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Research Article

Reassemblable quasi-chip free-flow electrophoresis with simple heating dispersion for rapid micropreparation of trypsin in crude porcine pancreatin

An increasing number of small biosamples (e.g. proteins and enzymes) need micropreparation in lab. However, neither large-scale free-flow electrophoresis (LS-FFE) nor chip FFE (C-FFE) could fit the growing demands. Herein, a simple quasi-chip FFE (QC-FFE) was constructed. In contrast to C-FFE, the features of QC-FFE are as follows: (i) its separation chamber is reassemblable and rewashable avoiding discard of C-FFE due to blockage of solute precipitation in chamber; (ii) its chamber size is 45 mm × 30 mm × (80–500) μm (108–654 μL volume) having function of micropreparation; (iii) there are up to 16 outlets in QC-FFE bestowing fine fraction for micropurification. The QC-FFE was used for the micropurification of model enzyme of self-digestible trypsin in crude pancreatin. Under the given conditions, the purification factor of enzyme was 11.7, the specific activity reached 6236 U/mg, the run time for 19 μL sample purification was 45 s and the throughput of trypsin was 3.34 mg/h, and the yield of pure trypsin was 55.2%. All of the results show the feasibility of enzyme micropreparation via QC-FFE. The developed device and procedure have potential use to other micropurification of protein or peptide sample.

Keywords:
Free-flow electrophoresis / Micropreparation / Micropurification / Porcine pancreatin / Trypsin
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1 Introduction

The micropreparation of biosample was of great importance for the concentration monitoring of analytes in vivo [1], protein analysis via mass spectrometry [2–4], and rapid analysis of protein interactions [5] as well as medical use [6]. Different techniques have been developed for the micropreparation, e.g. microdialysis sampling for continuously monitoring the extracellular concentration of compounds in vivo [1], microcolumn chromatographic separation [3, 6], electrophoresis-based techniques [7–9], etc.

Free-flow electrophoresis in large scale (LS-FFE) is a tool for continuous separation of solutes in a thin chamber aqueous environment enveloped between two parallel plates [10–12]. In free-flow electrophoresis (FFE), an electric field is applied perpendicularly to the flow to deflect charged analytes laterally into distinct streams based on their electrophoretic mobility. Four kinds of FFE techniques have been developed for bioseparation, including free-flow zone electrophoresis (FFZE), free-flow ITP (FFITP), free-flow IEF (FFIEF), and free-flow field step electrophoresis (FFFSE) [13–16]. FFE has the following merits [13–16]: (i) good preservation of biological activity due to mild aqueous circumstance, (ii) continuous sample preparation, (iii) high recovery, and (iv) low cost (except for free-flow IEF). These merits bequeath FFE with widespread potential application to bioseparation [13–16]. For example, Lanz et al. [17] and Gratz et al. [18] carried out the enantiomeric separation via LS-FFE. Loseva et al. [19], Kasicka et al. [20], and Malmstrom et al. [21] performed a series of significant investigations on separation of proteins or peptides via LS-FFE. Zischka et al. [22] implemented the separation and differential analysis of cerevisiae mitochondria by LS-FFE for proteomics study.

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Abbreviations: BPB, bromophenol blue; **C-FFE,** chip FFE; **FFE,** free-flow electrophoresis; **FFZE,** free-flow zone electrophoresis; **LS-FFE,** large-scale FFE; **QC-FFE,** quasi-chip FFE

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However, the commercial device was rarely used for the micropreparation of biosample owing to its large size.

Chip FFE (C-FFE) has been given great attention in the field of separation science in the last two decades [13–16]. In 1994, Raymond et al. [23] developed the first C-FFE device for bioseparation. In 2006–2010, Bowser and coworkers conducted a series of fundamental studies on the flow controlling [24], band and resolution optimizations [25], mitochondria mobility determination [26], and aptamer equilibria measurement [27]. A lot of works have demonstrated that C-FFE could be excellently used for the analyses of mitochondria [26], aptamers [27], and proteins [28] as well as the estimation of equilibrium constants [29]. Nonetheless, few of the works were reported on the micropreparation of biosample via C-FFE. For example, in 1994 Raymond et al. [23] introduced C-FFE as a tool of sample pretreatment. In 1996, they [30] fabricated a μ-FFE with 25 μL chamber volume for continuous microseparation. Last year, Han and coworkers [28] advanced cascade C-FFE for the microsorting of peptides and proteins. However, these issues hindered C-FFE for biosample micropreparation: (i) weak ability of preparation in virtue of tiny (20–134 μL) chamber volume; (ii) poor fraction due to a few of outlets used for sample collection; and (iii) easy blockage caused by precipitation (Supporting Information Fig. S1) in C-FFE chamber leading to the discard of C-FFE.

