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Development of an instrumental configuration for pseudo-electrochromatography–electrospray mass spectrometry

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Abstract

The development of an instrumental configuration for the application of the electric field in pseudo-electrochromatography is described. To increase detection sensitivity and selectivity, special attention has been paid to the hyphenation of PEC with electrospray ionization mass spectrometry. Problems encountered in previous instrumental configurations like formation of gas bubbles have been overcome by using liquid junctions to apply the electric field over the column. Some sulfonamides and a nonapeptide have been used as test compounds to compare pseudo-electrochromatography with micro-liquid chromatography. The characteristic features of pseudo-electrochromatography on retention behaviour and separation efficiency are demonstrated.

1. Introduction

The coupling of separation methods based on electromigration principles, like e.g. capillary zone electrophoresis (CZE) with electrospray ionization mass spectrometry (ESI-MS) is mostly concerned with the transfer of ions in solution to ions in the gas phase [1]. Because of its high efficiency, CZE is favoured for the analysis of complex mixtures. The combination of CZE with ESI-MS increases the selectivity and expands the application range of CZE. The increasing use of CZE–ESI-MS for the analysis of drugs [2], biochemicals [3] and biomacromolecular compounds (peptides, proteins and nucleotides) [4–7] might argue for this powerful combination. However, although the high efficiencies obtained in CZE result in high mass fluxes, the mass flows are still limited and, therefore, the sample-concentration detection limits are disappointing. This is caused by the small dimensions in CZE in conjunction with the low flow-rate induced by the voltage applied. ESI-MS is characterized as a mass-flow sensitive detection system functioning according to concentration-sensitive principles [8]. Combination of CZE with sample-concentration techniques or enhancement of loadability is necessary for the hyphenation of CZE with mass-spectrometric detection to reach its full potential.

Micro-packed bed liquid chromatography (μLC) can serve as an example of a micro-separation method which has acceptable loadabilities despite its small dimensions when compared with CZE and open-tubular LC. It has been demonstrated that a decrease of column

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diameter leads to increased mass sensitivity in sample-limited situations [9]. Although the loadability of μLC is higher when compared with CZE the separation efficiency of μLC expressed as a function of the theoretical plate number is almost two orders of magnitude lower than of CZE. Another limitation with regard to the use of μLC is that in the separation of complex mixtures, gradient elution conditions or the use of ion-pairing or ion-exchanging agents in the mobile phase are often needed to deliver acceptable analysis times and good resolutions. Such conditions are not always compatible with good mass-spectrometric performance. A technique which is a good compromise between the high separation efficiency of CZE and the high loadability of μLC is pseudo-electrochromatography (PEC). The strategy behind the development of PEC in our group was to increase selectivity without impairing possible hyphenation with mass-spectrometric detection techniques as has been demonstrated by Verheij et al. [10] and Hugener et al. [11]. The pressure-assisted variant of electrochromatography, which has been described by Tsuda [12,13], has been developed to suppress bubble formation in electrochromatography (EC) [14–16]. In EC the transport of the mobile phase in packed capillaries is induced by the same principles as known from CZE. An electroosmotic flow is generated by the application of a voltage over the column. Similar to CZE, in EC the possibility of reversal of the voltage polarity is limited by the electroosmotic flow in combination with the electrophoretic behaviour of the compounds of interest. Due to the corresponding reversal of the direction of the electroosmotic flow, electroneutral and positively charged compounds will not migrate in the direction of the column outlet in that case. Although the use of surface-modifying additives in the mobile phase like fluorinated hydrocarbons [17] enables to manipulate the direction of the electroosmotic flow, this leads to a decrease of electrospray ionization efficiency and contamination of the ion source.

However, by superimposing the electroosmotic flow on a pressure-induced hydrodynamic flow, the choice of applied voltage and its polarity is completely free. Moreover, there are no constraints upon the pH used. This approach results in a stable flow that is only limited by the back pressure of the column. This intrinsic packed-bed property does not limit the flow generated by a voltage applied along the column. The voltage applied introduces a plug-like flow profile that is superimposed on the parabolic hydrodynamic flow profile. Through the implementation of a plug flow in a hydrodynamic flow the efficiency can be increased. As a result, a decrease of analysis time while maintaining good separation performance for electroneutral compounds can be obtained.

