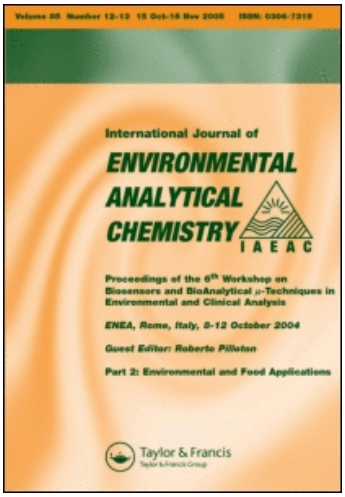

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Development of a capillary electrophoresis system coupled to sequential injection analysis and evaluation by the analysis of nitrophenols

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Development of a capillary electrophoresis system coupled to sequential injection analysis and evaluation by the analysis of nitrophenols

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A combination of a laboratory-made capillary electrophoresis system and a sequential injection analysis equipment is described. For characterization, the system was successfully applied to the separation and quantification of nitrophenols. A blue LED was used as light source, and hydrodynamic injection was carried out by using a pressure-stable solenoid valve and an inflatable pressure reservoir. A good reproducibility of migration time (0.5%) and peak heights (5%) were obtained. The calibration by using peak heights was found to be linear up to 776 $\mu mol\,L^{-1}$ for all three compounds. The system was robust and reliable for autonomous analysis without observation. All maintenance requirements including the conditioning of the capillary and flushing of both buffer reservoirs were carried out automatically. Instrumentation aspects of the capillary electrophoresis part are compared with former described hyphenated flow systems showing maximal operation versatility. Instrumental control and data evaluation were carried out using the software package AutoAnalysis.

Keywords: Nitrophenols; Capillary electrophoresis; Sequential injection analysis; Hyphenation

1. Introduction

Flow techniques such as flow injection analysis (FIA) [1] or sequential injection analysis (SIA) [2] are powerful tools for the automation of laborious procedures in analytical chemistry. The most noteworthy advantages of both techniques are the possibility of programmable handling of small liquid volumes reproducibly and precisely. In SIA, the sample treatment and analytical procedure are carried out according to an instruction protocol. In contrast to FIA, method optimization and adaptation can be carried out easily by modification of the flow protocol without the need for manual modification of the tubing manifold. By using a reliable control software and a robust instrumentation, SIA represents an ideal technique for monitoring purposes in

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industrial or biotechnological processes or for environmental research [3]. However, the selectivity of flow-technique approaches is generally limited to methodologies applying reagents or detection techniques, which are specific with respect to the analyte. The combination of flow techniques with a separation technique is therefore of great interest.

Capillary electrophoresis (CE) is a modern separation technique, which has gained considerable importance especially in biomolecular, pharmaceutical, and forensic analysis [4]. This can be attributed to its high separation efficiency, simple instrumentation, and variety of method modifications and the wide range of separable analytes, reviewed in a comprehensive synopsis [5]. Since CE does not require the implementation of high-pressure pumps, and aggressive solvents are seldom needed, CE is particularly suitable for coupling with flow techniques [6]. On the other hand, CE methodologies can benefit from this combination by the automation of required sample pretreatment [7], analyte derivatization [8], or calibration procedures [9] using flow techniques. Further, the main shortcoming of CE, the limited sensitivity, can be overcome applying on-line stacking [10, 11] or pre-concentration of the analyte on solid-phase columns [12, 13]. Two reviews have been published lately on this topic [14, 15].

A simple way of interfacing CE and flow techniques is the continuous exchange of the content of a vial on the sampler carousel of a commercial CE system [16, 17]. By using dialysis or gas diffusion membrane tube joints for on-capillary injection, the enrichment of gaseous or dissolved analytes and simultaneous separation from sample matrix was possible [18, 19]. Another way to connect a commercial CE instrument to flow techniques was described lately [20] with the use of a micro-injection valve to introduce a small section electrokinetically.

Fang and co-workers proposed a conical, permanently open capillary flow interface for FIA, where the sample was injected in electrokinetic mode [21, 22] or bias-free by traction by the electroosmotic flow (EOF) [23]. A permanently open split flow (SF) interface was used for coupling FIA [24, 25] or the SIA technique was used for home-made CE approaches [26–29]. However, since pressure application was not possible, manual maintenance was necessary for re-conditioning of the capillary, and only electrokinetic injection was possible.

By the intermediate blocking of the SF-interface outlet by using a valve [30] or a second peristaltic pump [11], electrokinetic as well as hydrodynamic injection was possible using FIA technique. Further, *in situ* flushing of the capillary is possible; however, the high-voltage buffer reservoir could not be flushed as necessary for long-term operation.