Furthermore, few of the works were described on the enzyme preparation via FFE. In 1989, Kuhn and Wagner [31, 32] used FFE for the purification of enzymes from E. coli cell extract. In 1990, Hoffstetter-Kuhn et al. [33–35] presented a scaleup FFE for the purification of alcohol dehydrogenase from a crude yeast extract. In 1990–1996, Weber and coworkers [36, 37] purified some enzymes from microorganism crude extract via FFE. FFZE was the most popular separation mode utilized for isolation of enzyme. The detail of principle can be found in [38]. However, these works [31–37] concerned large-scale separation rather than the micropreparation of enzyme.

To address the issues on the micropreparation of FFE [1–6], we developed a reassemblable and rewashable quasi-chip FFE (QC-FFE) device. It has 16 outlets for fractionation, hydrostatic-induced uniform background buffer and sample flows, and 108–654 μL chamber higher than that of C-FFE [39] but less than the one of LS-FFE [40–42]. The device could be well used for the micropurification of model enzyme of trypsin in crude sample. The following sections detail the relevant design and evaluation on QC-FFE, and the experiments on the micropreparation of trypsin.

2 Design of QC-FFE

2.1 Fractionation of reassemblable and rewashable QC-FFE

LS-FFE can provide very fine fraction, but its size is too large for micropreparation. C-FFE may offer a proper chamber size for micropurification [23, 28, 30] if its thickness is slightly increased, whereas the current C-FFE cannot present fine fraction collection because it is designed for the analytical objective rather than micropreparative purpose. Generally, it has three outlets for the drainage of electrolytic solution after passing through the C-FFE chamber [24–29]. To obtain fine fraction of QC-FFE herein, 16 outlets are designed as shown in Fig. 1. The 16 outlets used herein are greatly more than three outlets normally designed in C-FFE [13–16, 28, 30] and are the base for the fine micropreparation of biosample (Sections 4.3 and 4.4).

Figure 1 shows the simple disassembly of QC-FFE chamber. It was the modification of the previous LS-FFE chamber [40–42], The original LS-FFE device has been described in detail in [40–42]. The main modifications were that the size of separation chamber was 45 mm × 30 mm × (80–500) μm. The top and bottom plates could be clamped via C-shape clamps. Unlike a C-FFE chamber, the sandwich-like design makes the reassemblage and rewashing of chamber possible. Thus, solute precipitation in a run of QC-FFE might be removed by complete washing, and then the clean QC-FFE chamber could be easily assembled again. Obviously, the reassemblable and rewashable design can effectively avoid the discard of C-FFE chamber due to blockage induced by precipitation (Supporting Information Fig. S1).

In addition, the electrode baths were devised much deeper than the thickness of the separation chamber. The two electrodes were located near the bottom of electrode bath. The bubbles within the two electrode baths could be quickly removed via a pump, effectively avoiding the interference of bubbles to IEF run [43].

Supporting Information Figure S2 shows the simple but effective design on the cooling of QC-FFE chamber.

2.2 Adjustable volume of QC-FFE chamber

In Fig. 1A, a triple layer with 80–500 μm plastic film spacer champed between two glass plates. The size of separation chamber was 45 mm × 30 mm × (80–500) μm, corresponding to 108–654 μL chamber volume based on the thickness of plastic spacer. Actually, a thinner (e.g. 60 or 40 μm) or a thicker (600–1000 μm) plastic film can be chosen as a spacer of QC-FFE chamber. It is observed in our experiments that a thinner film used can easily result in the precipitation-induced blockage of chamber (Supporting Information Fig. S1). Evidently, the size of developed QC-FFE device was slightly higher than the size of C-FFE (20–40 mm × 20–56 mm × 20–60 μm, the corresponding volume 20–134 μL) [39], but greatly less than that of LS-FFE (e.g. 400 mm × 182 mm × (0.4–1.2) mm = 56.2–87.4 mL [40–42]).

The adjustable volume well fits the micropreparation of biosample. The volume adjustment is achieved by the factors: (i) thick adjustment of plastic film ranging from 80 to 500 μm (Fig. 1A), (ii) the PAG membrane located at the two sides of chamber (Fig. 2), and (iii) the battlement-like

