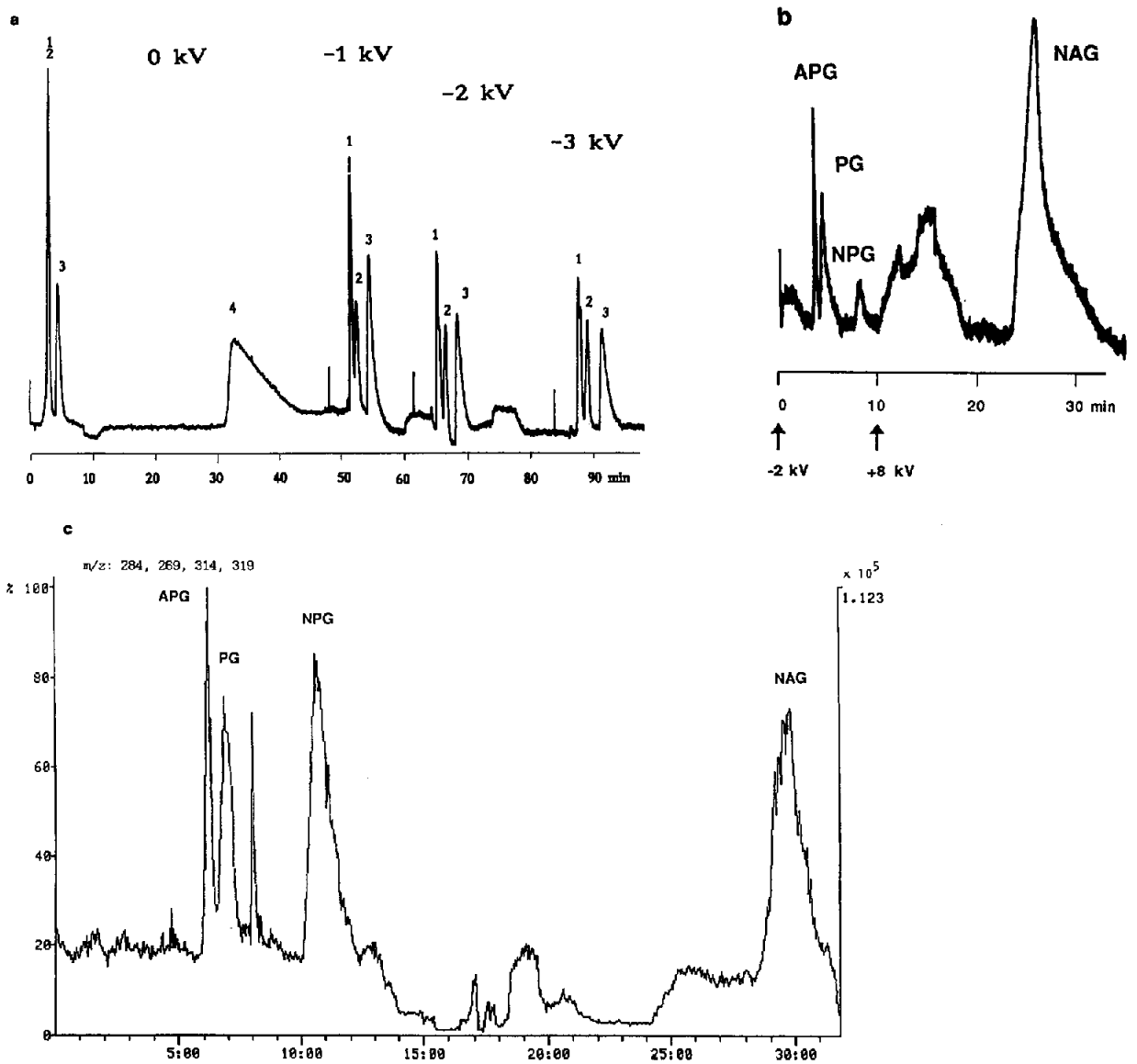

Fig. 5. Separation of aromatic glucuronides on a 20 cm × 220 μm I.D. fused-silica column packed with PRP-1 (12–20 μm). Mobile phase, 2 m*M* ammonium acetate (pH 7)–acetonitrile (95:5). (a) Influence of voltage on the retention time (1 = PG, 2 = APG, 3 = NPH, 4 = NAG; UV detection at 220 nm); (b) UV and (c) mass chromatogram of the four glucuronides; concentration, 100 μg/ml in water.

elute together, but if a negative voltage is applied to increase the retention time, the resolution of the three glucuronides becomes better with higher voltage (Fig. 5a). At −3 kV almost baseline separation is obtained. Unfortunately, NAG is retained in the same way, resulting in a very long analysis time. In Fig. 5b and c the advantage of PEC over ion pairs is demonstrated: to shorten the analysis time of NAG the voltage was changed from −2 to +8 kV after the elution of the third compound, whereby NAG was now accelerated instead of delayed.