For one reason, the flow manifolds are generally connected only to the grounded side to avoid instrumentation damage [31]. Three principles have been applied to overcome this problem: the intersection of a deionized water or air segment between the flow interface and the high-voltage side [32], construction of system with components, which are not affected by the high voltage [32, 33], or by use of a falling-drop interface. Here, the sample and background electrolyte (BGE) are provided dropwise, and the interface is flushed by gravimetric flow. A small liquid volume remains between the capillary tip and high-voltage (HV) electrode. Since the galvanic contact of the capillary and the flow manifold is avoided, the normal electrode configuration is applicable, allowing amperometric [34] or fluorescence [35] end-of-capillary detection and injection [36] on the high-voltage side.

The aim of the present work was the construction of a capillary electrophoresis system coupled to SIA, which allows autonomous operation applicable for process monitoring, including automated buffer replacement at both sides of the capillary and the possibility of hydrodynamic injection. By using an inflatable pressure reservoir, *in situ* reconditioning of the capillary was possible in background operation. A single blue LED was used as light source due to its high emission stability. A synopsis about the instrumentation and operation versatility of recently proposed flow systems coupled to CE including this work is given in table 1. Details on the described analytical procedures could not be given, since the systems were used for the quantification of distinct analytes.

The presented system was characterized by the separation of nitrophenols (NPs). NPs are ideal model analytes due to their high molar absorbance values in the ultraviolet and shorter visible spectral range and their negative charge at already moderate basic condition, thus allowing separation without the need to reverse the EOF. They present harmful compounds with considerable mutagenic, cyto and phytotoxic effects, which can be found in different environmental compartments such as atmosphere or surface waters [37]. Simple analytical techniques for their determination are therefore of great interest. By using flow techniques, the in-line concentration of the analyte was carried out successfully allowing multi-component spectrometric analysis [38, 39]. Only a few works have been published to date on the separation of NPs by means of CE. Among these, nitrophenols have been used likewise as model substances for novel separation techniques implying the step change of the BGE composition [40], the use of calixarenes [41] or cyclodextrines and polyvinylpyrrolidone [42] as BGE additives. In this work, we applied similar separation conditions as in a recent work where the full separation of *ortho*-, *meta*-, and *para*-nitrophenol (*o*-NP, *m*-NP, and *p*-NP, respectively) was achieved by borate buffer as BGE and methanol as an organic modifier [43].

2. Experimental

2.1 *Interfaces*

Individual interfaces, acting as buffer reservoirs for the capillary, were developed to connect both sides of the capillary to the tubing manifold (figure 1). For the injection side, a grounded SF-interface was used of a similar design as reported previously [24]. The interface on the detection side was connected to the HV, and the concept of falling drops was implemented to achieve a galvanic separation from the SIA manifold [34].

2.2 *Flow system*

The implemented SIA system coupled to CE is depicted in figure 2. A valve module VA1 + 1 equipped with a rotary eight-port selection valve, a syringe module Bu4 S [44], and an autosampler device (Micro sampler) from Crison Instruments S.A. (Allela, Spain) were used for the SIA system. The sampler was used during optimization experiments for the automated change of the BGE. The syringe module had four additional supplying ports for powering and control of external devices. It was equipped with a 2.5-mL glass syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland).

Table 1. Instrumental comparison of coupling SIA or FIA via flow interfaces with CE.[a]

Flow-technique	Type of interfacing	Control of buffer reservoirs	Electro-kinetic injection	Hydro-dynamic injection	*In situ* conditioning	Pressure adaptation	Comments	Reference
LOV	A2	1	+	+	+	−		[8, 28]
SIA	Microvalve							[20]
SIA	A1 + B	1 + 2	+	+	+ (BG)	+		Present work
SIA	A2	1	+	+	+	+		[29]
SIA	A2	1	+	+	+	−		[7]
SIA	A2	2	+	+	+	+	Commercial apparatus	[32]
SIA	A1	1	+	−	−	−		[26, 27]
FIA	A2 + A2	1 + 2	+	+	+	−	Hydrostatic pressure for FIA system	[46]
FIA	A1 + A2	1 + 2	+	−	+	+		[33]
FIA	A1	1	+	−	−	−		[21, 22]
FIA	A2	1	+	+	+	−		[30]
FIA	A2	1	+	+	+	+	Two peristaltic pumps	[11]
FIA	A1	1	+	−	−	−	Injection by bias-free EOF-traction	[23]
FIA	B	2	+	−	−	−		[35, 36]

[a]A1: permanently open flow through interface; A2: intermediately closed flow through interface; B: falling drop interface.
1: at grounded injection interface; 2: at high-voltage injection interface; BG: in background.

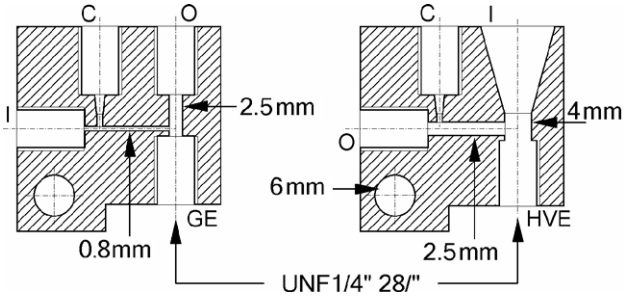

Figure 1. Grounded flowthrough interface (left) and high-voltage falling drop interface (right) with each of the four ports: flow in (I), flow out (O), capillary (C), grounded electrode (GE), and high-voltage electrode (HVE).