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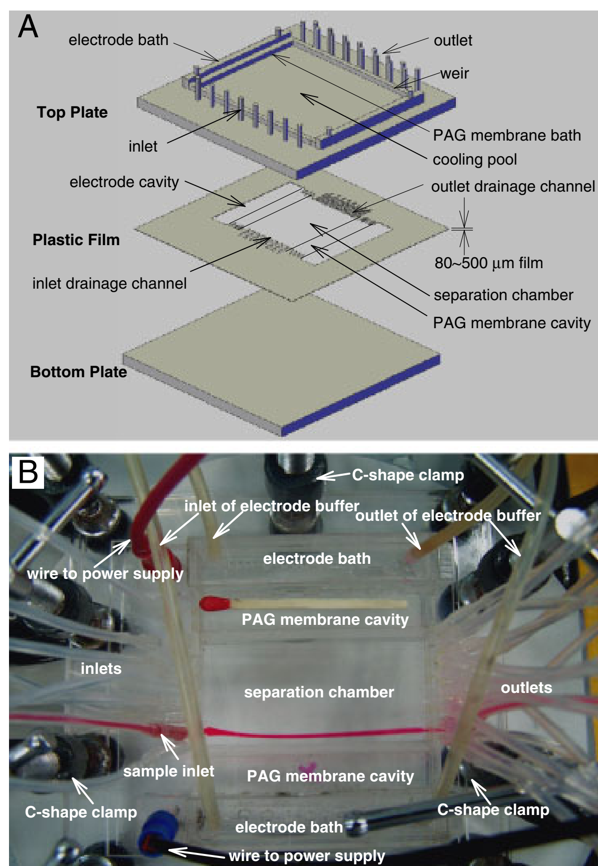

Figure 1. The schematic diagram (A) and the simple integration of QC-FFE separation chamber via several C-shape clamps (B).

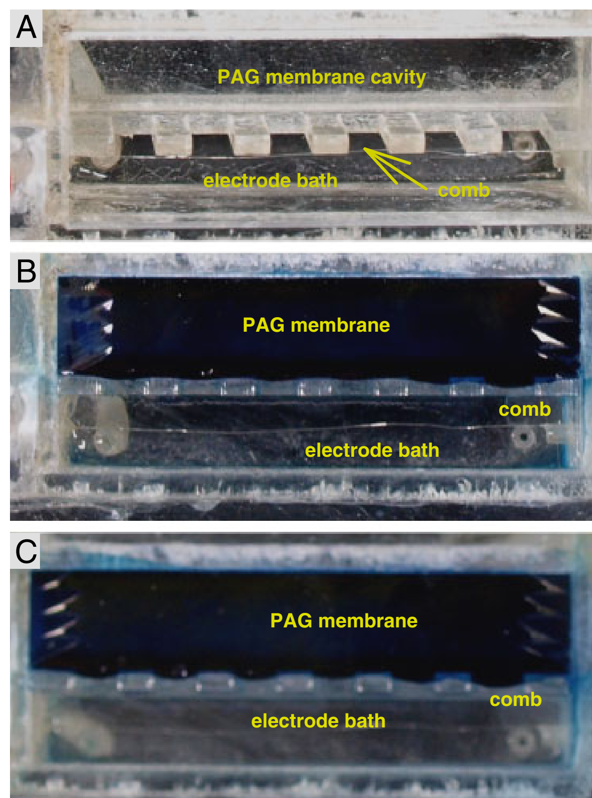

Figure 2. Adjustable volume of QC-FFE separation chamber via the thickness adjustment of the plastic film spacer, the whippy PAG membrane, the battlement-like comb and the pressure of C-shape clamp. (A) the electrode bath, the PAG membrane cavity and the comb within the top plate of QC-FFE; (B) one side of top plate assembled with the plastic film spacer and the PAG membrane highlighted with a little blue stain without using C-shape clamps; and (C) tight water-proof isolation between separation chamber and electrode bath via wave-like PAG membrane slightly out of its cavity through the comb, thanks to the use of bottom plate and C-shape clamps.

comb sat between the electrode and the PAG membrane cavities (Figs. 1 and 2). Figure 2A shows the design of electrode bath and PAG membrane cavity and the comb within the top plate. Figure 2B displays the assemblage of the PAG membrane highlighted with blue stain, the plastic film spacer, the top plate, and the bottom plate without use of C-shape clamp. It is manifestly shown in Fig. 2B that the PAG membrane keeps in good shape in its cavity. The pressure, however, presses the PAG as battlement-like membrane slightly out of its cavity through the comb, and the thickness of PAG membrane automatically fits the 80–500 μm plastic film spacer (Fig. 2C) if the C-shape clamp is used. Note herein, the height of PAG membrane over the top plate ought to be slightly higher than the thickness of plastic film (Fig. 2C), or the water-proof ability of PAG membrane becomes poor.

2.3 Whole QC-FFE system

Figure 3A shows the diagram of whole QC-FFE system. The chamber has 8 inlets and 16 outlets. Seven inlets (the blue ones) are set for the input of background buffer, and the remaining one (the red one) is used for sample injection. Through silicone hoses, the 16 outlets are connected to the 16 1.5-mL Eppendorf tubes used as the sample collectors. A 16-channel pump (BT-1001L, Longer Pump, Baoding, China) is used to drive the flows of background buffer and sample solution in whole FFE system. The anodic and cathodic buffers are flowed by a peristaltic pump (HL-2, Huxi Scientific Instrument, Shanghai, China). A power supply (DYY-6C, Beijing Liuyi Instrument, Beijing, China) is used for electrophoretic separation in the QC-FFE. Figure 3B further shows the real integration of FFE system.

