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Continuous full filling capillary electrochromatography - electrospraying chromatographic nanoparticles

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Abbreviations

CFF	Continuous full filling
IS	Ionic strength
NP	Nanoparticle
Neb	Nebulizing gas pressure
PF	Partial filling
PSP	Pseudostationary phase

Keywords

Continuous full filling capillary electrochromatography, nanoparticles, nebulizing gas pressure, pseudostationary phase, sheath liquid.

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Abstract

The influence of instrumental parameters affecting the ionization in continuous full-filling capillary electrochromatography / electrospray ionization mass spectrometry (CFF-CEC/ESI-MS) was investigated. The investigated parameters were the background electrolyte (BGE) and sheath liquid ion strength and organic modifier content, the nebulizer gas pressure, and the concentration of nanoparticles in the BGE. It was found that the nebulizer pressure had the largest influence on the separation efficiency and apparent retention. It was shown that even the lowest pressure investigated was sufficient to guide the nanoparticle flow away from the mass spectrometer inlet. A nebulizer pressure of 5 psi was found being optimal; increasing the pressure significantly decreased the separation efficiency due to the generation of a hydrodynamic flow. Generally, the ion strength of both the BGE and the sheath liquid were found to have very moderate effects on the separation of a homologous series of dialkyl phthalates. Whereas the ionization efficiency was found being unaffected by the nanoparticles, the separation efficiency was found to increase with increasing concentrations up to 3.8 mg/mL, where after it was observed to drop. The optimized method was linear over a wide concentration range and presented limits of detection and quantification more than three-fold lower than what have previously been reported using CFF-CEC/ESI-MS.

1. Introduction

High performance liquid chromatography (HPLC) is the most frequently used separation technique for analytes in the liquid phase. However, ordinary HPLC requires large sample volumes, consume large volumes of mobile phase, and necessitate long analysis times. To overcome these shortcomings, the instrumentation has continuously been miniaturized resulting in μ LC and later on also nanoLC [1, 2]. To improve the separation efficiency, the stationary phase particle size has in parallel to the instrumentation miniaturization been decreased, resulting in ultra high pressure liquid chromatography (UHPLC) [3]. However, with decreased column and particle diameters, the risk of clogging the column can in some applications increase. Furthermore, due to the increased back pressure with decreasing particle size, the efficiency limit of LC may soon be reached.

Capillary electrophoresis (CE) is an orthogonal technique to HPLC with the same miniaturization features as μ LC and nanoLC. Electrophoresis has since it was introduced by Tiseluis [4] during the 1930's and further adapted to the capillary format, CE, by Hjertén and others [5-7] developed into a robust separation technique. One example of the great performance of CE was demonstrated when the human genome was sequenced well ahead of schedule in 2001 [8]. Although being virtually resistant to clogging and offering exceedingly high separation efficiencies, CE suffers from other drawbacks such as an incapability of separating analytes with identical size to charge ratio. Capillary electrochromatography (CEC) [9], micellar electrokinetic chromatography (MEKC) [10], and microemulsion electrokinetic chromatography (MEEKC) [11] are modifications of CE that have mainly been developed to enable separation of isomers and neutrals. Thus, with these techniques charged analytes and analytes having the same charge-to-size ratio can be simultaneously separated based on the combination of electrophoresis and chromatography. The techniques utilizing a pseudostationary phase (PSP), MEKC and MEEKC, have the advantage over CEC that a new chromatographic phase is applied in every separation. Thus, these techniques would have unique features for separation of samples in complex matrices. However, coupling of MEKC and MEEKC with mass spectrometric detection has been shown to be a great challenge. The most commonly used surfactant in MEKC, sodium dodecyl sulphate (SDS), has been shown to cause massive ionization suppression, ionization source contamination and increased background signals in electrospray ionization (ESI) mass spectrometry (MS) [12, 13]. Nevertheless, a few papers have reported on the direct coupling of MEKC with ESI-MS utilizing SDS as surfactant [14, 15]. Even though they could obtain the required LOD for the application, significant ionization suppression was found. Several different approaches have therefore been applied to solve these problems. Partial filling (PF) MEKC [16] and reverse migrating micelles have been used to prevent the surfactants from entering the ionization source. These techniques therefore require application specific optimization, with large restrictions in the background electrolyte (BGE) composition. Other procedures reported have involved quite complicated capillary connections constructed to dilute or remove surfactants from the separation capillary effluent [17-19]. Yet other approaches have involved volatile surfactants or high molecular weight PSP's. Despite these technical developments, coupling of MEKC and MEEKC with ESI-MS

has so far been shown to require careful optimization as well as suffer from limitations in the selection of the interaction phase.