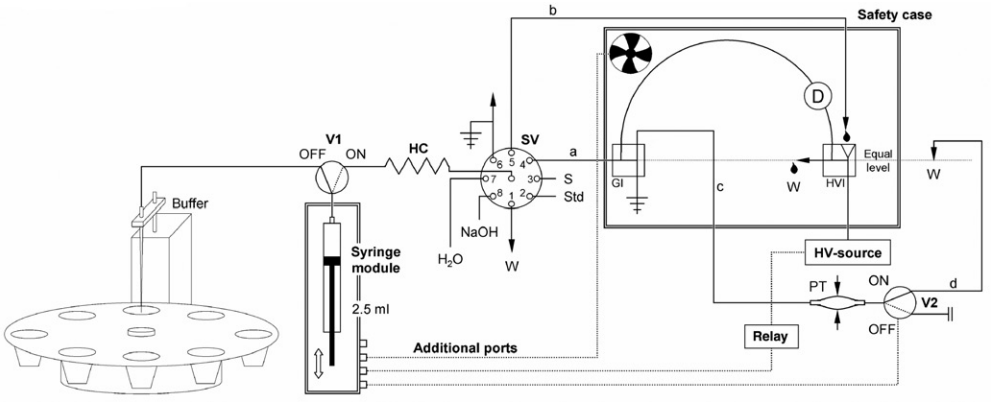

Figure 2. Scheme of the SIA–CE system. SV: selection valve (Pos. 6: air and electrical ground); GI: grounded interface; HVI: high-voltage interface; V1 and V2: solenoid valves; PT: pressure tube; HC: holding coil (175 cm, 0.8 mm i.d.); a: tube: 40 cm; 0.5 mm i.d.; b: tube 50 cm, 0.5 mm i.d.; c: tube 40 cm; 0.8 mm i.d.; d: tube 20 cm; 0.8 mm i.d.; W: waste; S: sample; Std: standard. Electrical connections are shown as dotted lines.

The head outlet of the syringe was connected to a multicommutation solenoid valve (V1) in order to enable the connection either to the manifold (ON, valve enabled) or to the carrier reservoir for refilling (OFF, valve disabled). A second solenoid valve (V2, shut in position OFF) was used to close the outlet of the SF-interface temporarily for pressure application for hydrodynamic injection or for flushing of the capillary for reconditioning, respectively. Both solenoid valves had a nominal pressure stability of up to 600 kPa (type MTV-3-1/4UKGH) and were purchased from Takasago Electric Inc. (Nagoya Japan). They were controlled via two supplying ports of the syringe module. Excessive valve heating was prevented by using protection circuits from Sciware SL (Palma de Mallorca, Spain). All flow instrumentation was controlled remotely by connecting both modules in series via an RS232C serial interface to a PC using the software package AutoAnalysis (see section 2.5).

The liquid contacted parts of the instruments and the tubing manifold were made of ethylene-tetrafluorethylen (ETFE), polyether-ether-keytone (PEEK), and polytetrafluoroethylene (PTFE). The tubing dimensions are given in figure 2. Narrow inner diameters were used for the tubes connecting the SIA selection valve to the grounded

and the HV-interface in order to reduce the dispersion of the sample (port 4, 0.5 mm) and to ensure the highest possible resistance (port 5, 0.5 mm) in the case of arcing, respectively. The dropping outlet of the tube connected at the selection valve port 5 was fixed about 2.5 cm above the miniature funnel of the HV-interface. Tubing with a wider inner diameter (0.8 mm) was used at the SF-interface outlet and for the holding coil to minimize backpressure while propelling the liquid through the interface and negative pressure during aspiration of solutions, respectively.

2.3 *Inflatable pressure reservoir*

A silicone tube (length 3.5 cm, 5.5 mm O.D., 1.5 mm i.d.) was placed between the grounded SF-interface and the valve V2. By propelling liquid to the SF-interface with V2 in position OFF (interface outlet closed), the silicone tube was expanded. As a result of the increasing tubing wall tension, pressure increased in the interface in relation to the dispensed volume.

2.4 *Capillary electrophoresis*

All experiments were performed using a 2127 Tachophor high-voltage supply (LKB, Bromma, Sweden). Remote control of voltage application was achieved by connecting the electrical contacts of the safety circuit of the HV-source to a relay, which was powered by one of the supplying ports of the syringe module.