2.4 Hydrostatic-induced uniform flow in QC-FFE

Figure 3A shows the principle for the uniform background flow and sample injection induced by the hydrostatic and the 16-channel pump. If the pump is turned on, the air in FFE chamber is removed and the subpressure is formed in QC-FFE chamber as shown in Fig. 3A. As a result, the

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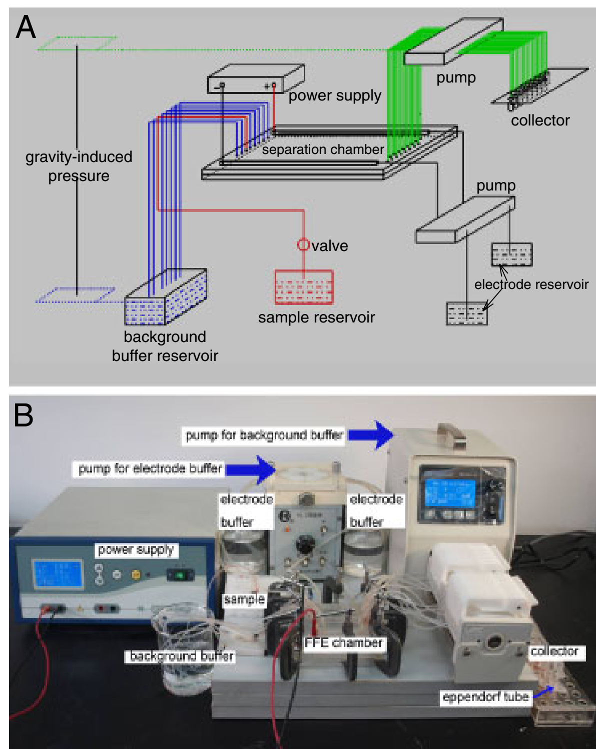

Figure 3. The schematic diagram of gravity-induced uniform background flow in QC-FFE device (A) and the assemblable QC-FFE system (B).

pressure difference is created by the hydrostatic between the liquid level in the background buffer reservoir and the gas/liquid level of the 16-channel pump. The pressure difference further leads to the simultaneous rising/falling of liquid level in inlets, or FFE chamber, or outlets, based on the direction of 16-channel pump. Consequently, uniform background and sample flows are established as soon as the separation is started (Fig. 1B and Supporting Information S4).

The use of hydrostatic herein is distinctly dissimilar to that in the previous works on the LS-FFE [40–42]. In the QC-FFE, the hydrostatic-induced pressure difference is formed between the liquid level of the background buffer reservoir and that of outlets positioned at the 16-channel pump (Fig. 3A), whereas in the LS-FFE with hydrostatic-induced uniform background flow, the pressure difference is induced between the liquid level in gas cushion injector and that in sample collecting tubes [40–42].

3 Materials and methods

3.1 Chemical reagents

Bromophenol blue (BPB, biological reagent [BR]) and Tris (biological reagent) were purchased from Sinopharm (Shanghai, China). Formic acid, sodium hydroxide, sodium dodecyl sulfate, and ammonium persulfate (analytical reagent grade, AR) were purchased from Shanghai Chemical Reagent (Shanghai, China). Acrylamide and Bis-acrylamide were bought from Sigma-Aldrich China (Shanghai, China). *N,N,N′,N′*-tetramethylethylenediamine (TEMED) was obtained from Sigma (USA). All other chemicals were of analytical reagent grade from local companies. Horse heart cytochrome *c* (CytC, Mr = 13 kDa, p*I* = 10.7) was obtained from International Laboratory, USA. Crude pancreatin of hog pancreas with some enzyme activity inhibitor was purchased from aladding-reagent (Shanghai, China). Protein molecular mass markers and Ncu-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Beyotime Institute of biotechnology (Jiangsu, China).

3.2 Instruments

All solutions were prepared with deionized water (5.5×10^{-6} S/m) which was first made by an automatic distiller (SZ-93, Yarong, Shanghai, China) and then by a commercial ultrapure water system (SG Isserauf-bereitung und Regenerierstation Gmbh, Germany) unless otherwise noted. A type-320 pH Meter (Mettler-Toledo, Switzerland) was used for the preparation of buffer. The pH Meter was calibrated with three kinds of standard solutions with pH values of 4.00, 6.86, and 9.18 (Degussa, dus, Nordrhein-Westfalen, Germany) in advance.