The aim of this study was the development of a trouble-free instrumental setup enabling to examine PEC and to couple this separation technique to ESI-MS. The described configuration has been used to evaluate the characteristic advantages of PEC in comparison with μLC using sulfonamides and a synthetic peptide as test compounds. Examples are given to demonstrate the potential of PEC in reducing the analysis time, increasing the selectivity and improving the efficiency.

2. Experimental

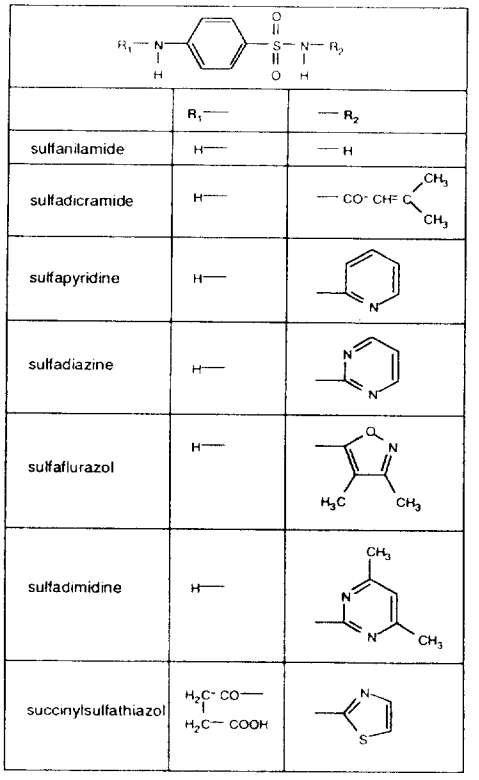
2.1. Materials

Analytical grade methanol, acetonitrile, acetic acid (HAc) and a solution of 25% ammonia (Baker, Deventer, Netherlands) were used. Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Trifluoroacetic acid (TFA) was purchased from Merck (Darmstadt, Germany). The sulfonamides (Table 1) came from Sigma (St. Louis, MO, USA). The nonapeptide was synthesized at the Biochemical Department of the University Hospital Leiden.

2.2. Equipment

In Fig. 1 the instrumental setup used for the PEC–UV and PEC–ESI-MS experiments is outlined. A micro-gradient pump (Brownlee Labs, Santa Clara, CA, USA) operating in the con-

Table 1
Molecular structures of some sulfonamides



	R_1—	—R_2
sulfanilamide	H—	—H
sulfadicramide	H—	—CO-CH=C(CH$_3$)$_2$
sulfapyridine	H—	
sulfadiazine	H—	
sulfaflurazol	H—	
sulfadimidine	H—	
succinylsulfathiazol	H_2C-CO— H_2C—COOH	

stant-pressure mode was used for solvent delivery. The pump was connected to a Valco CI4W internal volume (60/150 nl) micro-injector (Valco Instruments, Houston, TX, USA) by a PEEK capillary (400 mm × 308 μm I.D.) to protect the pump for electrical damage. The micro-injector was kept at ground potential to enable safe injections. The column used was a fused-silica capillary of 250 mm × 220 μm I.D., slurry packed with Nucleosil 100-5C8 (Macherey-Nagel, Düren, Germany). At both sides some packing material was removed with a micro-drill and glass-wool plugs were inserted serving as frits.

A 100 mm × 75 μm I.D. × 170 μm O.D. fused-silica capillary was used as transfer line between the injector and the column. The transfer capillary was inserted into the column and gently pressed against the glass-wool plug, thus minimizing extra-column band broadening (see Fig. 1). These connections were placed in high-pressure buffer vials containing platinum electrodes which were connected to a high-voltage power supply (CZE 1000R, Spellman, USA). The detection capillary was installed in the same way. Between the inner column wall and the external wall of the transfer and detection capil-

