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Chromatographic preconcentration coupled on-line to capillary electrophoresis via a Tee-split interface

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Abstract

Solid-phase extraction (SPE) and capillary electrophoresis (CE) are on-line coupled via a Tee-split interface, which provides hydrodynamic injection of the SPE eluate by flow splitting. The interface allows sample preconcentration independently from the CE separation and prevents sample matrix and washing solvents from entering the separation capillary. The effect of the Tee-split interface on the CE efficiency was examined using enkephalin peptides as model compounds. Most favorable plate numbers were obtained using a split ratio of 1:40. Breakthrough volume, desorption efficiency and elution volume for the C_{18} micro SPE column (5 mm × 0.5 mm i.d.) were found to be 750 μL, 65% and 1 μL, respectively. The performance of the complete system was demonstrated by the preconcentration and separation of an enkephalin mixture. Plate numbers up to 120 000 were obtained using a sample volume of 250 μL and a split ratio of 1:40. Enkephalin peak areas were linear (R^2 = 0.996) over the 10–1000 ng/mL range. UV absorbance concentration limits of detection (S/N = 3) were about 5 ng/mL. For 250 μL injections of 100 ng/mL, the relative standard deviation (n = 5) of peak area was lower than 10%.

Keywords: Solid-phase extraction; On-line preconcentration; Tee-split interface; Peptides

1. Introduction

Capillary electrophoresis (CE) is a microscale analytical technique providing highly efficient separation of analytes in an electric field according to their charge-to-size ratio. CE is a fast technique and especially useful for the separation of biomolecular compounds such as DNA, peptides and proteins. The total volume of a CE system depends on the internal diameter and the length of the capillary and is in the range of only a few microliters. This means that sample loadability is restricted (typically 5–50 nL) in order to avoid band broadening by injection. The small internal diameter of the capillary (25–100 μm) also implies a short optical path length when on-capillary UV absorbance detection is used. Overall, these characteristics lead to relatively high concentration limits of detection (CLODs), which are generally insufficient for (biological) samples with trace-level components. Improvement of the sensitivity can be achieved by either longer light path lengths for UV detection, more sensitive detection methods (e.g. laser-induced fluorescence or mass spectrometry), or analyte preconcentration.

Focusing on analyte preconcentration techniques in combination with CE, a distinction can be made in electrophoretically-based [1–4] and chromatographically-based methods. Chromatographically-based techniques can offer two to four orders of preconcentration [5], and therefore are attractive to improve the CLOD in CE. The challenge lies in the effective and efficient combination of the chromatographic preconcentration system with the CE separation system. For this purpose, off-line [6,7], at-line [8,9], on-line [10–14] or in-line [15–17] coupling methods have been designed. From these modes, the on-line and in-line approaches are preferred because they offer shorter total analysis times, minimum of sample handling and possibility of automation. In-line systems, in which the chromatographic preconcentra-

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tor is incorporated in the CE capillary, can provide effective preconcentration. However, in-line modes suffer from disadvantages like low sample loading speed due to the high back pressure caused by the precolumn and the capillary, interference of the precolumn with the CE separation process, and introduction of sample matrix in the separation capillary. Moreover, the preconcentrator's elution volumes often exceed levels that are acceptable in CE and, thus, additional stacking procedures are needed to achieve good separation.

On-line systems, in which the chromatographic precolumn is separated from the CE capillary, can circumvent these problems. It requires an interface that should allow both the introduction of proper eluate volumes into the CE and an undisturbed performance of the CE system. As the elution volume of the on-line chromatographic preconcentrator is usually higher than the acceptable CE injection volume, often only part of the eluate can be injected into the CE. Several designs for on-line interfacing chromatographic and CE systems have been developed. In most systems, the effluent is led past the inlet of the separation capillary [10,13,18–23]. Electrokinetic injection of a small part of the effluent is then carried out by applying a voltage over the capillary, while the rest of the effluent runs to waste. Several designs used a valve and/or a Tee to couple the separation capillary to the effluent stream [18–22], while others used small vials [10,13,23], in which the CE inlet, the electrode, the effluent capillary from the chromatographic system and a waste exit are positioned. The flow-gating concept [24–26] for coupling LC with CE uses a somewhat different approach. Herein, a cross is used as interface, having the chromatographic and CE system connected on opposite sides of the cross, and two capillaries of relatively large internal diameter connected perpendicularly. A flow of background electrolyte (BGE) through the latter two capillaries continuously flushes away the chromatographic effluent to waste. At a fixed time, this flow is stopped, which allows electrokinetic injection of a part of the chromatographic effluent. Subsequently, the BGE flow is started again and high voltage is applied for CE analysis. Although the flow-gating concept has been quite successful, the interfacing is rather complex and requires accurate timing of events.