3.3 Preparation of buffers

An aliquot of 500 mM formic acid solution was prepared and used for the acidic precipitation of crude pancreatin sample. About 60 mM formic acid was adjusted to pH 3.0 with 1.0 M NaOH solution and then double diluted to obtain 30 mM, pH 3.0, formic acid–NaOH buffer for these runs of QC-FFE. The running buffer used in SDS-PAGE was 62.4 mM, pH 6.8, Tris-HCl with 0.01% m/v BPB, 2% m/v SDS, and 350 mM DL-dithiothreitol (DTT). The sample loading buffer for SDS-PAGE was 62.4 mM, pH 6.8, Tris-HCl with 0.01% m/v BPB, 2% m/v SDS, 25% v/v glycerol, and 350 mM DTT. The glycerol used in the buffer was to increase the density of the sample buffers for stable sample loading before a run of SDS-PAGE. The enzyme assay buffer was 50 mM, pH 8.0, Tris-HCl with 1.0 mM BAEE (benzyl-L-arginine ethyl ester) and 50 mM $CaCl_2$.

3.4 Acidic precipitation and trypsin sample

The acidic precipitation for the purification of pancreatin was described in detail in [44, 45]. It was observed that the crude pancreatin precipitated greatly near the sample stream in the QC-FFE chamber if the crude sample was directly injected into the QC-FFE (Supporting Information

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Fig. S1). As the pH value of crude pancreatin was far from 3.0 of formic buffer used in the QC-FFE, the pH difference between the original sample and the background buffers might denature some proteins, resulting in the precipitation in Supporting Information Fig. S1. The crud sample was suspended in 30 mM, pH 3.0, formic acid–-NaOH buffer and a substantial amount of insoluble material was removed by centrifugation at $3000\times g$ for 10 min at room temperature. After then, 500 mM formic solution was added to the sample solution until pH 3.0 and the precipitation was eliminated via a centrifugation, then the supernatant was used as the trypsin sample for QC-FFE separation. The formic acid used has two effects: precipitating impure proteins with pI values near 3 and changing the crude sample as proper acidic matrix for the QC-FFE run.

3.5 SDS-PAGE

Classic SDS-PAGE was used for the purity analysis of sample fractions collected from the QC-FFE micropreparation. Samples (5 μL) collected from 16 fractions of QC-FFE were mixed with 5 μL sample loading buffer of 62.4 mM, pH 6.8, Tris-HCl with 0.01% m/v BPB, 2% m/v SDS, 25% v/v glycerol, and 350 mM DTT, and then the mixture solutions prepared were incubated at 25°C for 5 min before sample loading of SDS-PAGE. The run of SDS-PAGE was carried out with DYCZ-24DN electrophoretic system (Beijing Liuyi Instrument) at 90 V. After the run, the gels were stained with the Coomassie® Brilliant Blue R solution with 8% v/v acetic acid and 25% v/v ethanol, and destained with the solution with 8% v/v acetic acid, and 25% v/v ethanol.

3.6 Procedure of trypsin assay

Enzyme assay was performed for monitoring activity of different fractions collected from the QC-FFE. The assay was conducted in accordance with the method of Rick [46]. Three milliliter of 1.0 mM BAEE (benzyl-L-arginine ethyl ester, 50 mM $CaCl_2$, and 50 mM, pH 8.0, Tris-HCl) and 0.2 mL of diluted enzyme solution was added into the quartz cell. The change in absorbance at 254 nm was recorded every 30 s for 4 min via a UV-2450 UV–VIS (Shimadzu, Japan). The initial rate was calculated for determining the enzyme activity. An increase of absorbance by 0.001 absorbance units per minute is defined as 1 unit of tryptic activity.

3.7 Determination of protein concentration

The total protein concentrations of different enzyme sample matrixes and fractions collected from the micropreparation of QC-FFE were analyzed with a ST360 microplate reader for ELISA (Shanghai Kehua Bio-Engineering, China). The amounts of protease present in different fractions were calculated with BCA protein assay kit [47]. The BSA was dissolved in 30 mM, pH 3.0, formic acid–NaOH buffer, and then the standard curve was constructed with BSA in the wavelength of 570 nm. The BCA protein assay could be found in detail in the synopsis offered by Beyotime Institute of Biotechnology (Jiangsu, China).

4 Results and discussions

4.1 Evaluation of QC-FFE

Joule heating can result in the convection in FFE chamber and is one of the factors for instable run of FFE. Fonslow and Browser [48] have observed that: (i) a linear Ohm's plot of current versus voltage was present at a certain condition if the temperature in FFE chamber was under proper control; (ii) if excessive Joule heating was out of control, a positive deviation from the ideal linearity would be presented because of the decrease of viscosity and increase of electrolyte conductivity; and (iii) if there was excess controlling, a negative would occur.