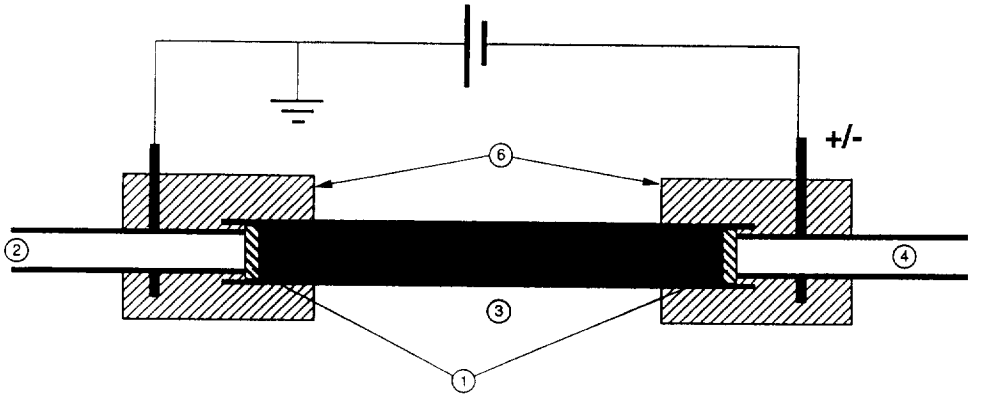


Fig. 1. Schematic diagram of the electrical contact between the power supply and the column with the use of liquid junctions and high-pressure buffer vials. (1) Glass-wool frit, (2) inlet capillary, (3) capillary column, (4) outlet capillary, (6) high-pressure buffer vial.

lary there existed an angular space of 25 μm width in which the buffer penetrates. In this way the electrical contact between the platinum electrodes and the column has been realized.

2.3. UV absorbance detection

An LKB 2151 (Bromma, Sweden) variable-wavelength detector set at 254 nm was used to monitor the column effluent. The detector cell was created by burning off the polyimide coating of the fused-silica capillary over a length of 5 mm. The detector cell was placed in a custom-made detector cell housing. The distance from the column end to the detector was 125 mm.

2.4. Electrospray mass spectrometric detection

Mass-spectrometric detection was performed using an SSQ 710 single-quadrupole instrument (Finnigan MAT, San José, CA, USA). Scanning was performed in the multiple-ion detection mode. Coupling with PEC was done using the Whitehouse–Fenn-type electrospray interface (Finnigan MAT). The detection capillary was inserted in the stainless-steel electrospray needle. The polyimide coating at the end of the fused-silica capillary was burned off over a length of 10 mm and polished to provide a stable spray. The sheath liquid consisted of a mixture of methanol and 0.5 *M* acetic acid (95:5, v/v) and provided the electrical contact between the fused-silica capillary and the stainless-steel electrospray needle, which was connected to ground. The sheath liquid was delivered at a flow-rate of 0.75 μl/min by a syringe pump (Harvard Apparatus, Edinbridge, UK). A nitrogen flow kept at 150°C was used as drying gas.

2.5. Packing procedure of the micro-columns

Columns were packed with an air-actuated pump (DSHF-302, Haskel, Burbank, CA, USA). Fused-silica capillary (220 μm I.D. × 350 μm O.D.) (SGE, Melbourne, Australia) was cut to the desired length. The end of the capillary was inserted into a piece of PEEK tubing (25 mm × 400 μm I.D.), which was tightened in a zero dead volume nut. A metal frit was used to prevent the packing material from leaving the column during the packing procedure. The other end of the column was connected to a custom-made slurry reservoir of 1.5 ml that was magnetically stirred. The slurry was prepared by dispersing 40 mg packing material in 5 ml acetonitrile. After the slurry was homogenized in an ultrasonic bath for 5 min, 1.5 ml was transferred into the mixing chamber, after which the column was connected with the outlet pointing upwards. After activating the stirrer the pressure was slowly increased to fill up the column with acetonitrile. After five minutes the column was turned upside down and the pressure was rapidly increased to 600 bar. It took about two minutes to reach the set pressure. Pressure was maintained for one hour, after which it was slowly released, over a period of almost one hour. Before use the columns were flushed with water followed by the buffer solution used in the described experiments.