In another approach, the preconcentrator is positioned inside a valve [27,28]. The precolumn can be placed on-line with the CE by valve switching. Very recently, an elegant set up in which SPE and interfacing are integrated inside a cross design was described [29,30]. The stationary phase is held in the cross by four semipermeable membranes, allowing loading and washing of the preconcentrator in one direction, and desorption of trapped analytes into the other direction (i.e. the CE capillary). With these designs the separation capillary is not contaminated with sample matrix during loading, but the preconcentrator remains part of the CE system during analysis, which may disturb the CE performance. Bonneil and Waldron [12] designed a system in which the complete SPE eluate is on-line injected into the CE via a Tee. A capillary from the CE inlet vial is connected via a shut-off valve to the Tee. The other two ports of the Tee hold the CE separation capillary and the capillary from the chromatographic system. Injection occurs hydrodynamically while the valve is closed. After injection, the valve is opened and analysis is started. Unfortunately, quite low plate numbers were obtained.

Based on studies on sample introduction into the CE via a special valve [31–33], we recently have developed an on-line SPE–CE system with an in-line positioned valve as interface [14]. In this system, the eluate from the precolumn is first partially captured in the loop of the valve. Then, partial loop injections into the CE occur electrokinetically by switching the valve to the inject position for a fixed period of time and then returning to the load position while the electric field is on. In the present study, a simple and straightforward on-line preconcentration–CE system is described, in which only a micro Tee is used as interface between the SPE column and the CE capillary. The preconcentration-CE procedure includes analyte trapping of a relatively large sample volume, analyte desorption, hydrodynamic flow-splitted injection of the eluate into the CE via a Tee and subsequent separation. A small C_{18} column with high loading capacity and small elution volumes is chosen for preconcentration. To minimize band broadening after preconcentration, the column is placed in the permanent flow path and not in the loop position of a valve in the preconcentration system. The Tee is used as an interface that splits the eluate flow, one part going into the separation capillary and the other part to waste. The various components of the system have been optimized and the performance of the total system is demonstrated.

2. Experimental

2.1. Chemicals

Boric acid, sodium hydroxyde (NaOH) and acetonitrile (all analytical grade or higher) were obtained from OPG Pharma (Utrecht, The Netherlands), Biosolve BV (Valkenswaard, The Netherlands) and Bufa BV (Uitgeest, The Netherlands), respectively. Acetates of Leu-enkephalin, des-Tyr^1–[D-Ala^2–D-Leu^5]-enkephalin and [Met^5]-enkephalin were purchased from Sigma (St. Louis, MO, USA). All solutions were made with deionized and filtered water from a Milli-Q system (Millipore, Bedford, MA, USA). The mobile phase in the SPE system and the BGE in the CE experiments were solutions of 50 mM borate adjusted to pH 8.0 with NaOH. For experiments without and with preconcentration, peptide samples of 100 μg/mL and 100 ng/mL in water were used, respectively, unless otherwise stated.