Separations were carried out in an uncoated fused-silica capillary of 75 μm i.d. × 59 cm total length (53.5 cm effective length) from Polymicro technologies LLC (Phoenix, AZ). For fixation of the capillary, the ends were inserted into PTFE tubing (2 cm, 0.5 mm i.d.) and squeezed into the conical inlet of the interfaces using commercial fittings. The sealing was sufficiently tight to apply the required pressure for capillary flushing. Platinum wire (0.5 mm) was used for both electrodes. The wire was cemented into commercial fittings with commercial adhesive, and silicon tube slides (5 mm × 1 mm) were used for sealing.

On-capillary photometric detection was carried out using a home-made detection cell (figure 3). This consisted of two aluminium blocks with centred connections for an optical fibre, optical window, and aligned groove. To obtain a detection window, the polyimide layer of the capillary was charred on a length of 1 cm, and rests were removed with methanol. The capillary was fixed with commercial adhesive (UHU, Bühl, Germany) into the groove of the detection cell to avoid any mechanical stress in the fragile detection zone. An aperture of about 70 μm in width and 200 μm in length was made in both parts of the detection cell by diminishing the diameter of a drilled opening of 0.3 mm using black nail polish.

The cell was connected to an USB-2000 miniature spectrophotometer via an optical fibre of 400 μm core diameter (both from Ocean Optics, Dunedin, FL). As light source, a blue LED was placed directly onto the detection cell and powered by a home-made constant current supply. All measurements were done at the emission maximum of the LED of 400 nm with the detection parameters frequency 2 Hz, averaging 22 × 20 ms, and wavelength integration interval of 4 nm.

The capillary, interfaces, and detection cell were encapsulated in a PMMA safety box, and the interfaces and respective outlets were adjusted to the same level in order

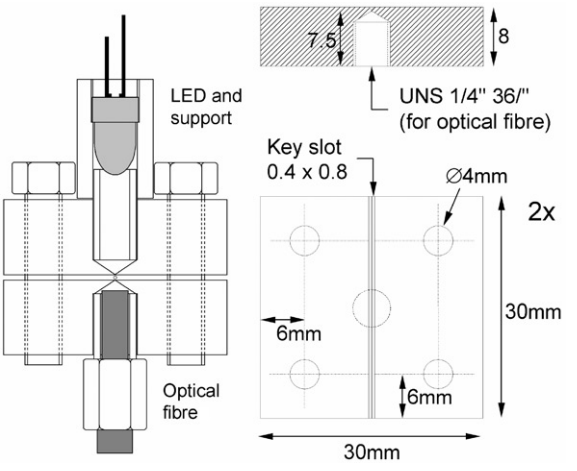

Figure 3. Detector device used for on-capillary application.

to avoid hydrodynamic flow in the capillary. By using an electrical switch, the remote control circuit of the source was interrupted, and the high voltage was turned off whenever the safety box was opened. The safety box was ventilated by using a computer processor fan for cooling to avoid a humid atmosphere.

2.5 *Software*

Instrumental control, data acquisition, and processing were performed using the software AutoAnalysis 5.0 (http://www.sciware-sl.com) [45]. The program involves a basic protocol to carry out an instruction method of instrumental operations. Specific dynamic link libraries (DLL) can be implemented when required in order to establish the communication with the assembled particular flow instrumentation and detector devices. The possibility of using variables, procedures, conditional definitions, loops, and waiting steps, and performing on-line data processing increases the versatility of the program. The analytical method applied in this work was made modular, incorporating specific procedures for flushing of the capillary, of each interface, injection, rising of the sample tube, removal of air bubbles in the grounded interface, and exchange of the BGE.

2.6 *Procedures*

Prior to the first use, the new capillary was pretreated with $1\,\mathrm{mol\,L^{-1}}$ of hydrochloric acid, $1\,\mathrm{mol\,L^{-1}}$ of NaOH and Millipore water, each for 30 min and flushed with BGE for 2 h.

For injection, a sample volume of $50\,\mu\mathrm{L}$ was propelled towards the grounded SF-interface. However, a sample volume of $100\,\mu\mathrm{L}$ had been aspirated into the holding coil prior to avoid sample dispersion. After discharge of the redundant sample from the holding coil, the sample slug was propelled to the capillary entrance with the outlet atmospherically open (V2 activated, ON). For hydrodynamic injection,

10 μL was dispensed with the SF-interface outlet closed (V2 deactivated, OFF). After 800 ms, the valve V2 was opened again, and the grounded interface was flushed with 200 μL of BGE. Finally, the high voltage was turned on by software commanded switch of the control relay. During separation, the interface outlet remained open (V2 activated) to ensure atmospheric pressure at both interfaces.