3. Results and discussion

3.1. Optimization of the instrumental setup

The first experimental configuration used for the PEC experiments was a modified version of the setup as described by Verheij et al. [10]. The micro-injector and the zero dead nuts were used as electrodes to apply the potential over the column. This did not result in a sufficiently stable system, the major problem being the generation of gasses due to electrolysis at the injector, the zero dead nuts and the metal frits. To circumvent the problems induced by the production of gasses, the electrical field was disconnected from the injector by use of a palladium decoupler while the zero dead nut was made from palladium. The high diffusion coefficient for molecular hydrogen in palladium enables the transport of any produced hydrogen from within the system to the ambient air, as has been successfully applied in CZE by Kok [18,19]. However, in our hands the use of such a decoupler did not lead to a significant improvement with regard to the

elimination of gas bubbles, probably due to the limited hydrogen-capture capacity of the Pd decoupler. For this reason, it was decided to investigate the potential of liquid junctions for the application of the electric field over the column. The open contact between the electrodes and the packing material in the column generates in fact an electrical split, and therefore a loss of analyte may occur. Nevertheless, with the configuration described here, a stable flow without disturbing gas bubbles could be realized.

3.2. Coupling to MS

Sulfonamides are ionizable compounds, which offers the possibility to apply a separation technique using the electrophoretic characteristics of these compounds in conjunction with the hydrophobic characteristics of this class of compounds. The analysis of sulfonamides using mass-spectrometric detection has been described by various authors who were using different separation techniques, such as CZE [20], SFC [21] and LC [20,22–24]. Because of their wide-spread use these compounds are often found in food products, like meat, milk and fish. The analysis technique of choice must be able to cope with the different sample matrices and to deal with the specific impurities. The majority of the papers dealing with the analysis of sulfonamides with LC–MS describes the use of a mobile-phase gradient to obtain acceptable analysis times and resolution [21–24]. The generation of reproducible gradient systems appeared to be problematic in case of miniaturized systems. Therefore, no comparison between PEC and gradient elution in μLC could be made. Besides, from our experience it was known that a mobile-phase gradient influences the electrospray process, and as a consequence the ionization efficiencies are affected. PEC offers the possibility to manipulate the separation process, to improve the selectivity and to speed up the analysis without modifying the composition of the mobile phase. In Fig. 2 it is demonstrated that with PEC it is possible to speed up the analysis without any loss in resolution. Fig. 2a shows a reversed-phase μLC–UV trace of three sulfonamides. Sulfaflurazol and sulfadicramide are retained twice as long as sulfanilamide. The long residence times of sulfaflurazol and sulfadicramide in the column lead to unacceptable band broadening. Manipulation of the voltage in PEC resulted in acceptable analysis times while a good resolution was maintained, as demonstrated in Fig. 2b. An additional but not less important effect of speed-