2.2. Equipment

For the on-line SPE–CE system (Fig. 1), a CE system from either Agilent (Waldbronn, Germany) or PrinCE Technolo-

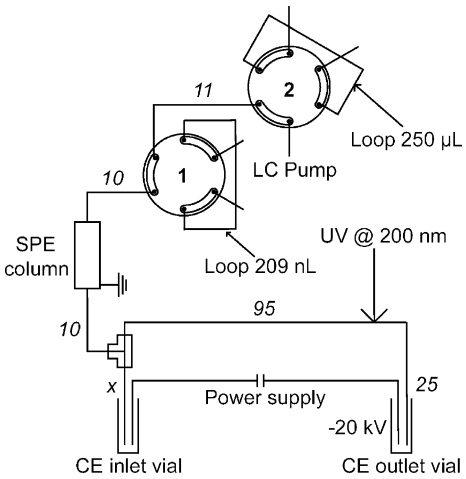

Fig. 1. Schematic diagram of the on-line SPE–CE system with the Tee-split interface. Lengths of fused silica (75 μm i.d.) are shown in italics (cm). Length *x* is varied (i.e. 3, 6, 9 or 12 cm). Valve 1 is a micro valve, valve 2 is a regular valve. See Section 2 for further details.

gies (Emmen, The Netherlands) was used, in conjunction with a small homemade inlet-vial assembly for external use. This assembly consisted of a 15-mL glass vial with plastic cap, in which holes were drilled for both an electrode and a fused silica capillary. In the case of the Agilent CE system, a dedicated cartridge was used to guide the separation capillary out of the apparatus. The electrode in the assembly was grounded, while a negatively charged voltage was supplied at the electrode inside the CE apparatus. UV detection was performed at a wavelength of 200 nm using the build-in photodiode array detector when using the Agilent system, or a model K-2501 detector (Knauer Wellchrom, Berlin, Germany) when using the PrinCE system. Fused silica capillaries with 75 μm i.d. × 375 μm o.d. (BGB Anaytik, Anwil, Switzerland) were used, unless otherwise stated. In the SPE system, a model LC10ADVP pump (Shimadzu, Kyoto, Japan) supplied eluent through the SPE column (PepMap C_{18}, 5 mm × 0.5 mm i.d., 5 μm particles with 100 Å pores, LC Packings—A Dionex Company, Amsterdam, The Netherlands). Either a 6-port or a 10-port micro valve (model M-435 or model V-472-1, Upchurch Scientific, Oak Harbor, WA, USA) was used for introduction of the sample (system without SPE column) or desorption liquid (system with SPE column). Port-to-port volumes of the 6- and 10-port valves are 44 and 51 nL, respectively. The length of the loop (fused silica, 50 μm i.d.) on the micro injection valves were 11.0 and 10.6 cm for the 10-port and 6-port micro valve, respectively, to ensure a constant injection volume of 260 nL. For introduction of 250 μL sample via a polyether ether ketone (PEEK) loop (Upchurch Scientific), a regular 6-port valve (model 7010; Rheodyne, Rhonert Park, CA, USA) was integrated into the system. As interface between the SPE and the CE, a micro Tee with a void volume of 29 nL (model P-775; Upchurch Scientific) was used. As safety precaution, the SPE column and the micro valve were grounded. Data acquisition was performed using the software supplied with the CE systems.

2.3. Procedures

Daily start-ups of the CE systems consisted of successively rinsing the capillaries with 0.1 M NaOH, water and BGE (each for 5 min at 1500 hPa). Each SPE–CE experiment was followed by a rinse of the separation capillary with water and BGE (each for 5 min at 1500 hPa) via the CE outlet, as well as a wash of the SPE column with three injections of 260 nL acetonitrile at a flow rate of 3 μL/min. The column was washed for 60 min with acetonitrile and water prior to first use.

The time schedule for operation of the system during an experiment is shown in Table 1. In brief, BGE was used to transfer the peptide sample (250 μL) from valve 2 to the SPE column via valve 1 and to rinse the SPE column (Fig. 1). Trapping and rinsing was done for 10 min at a flow rate of 50 μL/min, while applying 1500 hPa pressure on the BGE vial at the CE outlet. Desorption of trapped peptides was carried out at 3 μL/min with 260 nL acetonitrile, injected from valve 1 (Fig. 1). The desorbed peptides were partially injected into the CE system via the Tee; subsequently the pump was stopped and CE analysis was performed at −20 kV for 20 min.