Supporting Information Fig. S3 shows the Ohm's plots at different background fluxes of 0, 35.7, and 168.5 μL/min per inlet. It was observed in Supporting Information Fig. S3A–C that there was no obvious positive or negative deviation in the QC-FFE with the ice-water coolant in the pool (Supporting Information Fig. S2). We observed that no evident bubbles were formed in the QC-FFE chamber even if the voltage was up to 300 V and the run time was up to 1 h under the condition of ice-water cooling. Although if there was no ice-water cooling, numerous bubbles were produced in the chamber within 5 min, greatly affecting the microseparation. The results imply that the simple ice-water cooling could effectively disperse the Joule heating.

The background flow is quite stable. The flow line of red ink in Fig. 1B implies stable hydrodynamic environment within the QC-FFE chamber (without use of electric field). The results of crude pancreatin and its precipitation flows in Supporting Information Fig. S1 and Cyt *C* flows in Supporting Information Fig. S4 further manifest the stability of FFE chamber under the electric field. In Supporting Information Fig. S1, the evident protein precipitation caused by improper sample buffer did not affect the stable background flow and sample stream in the QC-FFE chamber. Supporting Information Fig. S4 further shows the stable hydrodynamic flows of Cyt *C* in QC-FFE without (Supporting Information Fig. S4A) and with (Supporting Information Fig. S4B) use of electric field.

4.2 Acidic precipitation

Herein, trypsin was used as a model enzyme for demonstrating the feasibility of micropreparation via QC-FFZE. However, the crude pancreatin sample is very complex, comprising numerous impure proteins, as shown by Lane II

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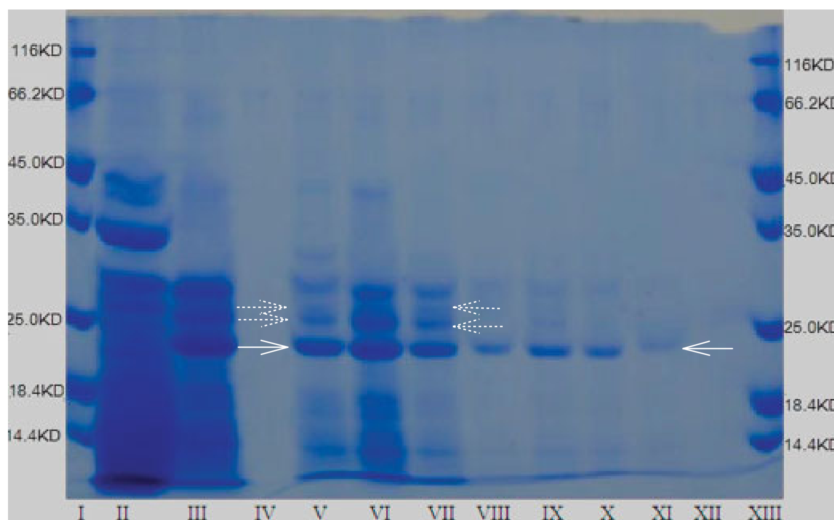

Figure 4. SDS-PAGE of the crude pancreatin and fractions collected from the QC-FFE. Lanes I and XIII indicate the protein molecular weight mark; Lanes II and III indicate the crude pancreatin and the enzyme sample collected after acid precipitation via 500 mM, pH 3.0, formic acid–NaOH buffer, respectively; Lanes IV–VI correspond to Fractions 4–6 collected from QC-FFE without use of electric field, respectively; Lanes VII–XII mean Fractions 7–12 collected from QC-FFE with use of 100 V/cm, respectively. The conditions of SDS-PAGE: 10 μL sample load for each lane, 90 V, 3 h run. The solid arrow indicates the trypsin band (23.8 kDa), the upper and lower break arrows mean the elastase (25.9 kDa) and chymotrypsin (25.0 kDa), respectively.

Table 1. Comparisons of total protein, total enzyme activity, yield, specific activity, and purification factor of crude pancreatin sample, the one after acidic precipitation and these ones from QC-FFE micropurification under different voltages

	Total protein (mg)	Total activity (BAEE units)	Yield (%)	Specific activity (BAEE units/mg)	Purification factor
Crude pancreatin	80	42 560	100.0	532.0	1.0
Acid precipitation	7.02	40 040	94.1	5703.7	10.7
33 V/cm (7, 8)	2.82	16 384	38.5	5834.3	11.0
67 V/cm (7–10)	4.43	20 846	49.0	4702.3	8.8
100 V/cm (8–11)	3.78	23 478	55.2	6227.6	11.7

in Fig. 4. Acidic precipitation has been used as a normal step for the purification of pancreatin [44, 45]. Lane III in Fig. 4 reveals the results of SDS-PAGE for the pancreatin sample after the acidic precipitation, showing the removal of some impure proteins from the crude sample. Table 1 further unveils that (i) the total protein is decreased from 80 to about 7.0 mg, (ii) the enzyme-specific activity is increased from about 532 to 5704 U/mg, and purification factor is up to 10.7, demonstrating the clear separation of enzyme from the impure proteins via the acidic precipitation.