An alternative approach to enable coupling of MEKC with MS detection involves the utilization of alternative ionization sources, such as atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) [20]. The ionization efficiency has been shown being unaffected by the surfactant SDS using both APCI and APPI [12, 15, 20]. However, ESI is the softest available atmospheric pressure ionization technique and therefore the most commonly used ionization technique today [21, 22]. Thus, it would be highly attractive to have at hand a technique enabling the coupling of PSP-based separations with ESI-MS detection.

In 2002, Viberg et al. [23] showed that it was possible to couple nanoparticle PSP-based CEC to ESI-MS with maintained ionization efficiency in absence of disturbing background signals and ion source contamination [24-26]. The technique was named continuous full filling (CFF), to distinguish it from PF that had previously been the only general technique available for coupling of PSP-based separations with ESI-MS detection (Figure 1).

Whereas the method parameters affecting the separation in CFF-CEC/ESI-MS have been thoroughly investigated [25, 26], the effects of parameters affecting the ionization efficiency have been left mainly uninvestigated. In the present study, a nanoparticle suspension is applied in CFF-CEC/ESI-MS for analysis of permanently neutral analytes optimized with emphasis on parameters affecting the ionization efficiency.

2. Materials and methods

2.1. Chemicals, sample solutions and nanoparticle suspensions

A dialkyl phthalate stock solution was prepared by dissolving dimethyl phthalate (23.8 g/L, 0.123 mol/L), diethyl phthalate (22.0 g/L, 0.098 mol/L), dipropyl phthalate (20.4 g/L, 0.082 mol/L), and dimethyl sulfoxide (DMSO) (66.0 g/L, 0.850 mol/L), all from Sigma-Aldrich (Schnelldorf, Germany), in methanol (Merck, Darmstadt, Germany). The stock solution was diluted 100-100 000 times in MilliQ water (Millipore, Bedford, MA, USA), to give samples with concentrations of approximately 1 000-1 μ M of each dialkyl phthalate. All chemicals were of p.a. grade or higher. All other solvents used were purchased from Merck (Darmstadt, Germany).

The BGE consisted of ammonium carbonate at pH 8.20 with an ionic strength of 50 mM (unless stated differently in the text).

Reversed phase nanoparticles were obtained from Nanosep AB (Lund, Sweden). The nanoparticles consist of a cross-linked hydrophobic polymer core and a surface consisting of a strong cation exchange material. The negative charge on the surface of the nanoparticle promotes the suspension stability of the otherwise hydrophobic particle. The diameter of the nanoparticles, determined by scanning electron microscopy (LEO 440), is 100 nm. The stock nanoparticle suspension in MilliQ water was diluted with acetonitrile and buffer to yield the nanoparticle BGE. In detail, 258.3 μ L nanoparticle stock (3.8 mg/mL), 371 μ L BGE, 371 μ L MilliQ water, and 500 μ L acetonitrile as organic modifier were mixed to yield a final organic modifier concentration of 33% (v/v) and an ionic strength

of 25 mM, unless stated differently in the text. The nanoparticle BGE was freshly prepared each day and ultrasonicated for 1 min prior to use in CFF-CEC-MS experiments.

2.2. Continuous full filling capillary electrochromatography

All experiments were performed on an Agilent Technologies HP^{3D} CE instrument (Agilent Technologies, Waldbroon, Germany) equipped with a 50 μm i.d. x 365 μm o.d. x 87 cm in length fused silica capillary column (Polymicro Technologies, Phoenix, AZ). The fresh capillary column was conditioned at 5 bar with 1.0 M sodium hydroxide, MilliQ water, and finally with separation buffer for 20 minutes each. The protruding distance between the nebulizing spray needle and the CE capillary tip was adjusted to 0.1 mm. Samples were injected hydrodynamically for 5 s at 50 mbar and separations were performed at ambient temperature with a field strength of 0.345 kV/cm. Between each run the capillary was rinsed for 2 min at 1 bar with nanoparticle BGE. The running buffer vial was replenished once every hour to avoid excessive buffer depletion. The sheath liquid consisted of 0.5 % (v/v) formic acid dissolved in methanol and MilliQ water (1/1, v/v) unless stated differently in the text. The sheath liquid was introduced with a flow rate of 20 $\mu\text{L}/\text{min}$ by a Jasco PU-980 HPLC pump (Jasco Inc., Easto, MD, USA) utilizing a fixed 1:9 split.

2.3. Mass spectrometry

Detection was performed in positive ESI mode using an electrospray G1603A Agilent Technologies sprayer on an Agilent Technologies series 1100 LC/MSD TOF mass spectrometer. The basic MS operating parameters were set according to following values: capillary voltage 4 000 V, nebulizing gas pressure 5 psi, drying gas temperature and drying gas flow of 250 °C and 7.0 L/min, respectively, unless stated differently in the text.