3. Results and discussion

The total system in which the SPE and CE systems are coupled by the Tee is shown in Fig. 1. The micro-Tee in the system is used for on-line sample introduction of the SPE eluate into the CE system by flow splitting. The flow supplied to

Table 1
Time schedule for operation of the on-line SPE–CE system

Start time (min)	SPE system	CE system
0	Analyte trapping and washing of the SPE column at 50 μL/min after injection of 250 μL sample using valve 2 (Fig. 1)	1500 hPa pressure on BGE vial at CE outlet
10	Flow from 50 to 3 μL/min and waste vial at CE inlet replaced by BGE vial	No pressure at CE outlet
11	Injection of 260 nL acetonitrile using valve 1 (Fig. 1) for desorption of analytes from the trapping column	Partial injection of the elution plug into the CE separation capillary via the Tee; main portion of the plug is directed to waste
13	Stop flow	
13.5		Start CE analysis (−20 kV, 20 min)

the Tee is split in two parts: one part going through the separation capillary towards the detector, the other part through a relatively short capillary, further referred to as waste capillary, towards the CE inlet vial. The ratio of the resistances of both flow paths leaving the Tee determines the flow splitting. For capillaries with identical diameters, as used here, this implies an inversely proportional relationship of the flow split ratio and the ratio of the lengths of both capillaries.

In this study, the main interest was the performance of the Tee-split interface. The column was placed in the permanent flow path and directly attached to the interface to minimize its contribution to band broadening. In case of the analysis of biological samples, however, it might be better to position the column in a loop over a valve. Such a set up can decouple SPE and CE and, thus, avoids any contamination of the CE system. An additional LC pump will then be required.

3.1. Capillary electrophoresis with injection via a Tee

First, studies were performed to investigate the effect of the micro Tee in the CE system. For this purpose, a grounded micro valve was connected to the Tee via a 30 cm capillary. The Tee was integrated in the CE system in a similar fashion as depicted in Fig. 1. A flow of BGE is supplied by a pump via the micro valve to the Tee and the CE system. The valve allowed introduction of 260 nL sample into the system.

A specific time is needed to transport the sample from the valve via the Tee into the CE system. This time can be calculated from the volume from the loop on the micro valve to the Tee (i.e. 1.7 μL) and the flow rate. When pumping 3 μL/min, a minimum of 34 s is needed. However, sample will also flow into the waste capillary, which will be part of the CE system during analysis. So extra time was needed to remove the sample from the waste capillary prior to CE analysis. A total delay time of 2 min was chosen for all experiments. When using a 3-cm waste capillary, this delay time allows a 20-fold volume rinse of the waste capillary, while the sample plug enters only 1.7 cm into the separation capillary. After the delay time, the waste vial at the CE inlet was replaced by a BGE vial and CE analysis was started.

To test CE performance with different split ratios, the length of the waste capillary was varied. The selected waste capillary lengths of 3, 6, 9 and 12 cm provide split ratios of 1:40, 1:20, 1:15 and 1:10, respectively, resulting in CE injection volumes ranging from 2.5 to 10% of the sample volume arriving at the Tee. It was established that the plug volume at the Tee after a 260-nL valve injection at 3 μL/min was about 1 μL. Actually, this means that the CE injection volumes ranges from 25 to 100 nL using the Tee-split interface. In each system with one of the waste capillary lengths, 260 nL of a solution of des-Tyr^1–[D-Ala^2–D-Leu^5]-enkephalin (100 μg/mL in water) was introduced via the loop on the micro valve towards the T-split interface at a flow of 3 μL/min. After replacing the waste vial for the inlet vial filled with BGE, CE analysis was carried out at −20 kV (n = 2). A linear relationship between the split ratio and the peak area of the enkephalin were observed (Fig. 2a). As can be expected, plate numbers for the enkephalin was decreasing from 140 000 to 55 000 using split ratios from 1:40 to 1:10, respectively (Fig. 2b). With a normal CE system applying the same separation conditions and capillary length and diameter, injections of 25 nL yielded plate numbers for des-Tyr^1–[D-Ala^2–D-Leu^5]-enkephalin and [Met^5]-enkephalin of 210 000 and 200 000, respectively. This indicates that for a split ratio of 1:40 the Tee causes some band broadening, but the plate numbers are still rather high. For all further experiments, a split ratio of 1:40, i.e. a 3 cm waste capillary, was used to achieve favorable efficiencies. Of course, higher sensitivities could be obtained by using a lower split ratio and, consequently, introducing a larger sample volume, but at the expense of separation efficiency.