Before each injection, the capillary was re-conditioned by flushing for 2 min with BGE, and the buffer in the HV interface was exchanged. For *in situ* cleaning or flushing of the capillary, analogue to the hydrodynamic injection protocol, 300 μL of the cleaning solution or carrier was dispensed towards the SF-interface with the outlet closed. By inflation of the silicon tube, a pressure of approximately 3 bar was applied at the capillary entrance for about 10 min. Therefore, a continuous operation of the syringe pump as performed in former works [7, 28] was not required. Thus, syringe refilling (V1 in position OFF) or flushing of the HV interface could be carried out in the mean time (background mode). The pressure was relieved by activation of V2, thus opening the SF-interface outlet. Since an adequate miniature sensor was not available, the relation between the dispensed volume and the resulting pressure was estimated from the resulting capillary effluence.

Flushing of the HV interface was carried out by gravity flow entering dropwise through the miniature funnel. Afterwards, the tube connecting the selection valve port 5 to the drop outlet was emptied for safety precautions.

2.7 *Reagents*

Millipore® cartridge (Billerica, MA) filtered water was used throughout. All chemicals were of analytical-reagent grade. The BGE was prepared from a stock solution of 0.5 mol L^{-1} boric acid, brought to an apparent pH of 8.4 with sodium hydroxide. All stock solutions were filtered through 0.45 μm nylon membrane filters prior to use. Stock solutions of *o*-NP, *m*-NP, and *p*-NP (360 mg L^{-1}) were prepared in 1 mmol L^{-1} of NaOH and stored in the dark. Nitrophenols working solutions were prepared by serial dilution of the stock standard solution. For optimization experiments, a solution of 14.4 mg L^{-1} *o*-NP, 50.4 mg L^{-1} *m*-NP, and 7.2 mg L^{-1} *p*-NP in 1 mmol L^{-1} NaOH was used in order to avoid any great differences between the peak heights. Methanol (1% v/v) was added to mark the EOF.

3. Results and discussion

3.1 *Flow system operation*

To achieve high pressure stability and reliability in the flow system, the original syringe head valve was replaced by a more robust valve used as well at the grounded interface outlet. The flow channel of the SF-interface had a diameter of only 0.7 mm to reduce the sample dispersion and the required BGE volume for flushing. For the HV-interface, a wider flow channel was advantageous to ensure the electrical contact of the electrode and the capillary outlet in spite of gas bubbles generated by hydrolysis.

Flushing of the interfaces required 600 μL each; for injection, 500 μL of BGE was needed. A difference in level of 1 cm for both interface outlets did not show a significant influence on peak resolution. The system was found to work reliably without the need

for any intervention from the analyst, and most optimization experiments were carried out overnight. BGE of different composition to estimate the influence of each component concentration were placed on the autosampler. The SIA system was flushed four times, and the capillary was conditioned for 15 min with each new BGE solution during optimization. All experiments were carried out at least in triplicate. The standard deviations of repeated experiments are given in the tables.

3.2 *Selection of detection wavelength*

Taking into account the absorbance characteristics of the analytes, a detection wavelength of 400 nm was applied using a blue LED. Although the molar absorptivities of the three nitrophenols are considerably higher, applying deep ultraviolet wavelengths [43], the advantages of using 400 nm and an LED as light source were predominating as the high emission stability resulting in a very low baseline noise and detection selectivity for the analytes.

3.3 *Influence of BGE borate concentration*

The optimization was done with aqueous borate BGE, without and with addition of 10 v/v% methanol and apparent pH 9.4 in both cases. The influence of the borate concentration was studied in the range of 10–60 mmol L^{-1} (table 2). The migration time and the peak resolution increased for higher borate concentrations. However, baseline separation of *p*-NP and *o*-NP was only achieved using the organic modifier methanol. The highest peaks were found for a borate concentration of 40 mmol L^{-1}, and the current was acceptably low (40 μA), so this concentration was used for further experiments. Borate-carbonate was tested as an alternative buffer system [43], but the electrical current was considerably higher without any notable improvement on the peak resolution.

3.4 *Influence of buffer pH*

The EOF rate as well as the dissociation and mobility of the nitrophenols increase strongly with the pH. The effect of the apparent pH was studied only in the range of pH 8.5–10, where the analytes show high absorbance values at the selected detection wavelength (table 3). The experiment was run with a methanol content of 12.5% v/v methanol to ensure separation of both peaks for each pH value. The migration times

Table 2. Influence of the borate concentration of the BGE on the migration time of the solutes.[a]

Migration time (min) with SD ($n=3$)	Borate concentration (mmol L^{-1})					
	10	20	30	40	50	60
Meta-NP (a)	4.49 ± 0.01	4.89 ± 0.04	5.01 ± 0.14	5.39 ± 0.03	5.54 ± 0.09	5.76 ± 0.04
Para-NP (a)	4.77 ± 0.02	5.31 ± 0.04	5.51 ± 0.15	5.95 ± 0.04	6.16 ± 0.12	6.45 ± 0.05
Ortho-NP (a)	4.85 ± 0.01	5.41 ± 0.04	5.63 ± 0.16	6.07 ± 0.04	6.29 ± 0.14	6.59 ± 0.06
Meta-NP (b)	6.37 ± 0.05	8.47 ± 0.05	9.26 ± 0.05	9.56 ± 0.06	9.56 ± 0.16	10.47 ± 0.03
Para-NP (b)	7.02 ± 0.07	9.81 ± 0.08	10.71 ± 0.07	11.24 ± 0.07	11.24 ± 0.18	12.76 ± 0.03
Ortho-NP (b)	7.19 ± 0.06	10.15 ± 0.08	11.12 ± 0.07	11.71 ± 0.07	11.66 ± 0.17	13.30 ± 0.05

[a]Conditions: apparent pH 9.4; (a): without methanol; (b): 10 v/v% methanol.