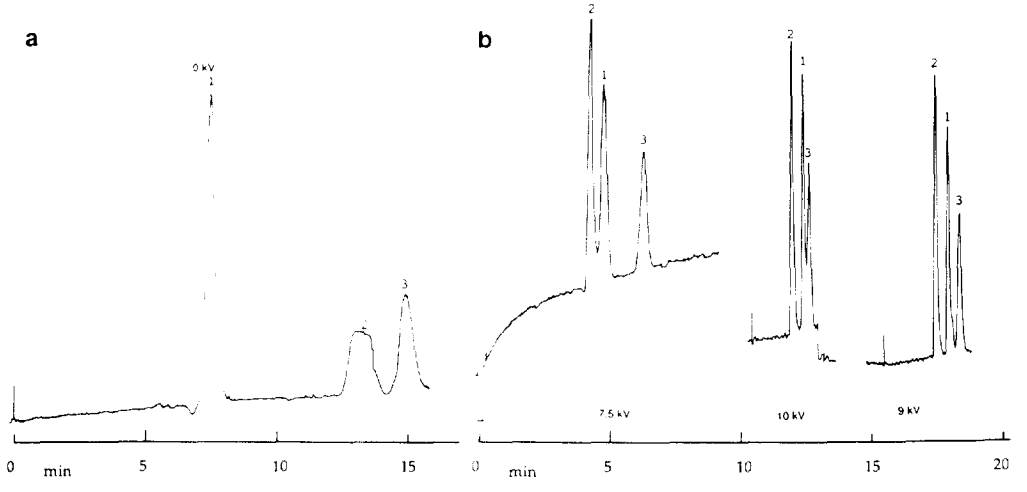


Fig. 2. Chromatograms showing the improvement of peak shapes after applying different voltages in the PEC mode: (a) without and (b) with the application of a voltage. Peaks: 1 = sulfanilamide, 2 = sulfaflurazol, 3 = sulfadicramide. Conditions: mobile phase 4 m*M* ammonium acetate–methanol pH 5 (60:40, v/v) (adjusted with trifluoroacetic acid), column length 23.5 cm, flow-rate 1 μl/min, pressure drop 50 bar, UV-absorbance detection at a wavelength of 254 nm.

ing up the analysis is that the mass flow of the analytes is increased, resulting in lower concentration-detection limits. The different voltages applied over the column were set at their value just prior to injection and switched off when the analysis was done. In this way reproducible runs could be performed. Nevertheless, it has been observed that with longer analysis times some time might be needed before reproducible retention times are obtained. This effect has also been described in the literature [11,25]. Still, PEC appeared to be a good alternative for gradient elution in reducing the analysis times, particularly when mass-spectrometric detection is involved.

In Fig. 3 the coupling of PEC to the mass spectrometer is demonstrated. In the described configuration, there exists a voltage drop over the transfer capillary from the column to the mass spectrometer. This voltage drop is of the same magnitude as that applied over the column and opposite relative to the mass spectrometer. The voltage over the transfer capillary induces a local electroosmotic flow that can be ten times higher than in the packed bed of the column [26]. As a consequence, the mobile phase is more or less dragged out from the packed bed, thus reducing the back pressure of the column. Operating the pump in the constant-flow mode increased the baseline noise under these conditions, for which reason the constant-pressure mode was preferred. As a result the hydrodynamic contribution to the total flow through the column has been increased. The reduction of the analysis time is thus an indirect effect of the voltage applied and is not due to the electrophoresis of the sulfonamides that electromigrate in opposite direction. This electrophoretic behaviour in the detection capillary is not of major concern for the separation process, which can be seen by considering the elution order of the compounds. Although its charge is less positive, the acidic succinylsulfathiazol elutes in front of sulfanilamide and sulfathiazole when the voltage is applied. When the migration order would be determined by the voltage drop existing in the detection capillary, this cannot be the case. This demonstrates that the selectivity in PEC can be tuned using both the hydrophobic and electrophoretic properties of the compounds to manipulate the separation process.

As mentioned above, not only increased loadability and tunable selectivity belong to the features of PEC, but also the improvement of the separation efficiency [15]. Because an electroosmotic flow is most effectively generated at higher pH values, an alkaline mobile phase is chosen to demonstrate this effect in PEC. Combination of both flow profiles decreases the contribution of convective mixing effects, resulting in less band broadening in comparison with the normal μLC mode. The plate height to velocity curve is shifted to higher velocities and presumably has a larger optimum value [26]. In the example shown in Fig. 4, a nonapeptide with a negative charge of minus three at pH 8 has been used. Because of its high charge the electrophoretic velocity of the nonapeptide almost counterbalances the electroosmotic flow. Therefore, the change in retention is minimal, implying that the increase of the plate number by a factor of two in PEC is mainly caused by the effect of the electroosmotic flow on the flow profile. Unfortunately, a substantial electrical split takes place at the liquid junction on the injection side of the column caused by the high electrophoretic mobility of the nonapeptide. As a consequence, the increase of efficiency as demonstrated in Fig. 4 did not lead to improved detection limits. In a closed separation system without liquid junctions, no electrical split will take place, and the gain in efficiency will also result in an improved concentration sensitivity.