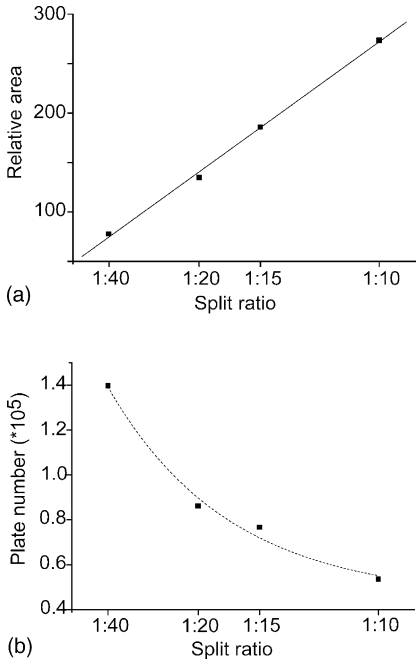

Fig. 2. Relative peak area (a) and plate number (b) of des-Tyr^1–[D-Ala^2–D-Leu^5]-enkephalin (100 μg/mL) versus split ratio (a linear scale for the x-axis is used) obtained with the CE system with the Tee-split interface. Analyses are performed at −20 kV. Shown data points are mean values of two measurements. See Section 2 for further details.

3.2. On-line solid phase extraction-capillary electrophoresis

For the coupling of chromatographic preconcentration techniques and CE several aspects are essential. Ideally, the precolumn should provide high volume loadability and allow high loading speed. Moreover, small elution volumes are desirable to achieve high preconcentration factors and to allow a maximum amount of analyte(s) to be injected in the CE system. Using a setup in which the micro column is directly connected to the UV detector, preliminary studies have been performed with the SPE column to investigate the volume loadability, elution volume and analyte recovery, in a similar

fashion as previously reported [14]. The breakthrough volume of the preconcentration column at a flow of 50 μL/min was found to be 750 μL. The elution volume was determined to be approximately 1 μL after a 260-nL acetonitrile desorption and the recovery was 65% using a desorption flow of 3 μL/min.

The complete on-line SPE–CE system (Fig. 1) was constructed and the system performance was tested. The total volume of two 10-cm capillaries and the dead volume of the SPE column between the micro valve and the Tee was similar to that of the previously selected 30-cm capillary. Therefore, the earlier selected delay time can be used in the SPE–CE system as well. During trapping of the analytes the sample matrix goes towards the Tee, where the total flow is split. This means that the sample matrix would fill the separation capillary. To prevent this, pressure (1500 hPa) was applied at the CE outlet vial during sample trapping and precolumn washing, flushing sample matrix to the waste vial.

The concentration and separation of a solution of 250 μL of two closely related enkephalins was performed as described in the Section 2. Reproducible baseline separations for samples with des-Tyr^1–[D-Ala^2–D-Leu^5]-enkephalin and [Met^5]-enkephalin were obtained (n = 5) with plate numbers of 120 000 and 105 000, respectively (Fig. 3). The relative standard deviation for enkephalin peak areas was 10% (n = 5). Probably, this rather high relative standard deviation is mainly caused by the SPE procedure. Comparison of the plate numbers with the data obtained with injection via the Tee but without the SPE column shows that the presence of the micro column is not contributing to the band broadening. Using nine solutions of des-Tyr^1–[D-Ala^2–D-Leu^5]-enkephalin with concentrations in the range of 10–1000 ng/mL, a linear relationship (R^2 = 0.996) between concentration and peak area was found. Fig. 4 shows the electropherogram obtained by the analysis of a mixture of enkephalins with a concentration of 10 ng/mL using the complete on-line SPE–CE system and this demonstrates that the CLOD is about 5 ng/mL. This means a gain in sensitivity of a factor of 200 compared to direct CE injection.