Table 3. Influence of the apparent pH value on the migration time of the solutes.[a]

Migration time (min) with SD ($n=3$)	Apparent pH					
	8.5	8.8	9.1	9.4	9.7	10.0
Meta-NP	5.92 ± 0.04	7.23 ± 0.01	8.85 ± 0.01	10.49 ± 0.05	12.19 ± 0.04	13.43 ± 0.08
Para-NP	8.40 ± 0.05	9.63 ± 0.01	11.06 ± 0.02	12.38 ± 0.06	13.83 ± 0.04	14.87 ± 0.10
Ortho-NP	8.57 ± 0.05	9.91 ± 0.01	11.47 ± 0.02	12.89 ± 0.06	14.47 ± 0.04	15.59 ± 0.11

[a]Conditions: 40 mmol L^{-1} borate, 12.5 v/v% methanol.

Table 4. Influence of the BGE methanol content on the migration time of the solutes.[a]

Migration time (min) with SD ($n=3$)	Methanol content (v/v%)						
	0.0	2.5	5.0	7.5	10.0	12.5	15.0
Meta-NP (a)	6.19 ± 0.03	6.87 ± 0.04	7.86 ± 0.13	8.86 ± 0.04	9.92 ± 0.08	11.26 ± 0.04	13.05 ± 0.07
Para-NP (a)	6.70 ± 0.03	7.49 ± 0.04	8.71 ± 0.15	9.81 ± 0.05	11.11 ± 0.08	12.72 ± 0.04	15.08 ± 0.07
Ortho-NP (a)	6.86 ± 0.03	7.69 ± 0.05	8.99 ± 0.16	10.15 ± 0.05	11.55 ± 0.08	13.29 ± 0.04	15.90 ± 0.08
Meta-NP (b)	7.44 ± 0.03	7.71 ± 0.12	8.16 ± 0.05	8.89 ± 0.07	10.63 ± 0.02	11.09 ± 0.03	12.37 ± 0.15
Para-NP (b)	8.37 ± 0.04	8.81 ± 0.15	9.29 ± 0.06	10.29 ± 0.12	12.54 ± 0.03	13.19 ± 0.05	14.78 ± 0.18
Ortho-NP (b)	8.60 ± 0.03	9.08 ± 0.16	9.58 ± 0.06	10.68 ± 0.12	13.10 ± 0.04	13.83 ± 0.04	15.49 ± 0.19

[a]Conditions: 40 mmol L^{-1} borate; (a): apparent pH 9.7; (b): apparent pH 9.4.

Table 5. Influence of the separation voltage on the migration time of the solutes.[a]

Migration time (min) with SD ($n=3$)	Separation voltage (kV)			
	15	20	25	30
Meta-NP	22.98 ± 0.76	16.40 ± 0.40	11.80 ± 0.11	8.92 ± 0.10
Para-NP	26.90 ± 1.10	19.05 ± 0.45	13.77 ± 0.10	10.35 ± 0.09
Ortho-NP	28.24 ± 1.24	19.94 ± 0.46	14.46 ± 0.07	10.85 ± 0.10

[a]Conditions: 40 mmol L^{-1} borate, apparent pH 9.7, 10 v/v% methanol.

as well as the resolution of the *p*-NP and the *o*-NP peak increased considerably with the apparent pH, and a value of 9.7 was finally chosen as a compromise.

3.5 *Influence of organic modifier*

To separate the three NPs by decreasing the EOF rate and, thus, increase the separation time, methanol needed to be added as an organic modifier to the BGE, as reported previously [43]. The influence of methanol content was tested in the range of 0–15% v/v (table 4). The peak resolution increased considerably with higher methanol contents, and baseline separation was achieved at methanol concentrations higher than 10% v/v. A methanol concentration of 10% v/v was finally chosen for future experiments as the peak width increased notably with increasing migration time.

3.6 *Influence of separation voltage*

The influence of separation voltage was studied at 15, 20, 25, and 30 kV (table 5). The peak resolution decreased slightly for 25 kV and evidently at 30 kV, whereas

Table 6. Influence of holding time after injection pulse on the peak heights.[a]

	Holding time (ms)					
Peak height (m AU)	200	400	600	900	1200	1500
Meta-NP	1.57	1.84	2.05	2.42	2.66	2.85
Para-NP	5.50	6.50	7.52	8.80	9.99	10.78
Ortho-NP	0.64	0.74	0.82	0.99	1.13	1.13

[a]Conditions: 40 mmol L^{-1} borate, 25 kV, apparent pH 9.7, 10 v/v% methanol.

the migration times increased substantially for 20 and 15 kV. A voltage of 25 kV was chosen as a compromise between migration time and resolution. A significant increase in the baseline noise with the applied voltage as reported was not observed [43].