4. Conclusions

PEC appears to be an interesting alternative for isocratic as well as gradient μLC, especially when mass-spectrometric detection is involved. In contrast with LC gradient elution, no compatibility problems with respect to the performance of the electrospray ionization process have been observed. The use of a configuration with liquid junctions in PEC–UV allows to study the net effects of the application of an electric field

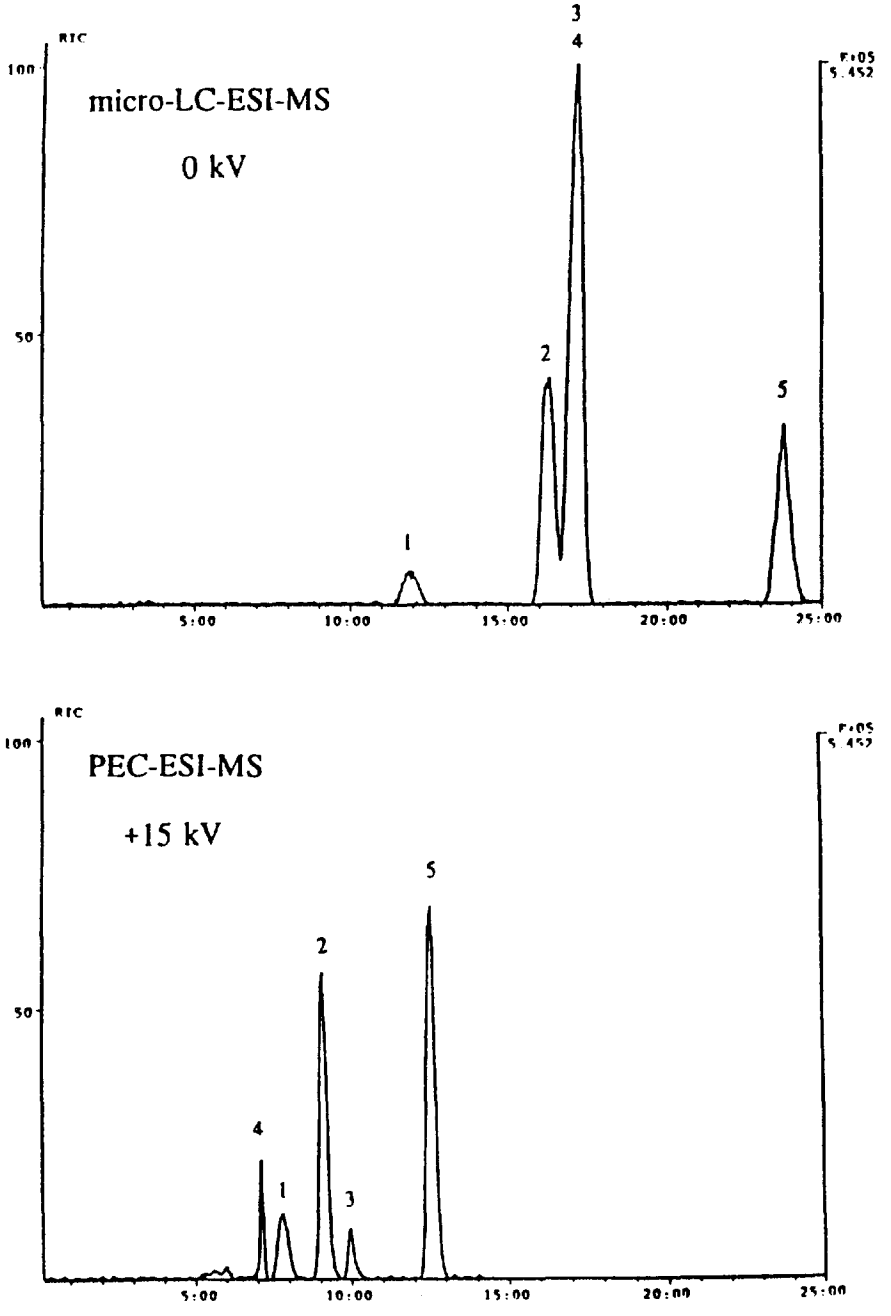


Fig. 3. Comparison of μLC with PEC applying mass spectrometric detection. Conditions: column length 21 cm, all other conditions as described in Fig. 2. Peaks: 1 = sulfanilamide, 2 = sulfathiazol, 3 = sulfapyridine, 4 = succinylsulfathiazol, 5 = sulfadimidine.