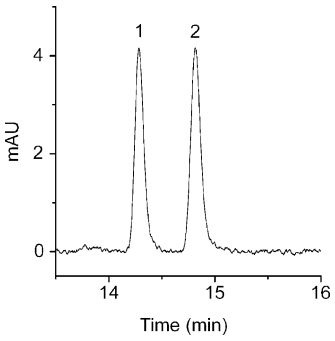

Fig. 3. Electropherogram of a mixture (100 ng/mL each) of des-Tyr^1–[D-Ala^2–D-Leu^5]-enkephalin (1) and [Met^5]-enkephalin (2) using the on-line SPE–CE system. Sample volume, 250 μL; split ratio, 1:40; analysis voltage, −20 kV. For further conditions, see Section 2.

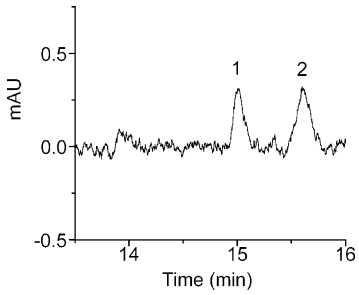

Fig. 4. Electropherogram of a mixture (10 ng/mL each) of des-Tyr^1–[D-Ala^2–D-Leu^5]-enkephalin, (1) and [Met^5]-enkephalin (2) using the on-line SPE–CE system. For other conditions, see Fig. 3.

4. Conclusions

It has been demonstrated that a Tee-split interface can be used for introduction of the eluate of a preconcentration column into a CE system in an on-line fashion. The design prevents the sample matrix from entering the CE separation capillary by applying pressure at the CE outlet during SPE. On-line preconcentration of relatively large sample volumes (250 μL) is accomplished, leading to CLODs of 5 ng/mL for enkephalin peptides. In principle, larger sample volumes can be loaded (up to the breakthrough volume (750 μL) of the SPE column), thereby further improving the CLODs. When the elution volume could be decreased by using smaller SPE columns, in principle a larger part of the eluate can be injected into the CE while maintaining high CE separation efficiency. This may lead to an improvement of the CLOD, although one has to consider the fact that smaller SPE columns also imply smaller breakthrough volumes (and thus smaller sample loads). Furthermore, when reducing elution volumes it should be noted that void volumes of valves and connections might become critical and limiting factors.

Overall, it can be concluded that the presented Tee-split interface provides an SPE–CE system that is relatively simple and easy to use. The set-up is well suitable for automation because disconnections or other manual steps are not required. The SPE desorption flow is split according to the relative resistances of the CE capillary and the waste capillary, so that the volume injected into the CE is representative for the whole SPE eluate. This is an important difference with the zone-cutting approach for the on-line SPE–CE system with an in-line injection valve, which has recently been developed in our group [14]. Although the latter allows injection of the most concentrated part of the SPE eluate zone, timing is less critical when using the Tee-split interface since this is less sensitive to variations in the desorption flow rate.

The detection limits (low-ng/mL range) achieved with the present set-up are low compared to similar designs [10–13,20,23,27], especially considering the relatively small sample volumes used. During CE analysis, the Tee-split interface does not cause serious band broadening and plate numbers of more than 100 000 have been obtained, which

is quite high when compared to other SPE–CE systems [8,10,12,27].

In the near future, the system will be used for the analysis of biological samples that will be extracted and concentrated by on-line SPE and separated by CE. Increased sensitivity and selectivity may be achieved by the use of special precolumns, e.g. immobilized antibodies and metal-affinity materials, and mass spectrometric detection. Another interesting approach is to use this set up for coupling LC with CE. A fraction of the LC eluate can be trapped on a micro column, which is then desorbed towards the CE.

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