4.3 Concise optimization on trypsin separation in QC-FFE

There were several valuable proteases in crude porcine pancreatin, including trypsin, chymotrypsin, and elastase [44]. There are similar physical, chemical, and biochemical properties and substrate specificity among the three enzymes. The molecular weights of trypsin, chymotrypsin, and elastase are 23 800, 25 000, and 25 900, respectively, and their p*I* values are 10.8, 8.1, and 8.1, respectively [44]. Trypsin has an anionic subtype in porcine pancreatin [44]. It is stable and has low activity of self-digest in pH 3.0, whereas becomes unstable and has obvious change (including self-digest change) if the pH over 5.0 or below 2.0, leading to loss of enzyme activity [45]. Thus, in the QC-FFE run, 30 mM, pH 3.0, formic acid–NaOH was chosen as the background buffer, excellently fitting the pH 3.0 formic buffer used in the acidic precipitation (Section 4.2). The second benefit of low pH value is that the valuable proteases take a lot of positive charges, leading to their cathodic deflection in the electrical field, whereas the unwanted proteins carry little of positive charges, resulting in a small cathodic movement in FFE chamber. This further improves the separation of trypsin from the unwanted. Third, the electroosmotic flow (EOF) under pH 3.0 is nearly zero in the QC-FFE of PMMA [49], improving the resolution of protein separation in FFE (our unpublished data on increasing resolution via control of EOF). Fourth, the Joule heating was low. The pK_a value of formic acid was 3.75. It was noticed that if pH 3.5–4.5 formic buffers were used in the runs of FFE, more bubbles in the chamber were created due to the obvious increase of buffer conductivities and production of more Joule heating.

The deflection of Cyt *C* in QC-FFE was vividly used for the design of sample injection and fraction collection. Cyt *C* is a red protein with the molecular weight 13 kDa and p*I* 10.3 [44]. Cyt *C* runs slightly faster than trypsin in pH 3.0 buffer environment in capillary electrophoresis [50]. Herein, Cyt *C* can be used to estimate trypsin deflection in QC-FFE (Supporting Information Fig. S4). Supporting Information

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Fig. S4B unveils the deflection of Cyt *C* in QC-FFE with an electric field, the sample injection of 19.2 μL/min flow rate via the 3rd inlet results in the collection of Fractions 12–16 under 100 V/cm electric field. Thus, in the next separation of QC-FFE, the enzyme sample was injected via the 3rd inlet at a flow rate of 19.2 μL/min and background flow rate of 74 μL/min per inlet.

The voltage used has obvious effect on the separation of QC-FFE. Figure 5A shows the protein concentration distribution among the fractions of No. 1–16 under 33 V/cm electric field, unveiling the high protein concentration of No. 5–8 (especially No. 6). Figure 5B shows the enzyme activity distribution among the 16 fractions, demonstrating the high enzyme activity at Fractions 7 and 8, especially at Fraction 6, whereas SDS-PAGE revealed that Fraction 6 contains numerous impure proteins with high content, and Fractions 7 and 8 have the band of trypsin with quite high purity (not shown herein). Hence, Fraction 6 was removed, and Fractions 7 and 8 were collected for the assay of enzyme activity (Supporting Information Table S1). Table 1 further reveals the total protein amount, enzyme activity, yield, specific activity, and purification factor of Fractions 7 and 8. The data in Table 1 summarize the slight microseparation of enzyme from the impure proteins with the low yield and poor purification factor under the condition of 33 V/cm electric field.

If 66.7 V/cm was chosen, as shown in Fig. 5A, the high protein contents were observed at fractions of No. 5–10 (especially No. 6–9). As compared with concentration profile of 33 V/cm, the profile of 66.7 V/cm shifted toward the cathode. Figure 5B shows the high enzyme activity in Fractions 6–10, especially in Fraction 6. Nevertheless, the results of SDS-PAGE in Supporting Information Fig. S5 reveal that Fraction 6 had some impure proteins, Fractions 7 and 8 hold the three pancreatins of trypsin, elastase, and chymotrypsin, and Fractions 9 and 10 have a single trypsin zone. Therefore, Fractions 7–10 were analyzed for the assay of enzyme activity (Supporting Information Table S2). Table 1 further unveils the total protein quantity, enzyme activity, yield, activity, and purification factor of Fractions 7–10. The data in Table 1 indicate the obvious separation of enzyme from impure protein still having low yield and poor purification factor under the given conditions.