However, as can be seen, after the change in the voltage, the baseline is disturbed, resulting

from a redistribution of the acetate buffer. Acetate anions are now accelerated while ammonium is retarded. This phenomenon can also be seen by simply changing the voltage without injection. Tsuda [17] already described that after applying a voltage to a packed capillary, an equilibration time of around 20 min was necessary before the retention time became constant. The same happened when the voltage was switched off. This phenomenon was explained by a change in the surface and with release or saturation of adsorptive materials from or to the surface of the column support, originally present in the mobile phase.

A closer investigation of this problem shows that this equilibration time is dependent on different factors such as mobile phase composition, flow-rate and especially the nature of the packing material. In order to obtain more information about this equilibration time the following experiment, also described by Tsuda [2], was carried out. Repeated injections of a solution of E124 were made every 3 min on to a column filled with Nucleogel RP100-5/150 and with E110 on to a column of Nucleosil 100-5C_{18} (Fig. 6a) under identical conditions. Different compounds have to be used in order to obtain peaks with comparable retention times, because of the different polarities of the two stationary phases. A voltage of +3 kV was applied 16 min after the first injection and switched off after 41 min. For the polymer packing no equilibration time was observed. The first injection, after the voltage had been applied (at 18 min), already shows the same retention time as the samples injected later (Fig. 6b). The injections at 12 and 9 min are different, because these compounds were already in the column when the voltage was switched on. The sample injected at 9 min shows a peak that is much broader than the preceding peaks and with a retention time that is longer, instead of being shorter as expected (Fig. 6a). There is no good explanation for this observation, but the change in the buffer concentration in the column, caused by the high voltage, is certainly one factor contributing to this phenomenon. When the voltage is switched off, the retention time returns to the initial value as in the beginning of the experiment.

With the ODS stationary phase an equilibrium time of *ca.* 8 min is measured after the voltage has been applied. Again the first compound which is in the column when the voltage is changed has a longer retention time than without a voltage. When the voltage is switched off, ODS material needs a very long time to regain the original surface properties, which is measured by the retention time of the food colour. Even 1 h after the voltage has been switched off, the retention time is still shorter (400 s) than at the beginning (444 s). This remains in contradiction with the observations of Tsuda [2], who described an equilibrium time of 20 min when the voltage was applied, but only 10 min when the voltage was switched off. It is obvious that the nature of the ODS stationary phase, in contrast to the polymer phase, is changed on applying a voltage. This must be ascribed to the presence of free hydroxyl groups still present in silica-based supports and the easier adsorption of polar compounds by these groups.

The mass spectrum with continuous-flow injection of the four glucuronides in the positive- and negative-ion modes is shown in Fig. 7. The intensities of the $[M-H]^-$ ions are almost identical in the negative-ion mode and the spectrum shows no other ions except for some addition clusters of two glucuronides $[M_1 + M_2 - H]^-$ between *m/z* 500 and 600 with low intensity. In the positive-ion mode, $[M+Na]^+$ ions are formed and APG as the only compound forms a very intense $[M+H]^+$ ion (100%). The relative intensity of the ions reflects the electron affinity and the pK_a of the aglycone. This is also observed in the FAB mass spectra of aromatic glucuronides, where the sensitivity for NPG is 100 times lower than for 8-hydroxyisoquinoline glucuronide [18]. No fragmentation was observed and the base peak was $[M+H]^+$ or $[M+NH_4]^+$, again depending on the basicity of the aglycone.