2.4. Software

Buffer compositions for BGE were calculated in PHoEBuS version 1.3 (Analis, Orleans, France). HP ChemStation was used for MS-data collection. The MS-data were then evaluated in AnalystQS Build 9865 (Applied Biosystems | MDS SCIEX). Plots were created in Microsoft Office Excel 2007 and the data of the sheath liquid composition was evaluated in Minitab 15.1.30.0.

3. Results and discussion

Nebulizer pressure and ionic strength

Previously, CFF has been performed in conjunction with high nebulizing gas pressure [25, 26]. The rationale for this has been to aid the flow of nanoparticles in the capillary direction, i.e. orthogonal to the mass spectrometer inlet. However, using a high nebulizing gas pressure may introduce a hydrodynamic flow in the capillary [27], which obliterates the efficiency gain obtained with the flat flow velocity profile generated by the electroosmotic flow (EOF). In the present investigation, the nebulizing gas pressure and the ion strength were varied to investigate the effects of these parameters on the separation efficiency (Figure 2). Whereas the spray formation was found to be stable within the parameter range investigated, a large effect of the nebulizing gas pressure on the separation efficiency

was found. The efficiency was found to be increased approximately 5-fold when decreasing the pressure below 18 psi. Within the investigated range, no significant effect of ionic strength was found. The mass spectrometer inlet was investigated after several months of analyses performed at the lower nebulizer pressure, but no indications of that nanoparticles had entered the mass spectrometer could be noticed within this excessive time frame. Also the capacity factors were found to be in general unaffected by the ion strength (Figure 2b). Thus, from the present investigation, it can be concluded that the nebulizing gas pressure should be kept low whereas the ionic strength is a less important parameter with respect to the performance of reversed phase CFF.

Nanoparticle concentration

The nanoparticle concentration is likely to affect both retention and separation efficiency. Increasing the nanoparticle concentration decreases the distance between the nanoparticles, thus improving mass transfer resulting in an improved performance. At the same time, the ratio of the phase volumes are increased, thus increasing retention. However, it was not known whether a very high nanoparticle concentration may have negative influence on the ESI. To investigate the influence of the nanoparticle concentration on the CEC and ESI performances, the concentration was varied between 0.9 mg/mL and 7.5 mg/mL. As expected, the elution time as well as the apparent retention increased with the nanoparticle concentration (Figure 3a-b).

Next, the influence of the nanoparticle concentration on the efficiency was investigated. It was found that the efficiency was highest at 3.8 mg/mL where after it decreased (Fig 3c). The increase in efficiency with increased nanoparticle concentration observed in the lower concentration range could be expected as a result of improved mass-transfer. The drop in efficiency above 3.8 mg/mL is somewhat unexpected although it might be related to some alteration in the nanoparticle phase behavior, e.g. aggregation or coating of the capillary inner wall. Alternatively, the cause of this drop could be related to compatibility problems between the sheath liquid and the nanoparticle containing BGE. The peak areas were unaffected by the nanoparticle concentration (Figure 3d), indicating that the ESI process is not disturbed by the nanoparticles. The signal-to-noise ratio (Figure 3e) followed the efficiency curve (Figure 3c), as would be expected from an unaltered peak area. Thus, from these studies it can be concluded that the optimal nanoparticle concentration is 3.8 mg/mL, and that the presence of nanoparticles in the electrospray does not influence the ionization efficiency. In a previous study on CFF-CEC/ESI-MS, Nilsson et al indicated that for dextran coated polymer nanoparticles, the optimal particle concentration was 5 mg/mL [24]. However, the data was not replicated in that study and no thorough analysis of the data was performed.

Sheath liquid and organic modifier

The composition of the BGE and the sheath liquid could, besides the influence on the chromatographic performance and the ionization efficiency, respectively, potentially affect the desorption of analytes from the nanoparticles in the ESI source. To investigate this, the organic modifier content in both the BGE and the sheath liquid was varied. It was found that a large variation in peak area and peak width

was achieved by altering the acetonitrile percentage in the BGE and sheath liquid (Figure 4). The peak area showed a local plateau at 25% acetonitrile in both the BGE and the sheath liquid for all analytes. However, a trend towards higher peak areas with increased acetonitrile content in the sheath liquid could be observed for diethyl phthalate and dipropyl phthalate. Thus, for the most retained analytes, an increased concentration of acetonitrile in the sheath liquid is potentially improving desorption of the analytes from the nanoparticles in the electrospray. Figure 4 indicate that greatly differing acetonitrile concentrations in BGE and sheath liquid may decrease the peak area. Thus, these results indicate that the acetonitrile concentration in the BGE and the sheath liquid should be similar to yield higher peak areas.