3.7 *Influence of injection parameters*

The injection parameters holding time, flow rate, sample volume, placement, and injection pressure were software-controlled and easy to manipulate for optimization. The hydrodynamic injection was carried out with a pressure pulse volume of 10 μL. Higher volumes required shorter and less reproducible holding times, whereas the reproducibility was likewise reduced at smaller dispensing volumes. The influence of the holding time after the pressure buildup was studied in the range of 200–1500 ms (table 6). Peak resolution and plate number decreased with longer holding times, whereas the sensitivity increased about 180% over the total range. A holding time of 800 ms was chosen to ensure baseline resolution for *o*-NP and *p*-NP at a maximal peak height.

3.8 *Influence of flow parameters*

The influence of flow rates and volumes applied for sample propulsion and injection at the peak heights was studied. At flow rates within the range of 1.5–3 mL min^{-1}, no significant influence was observed, whereas slightly wider peaks resulted in a flow rate of 1 mL min^{-1}. This was reduced to higher sample plug distortion due to the stronger contribution of the pulsation of the syringe pump. A flow rate of 1.5 mL min^{-1} was finally chosen. A minimal volume of 200 μL was used for rinsing the SF-interface after injection. Using lower volumes, the baseline increased notably beyond the last peak (*o*-NP). The dispense volume for the placement of the sample plug at the capillary entrance prior to the injection pressure pulse was found to be optimal with 40 μL.

4. Characteristics of quantitative analysis

4.1 *Calibration range and detection limit*

The linear response for all three analytes was found up to at least 776 μmol L^{-1} using peak heights for calibration. Since an LED was used as light source, the baseline noise was very low with a typical amplitude of 0.2 mAU. The detection limits, calculated as the concentration resulting in a peak height of three times the noise amplitude, were 22.5 μmol L^{-1} (*o*-NP), 68 μmol L^{-1} (*m*-NP), and 5.1 μmol L^{-1} (*p*-NP), respectively.

The high signal-to-noise ratio of the light source used compensated the loss of sensitivity applying detection at 400 nm compared with detection at deep ultraviolet. Thus, the detection limits were only 1.25, two, and seven times higher for *p*-NO, *o*-NP, and *m*-NP compared with values obtained with a commercial instrument applying detection at 191 nm [43], although the adsorptivity values were about four, 26, and 44 times lower, respectively. Nevertheless, pre-concentration is required for analysis of samples from environmental compartments.

4.2 *Reproducibility and recovery*

The method was found to be reproducible regarding migration times, peak heights, and peak widths with relative standard deviations of $<0.5\%$, $<5\%$, and $<8\%$, respectively ($n = 15$). The relative standard deviation using the peak area for calibration was about 33% higher than the relative standard deviation using the peak heights. Air bubbles stacked in the grounded SF-interface were removed by aspiration of air from the selection valve port 6 and propelling through the grounded interface, as they were of great concern to achieve a high reproducibility.

4.3 *Real samples*

For the evaluation of matrix influence, samples from a wastewater treatment plant were used. The samples were collected at the plant entrance (E), the outlet of the mechanical treatment (S1), and the final outlet after biological treatment and sedimentation (S3). The wastewater samples were filtered through nylon-membrane filters of 0.45-μm pore size prior to analysis. The concentration of the nitrophenols was below the detection limit for all samples. Thus, the samples were spiked with aqueous standards with final concentrations of 103.5, 362, and 51.8 μmol L^{-1} for *o*-NP, *m*-NP, and *p*-NP, respectively. The recovery values calculated using peak height data compared with aqueous standards are given in table 7. They were satisfactory for the sample S1 and S3 but reduced for sample E for *m*-NP and *o*-NP. The lower values found for the sample E for *m*-NP and *p*-NP can be explained by the higher content of dissolved organic matter which caused prolonged migration times of 2–3 min for the three samples and consequently broader peaks (figure 4). The capillary had to be cleaned with 0.1 M NaOH solution and water, and reconditioned with BGE after each electrophoretic run as described in section 2.6. In this way, the influence of the complex matrix on the analytical characteristics as alteration of the EOF by adsorption of matrix components on the inner walls of the capillary was reduced. The outcome of this is that the possibility of automated and *in situ* flushing of the separation capillary as well as the buffer reservoir control on both sides are important characteristics for a hyphenated CE system as presented (compare table 1).