Whereas molecular ions of glucuronides cannot be detected with a particle beam interface in either the electron impact or chemical ionization mode [19], the best results in TSP are obtained with the filament on and buffer ionization in the negative-ion mode [20]. In positive-ion TSP with buffer ionization only the ammonium adduct

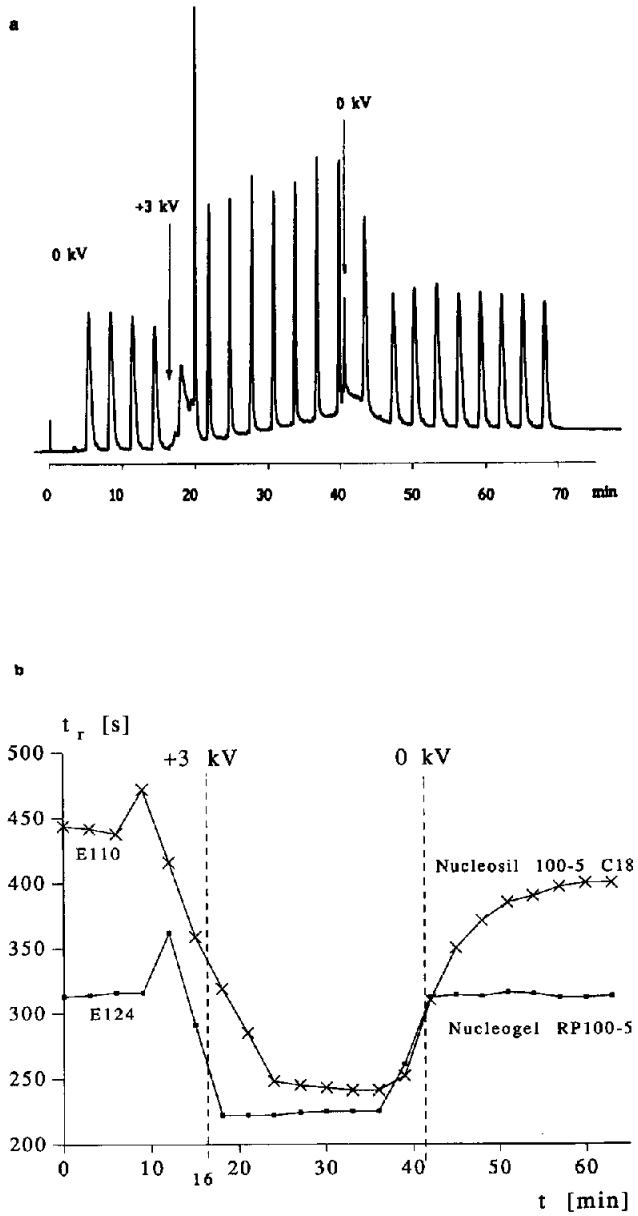

Fig. 6. Repeated injection of food colours. Mobile phase, 10 m*M* ammonium acetate (pH 7)–methanol (7:3); column, 20 cm × 250 μm I.D. PEEK column packed with polymer Nucleogel RP100-5/150 and silica-based Nucleosil 100-5C_{18}; UV detection at 220 nm. (a) Diagram of retention time with and without applied voltage; (b) multiple injection chromatogram of E124 on polymer support.

$[M + NH_4]^+$ was registered, whereas Liberato *et al.* [21] observed significant fragmentation in the filament-off mode, with the sugar fragments at *m/z* 194 or 177 as base peaks. Watson *et al.* [22,23] analysed steroid glucuronides and monosulphates in negative-ion TSP and obtained almost only the deprotonated anions without fragmentation in LC–MS, using a water–acetonitrile gradient without any ammonium acetate.