The surface plots generated for separation efficiency, clearly show that the optimal conditions would be to use high organic modifier content in the BGE and low in the sheath liquid. Although the efficiency was highest at the highest investigated acetonitrile concentrations, the retention and resolution was very poor at these conditions. For this reason, 25% acetonitrile in both BGE and sheath liquid was found being optimal, presenting a local maximum in the surface plots (Figure 4). Although the composition of the sheath liquid previously has remained uninvestigated the effects of the organic modifier concentration in the BGE has been investigated in two previous studies [24, 25]. These studies showed, as also shown here, that the efficiency increased while the resolution decreased with increasing acetonitrile concentration in the BGE.

Validation

Finally, the performance of the method was assessed in terms of repeatability, linearity, limit of detection (LOD) and limit of quantification (LOQ). The repeatability of CFF-CEC/ESI-MS is theoretically very high due to the application of a new interaction phase suspension in every new analysis. Thus, no sample adsorption, aging processes and packing variability should affect the performance. In a previous study on nanoparticle CFF-CEC/ESI-MS, the repeatability, expressed as %RSD (k'), was found to be 6.8 % in average [26]. In the present investigation the repeatability was found to be on average 5.0 % which is slightly lower than previously reported. The relative standard deviation for retention time, expressed as %RSD (t_R), is in the same range of what traditional CE can achieve (Table 1). The repeatability calculated should not be considered being a run-to-run repeatability, but rather being a column-to-column repeatability, as a new interaction phase is used in every separation. Thus, the repeatability presented here corresponds to 8 column changes performed during one working day when applying traditional CEC.

The linearity of the model indicates that no overloading is present on the nanoparticles in the evaluated concentration range (0.2-200 $\mu\text{g/mL}$). The LOD and LOQ were ranging between 0.16-1.52 $\mu\text{g/mL}$ and 0.52-5.07 $\mu\text{g/mL}$ respectively. This corresponds to 39, 13, and 3 fmol when injecting 5 nL for dimethyl phthalate, diethyl phthalate and dipropyl phthalate respectively. These results are in line with those from the previous investigation by Viberg et al. [26]. However, in the present and the previous study, different mass spectrometers were applied. Furthermore, in the previous study the LOD was defined as $S/N=2$, whereas we here apply $S/N=3$. Applying the previously suggested settings on our instrumentation showed that we have obtained a more than three-fold enhancement in the LOD.

4. Concluding remarks

In the present study the performance of reversed phase CFF-CEC/ESI-MS was significantly improved by a careful investigation and optimization of instrumental parameters related to the ESI-source. Extensive improvements in column to column variability and detection limits were achieved by fine-tuning the nebulizer pressure, nanoparticle concentration and sheath liquid composition.

The major benefits of CFF-CEC/ESI-MS are related to the ability to use a continuously regenerated stationary phase in combination with ESI-MS. This characteristic of the method is expected to be of great interest for applications in which samples in complex matrices, such as blood plasma, are analyzed. The ability of the method to completely avoid problems related to matrix adsorption and column aging in the analysis of complex samples is currently under investigation.

5. References

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Figure 1

Schematic picture showing the principle of CFF-CEC/ESI-MS. The nanoparticles in the BGE are transported through the open capillary allowing for a separation also of neutral analytes based on their differing partitioning to the nanoparticles. Despite the anodic migration of the nanoparticles, the EOF is sufficiently high to generate a net transport towards the cathode.

Figure 2

Effects of nebulizer gas pressure and ionic strength on the separation efficiency (a) and on the apparent retention of dimethyl phthalate, diethyl phthalate and dipropyl phthalate (b). The parameter settings are given on the x-axes.

Figure 3

Effects of nanoparticle (NP) concentration on elution time (a), apparent retention (b), separation efficiency (c), peak area (d) and Signal-to-noise ratio (e).

Figure 4

Surface plots indicating the influence of organic modifier in BGE and sheath liquid.

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Table 1

Estimated relative standard deviations (%RSD), linearity of models (R^2), detection- and quantitation limits (LOD and LOQ respectively) for the optimized conditions.

	%RSD (k')	%RSD (tR)	Linearity (R^2)	LOD (ng/mL)	LOQ (ng/mL)
DMP	8.34	1.0253	0.993	1.52	5.07
DEP	4.12	1.0437	0.982	0.56	1.88
DPP	2.44	1.0450	0.996	0.16	0.52

* LOD and LOQ are estimated from the lowest two concentrations applied. The calculations are based on a signal-to-noise ratio of 3 and 10 for LOD and LOQ, respectively.

a)

b)