4.4 *Peak widths*

Elevated peak widths were found throughout and independently from the sample concentration and the separation efficiency was relatively low with a theoretical plate number of 10^4. A decrease in the peak widths would lead to an equivalent increase

Table 7. Results of the determination of nitrophenols in spiked wastewater samples.[a]

Sample	Found nitrophenols (μmol L^{-1})			Recovery (%)		
	o-NP	*m*-NP	*p*-NP	*o*-NP	*m*-NP	*p*-NP
Plant entrance (E)	98.6 ± 9.3	311.0 ± 15.5	45.5 ± 4.1	95.3	85.9	87.8
Outlet of mechanical treatment (S1)	98.2 ± 9.7	336.2 ± 46.1	48.1 ± 5.3	94.9	92.9	92.9
Final outlet (S3)	103.4 ± 7.3	352.7 ± 24.0	50.2 ± 1.2	99.9	97.4	96.9

[a]Results are expressed as the mean of three determinations with standard deviation. Added nitrophenol standard 103.5 (*o*-NP), 362 (*m*-NP) and 51.8 μmol L^{-1} (*p*-NP) to each sample.

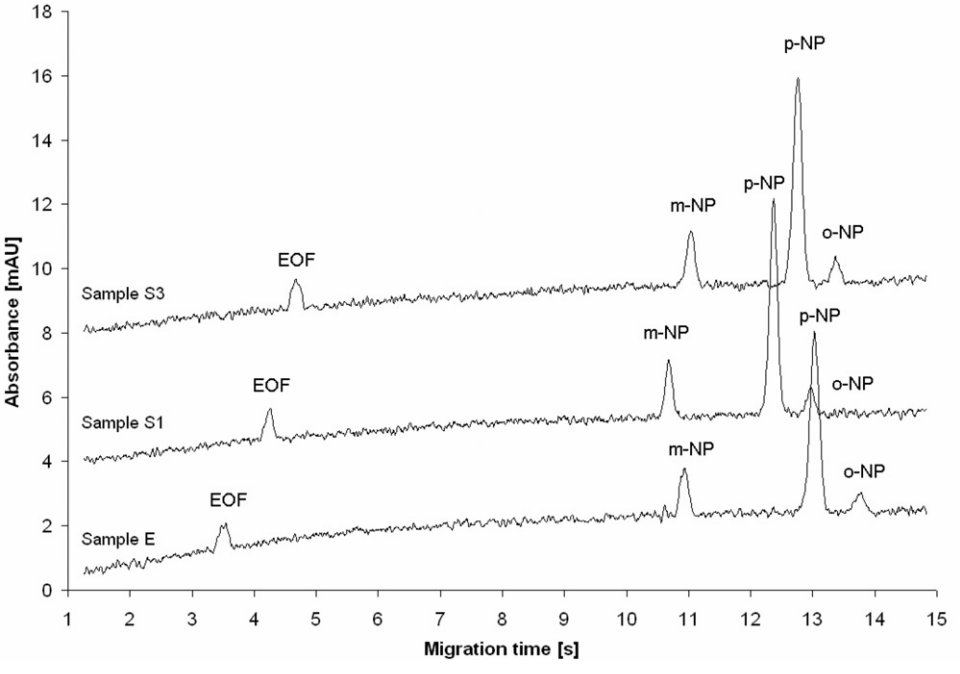

Figure 4. Capillary electrophoretic separation of *ortho*-, *meta*-, and *para*-nitrophenol (each 78.3 μmol L^{-1}). Baselines of samples S1 and S3 were shifted in 4 and 8 m AU, respectiviely.

in the sensitivity. Thus, attempts were made to ascertain possible causes for peak-broadening. Although finally no explanation can be given for peak broadening, the following points were excluded by experiments: laminar flow in the capillary by hydrostatic pressure, flow pulsation from the syringe pump, and pressure pulse from valve V2 on switching. Since the temperature in the safety box did not differ significantly from the room temperature of 25°C and the separation current was relatively low with about 40 μA, diffusion effects were supposed to be low.

Sample stacking by prior injection of water was tested; however, peak heights increased only about 30%, and the decrease in the peak widths was small, even after optimization of the water volume (10 μL pressure pulse, 100 ms holding time for the valve closure). Since stacking is affected by the ionic strength of the sample, the stacking procedure was not considered for quantification.

5. Conclusion

The proposed hyphenated system of SIA and CE was suitable for the separation and quantification of nitrophenols. *In situ* maintenance of the capillary and control of both buffer reservoirs and interfaces to the SIA apparatus was possible and automated. In comparison with previously described capillary electrophoresis flow systems, a maximal apparatus versatility has been achieved. All parameters of the performed hydrodynamic injection were software-controlled and adaptable. The system showed a satisfactory reliability, which allowed autonomous operation as required for monitoring purposes. The analytical method was reproducible, and the analyte recovery was satisfactory. Detection was done at a visible wavelength where nitrophenols show a reduced absorbance; however, using a highly stable LED and low baseline noise, the limits of detection were in the same range as those obtained for detection at deep ultraviolet wavelengths.

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