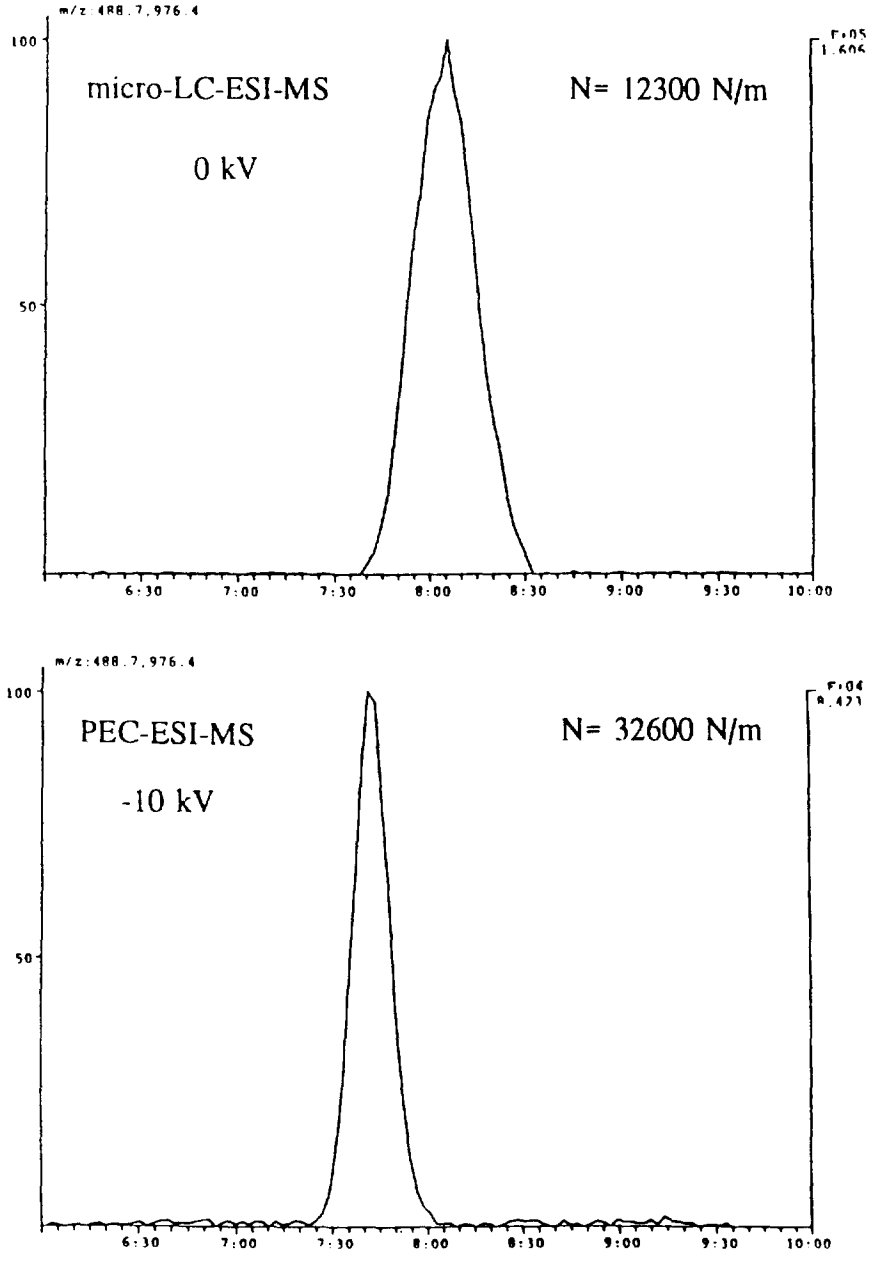


Fig. 4. Effect of the application of a negative voltage of −10 kV over the column in the PEC–ESI-MS mode (lower trace) in comparison with μLC–ESI-MS (upper trace). Conditions: pH 8 (adjusted with ammonium hydroxide), all other conditions as described in Fig. 3.

on the retention behaviour. Our findings indicate that decoupling of the electric circuit used for PEC is not necessary to obtain a stable electrospray. This offers the possibility to insert the column outlet directly into the atmospheric-pressure ionization source as described by Hunt et al. [27], thereby minimizing extra-column band broadening introduced by connection devices

and possible electrical split at the liquid junctions. A closed configuration for PEC is currently under investigation.

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