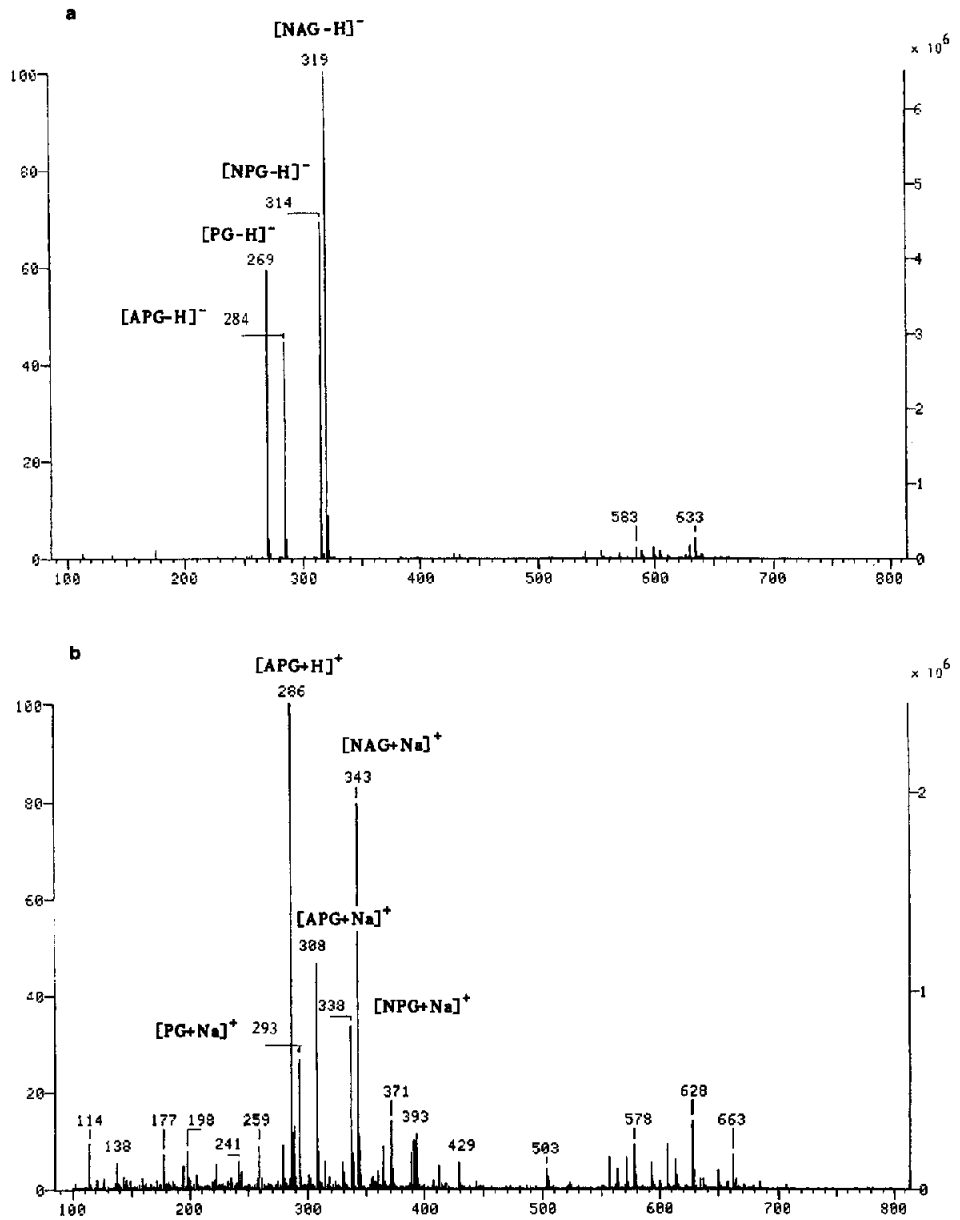

Fig. 7. Mass spectra of a mixture of four aromatic glucuronides. Constant infusion of 100 μg/ml of each compound in 2 m*M* ammonium acetate (pH 8.5) (2 μl/min). (a) Negative-ion mode; sheath flow, 2 μl/min of 2-propanol–2 m*M* ammonium acetate (pH 8.5) (9:1); (b) positive-ion mode; sheath flow, 2 μl/min of 1% acetic acid in 2-propanol–water (9:1).

CONCLUSIONS

It was shown that charged compounds can be both accelerated and retarded by changing the sign of the high voltage in the same run. This attractive feature was applied to some food colours (sulphonated azo dyes) and aromatic glucuronides in the on-line combination of PEC and MS. The coupling of electrospray mass spectrometry and PEC does not lead to more problems than with micro-LC. To prevent the high voltage from affecting the spray, a con-

nection to ground has to be placed in front of the electrospray interface. In addition, the capillary has to be narrow and not too short, otherwise the current passing through it is too high. Another possibility is to connect the injector to the high voltage and the outlet of the column to ground. In this case no zero dead volume union has to be placed between the column and electrospray interface. For security reasons, an automatic injector has to be used in that event. PEC–ESP-MS has been demonstrated to be a useful tool for the separation of charged compounds.

The interest in micro-LC is still growing because of the advantages of low solvent consumption and the smaller amount of packing material needed, which is an important factor when expensive supports are used, especially in separations using chiral phases. Here, PEC offers an alternative to microgradient systems, where the technique is still in development.

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