However, if 100 V/cm was used, the high protein concentrations were observed at Fractions 8–11, as shown in Fig. 5A. As compared with the profiles of 33 V/cm and 66.7 V/cm, the profile of 100 V/cm further shifted toward the cathode. Figure 5B proves the high enzyme activity in Fractions 7–11, but some impure proteins exist in Fraction 7 (see Lane VII of SDS-PAGE in Fig. 4). Hence, Fractions 8–11 were collected for enzyme analysis (Supporting Information Table S3). Table 1 further summarizes that the total protein quantity, enzyme activity, yield, specific activity, and purification factor of Fractions 8–11 were 3.78 mg, 23 478 U, 55.2%, 6227.6 U/mg, and 11.7, respectively, implying the good separation of enzyme from impure protein (Lane VIII, IX–XI in Fig. 4), the quite high yield, obvious increase of purification factor under the condition of 100 V/cm. Theoretically, the further increase of voltage (e.g. 133 V/cm, 400 V cross the chamber) could improve the separation of trypsin, whereas more air bubbles were formed in the chamber within about 10-min run, interrupting the separation of trypsin via the QC-FFE.

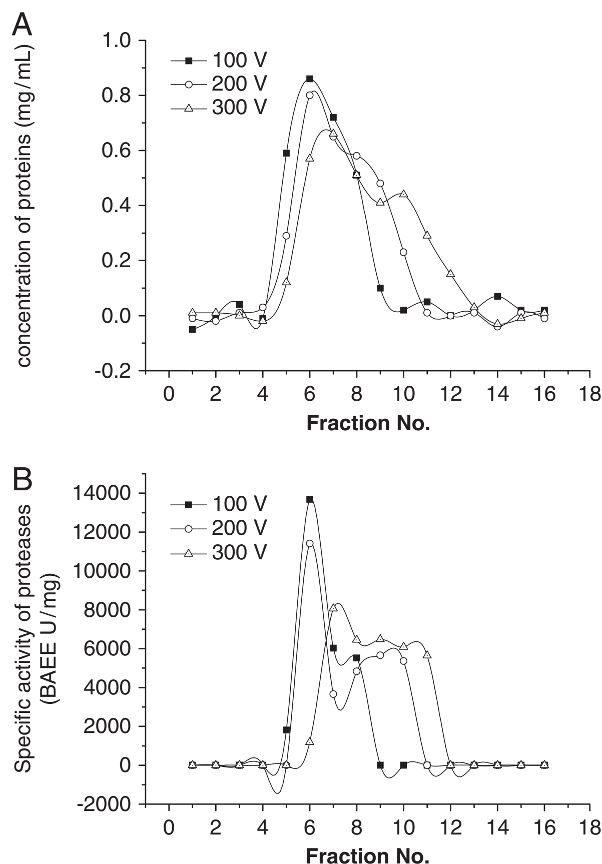

Figure 5. Protein concentration (A) and specific activity of proteases (B) after the microseparation of porcine pancreatin in the QC-FFE under different electric fields of 33, 66.7, and 100 V/cm. Experimental conditions of QC-FFE: 30 mM, pH 3.0, NaOH–HCOOH as both background buffer and electrode buffer, the sample injection via the 3th inlet tube, 19.2 μL/min per tube flow of sample, and 74 μL/min per inlet flow of background buffer.

Therefore, we could obtain the following experimental conditions from the design and brief optimization above for the micropurification of pancreatin via QC-FFE: (i) 30 mM, pH 3.0, formic acid–NaOH used as the running and electrode buffer; (ii) sample injection via the 3rd inlet at a flow rate of 19.2 μL/min; (iii) background flow rate at 74 μL/min per inlet; (iv) 100 V/cm electric field; and (v) ice-water cooling.

4.4 Micropurification of trypsin from pancreatin via QC-FFE

Figure 4 shows the results of micropurification of trypsin monitored with SDS-PAGE. Lane III is the run of

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pancreatin after the acidic precipitation, unveiling these impure proteins with molecular weight higher than 2.6 kDa and lower than 2.3 KDa. When 100 V/cm was used for the enzyme separation via the device, the most of trypsin was separated from the impure proteins, including other two pancreatins of elastase and chymotrypsin. Lane VII is the SDS-PAGE result of Fraction 7 collected from the QC-FFE, showing the coexistences of trypsin, elastase, and chymotrypsin as well as a few of impure proteins with molecular weight 30 and 14.4 kDa. Lane XII is the SDS-PAGE of Fraction 12, revealing the absence of any protein band. Lanes VIII–XI are the analyses of Fractions 8–11, respectively. The analyses clearly manifest that (i) a single band of trypsin almost existed in Lanes VIII–XI, viz., Fractions 8–11; (ii) the impure proteins of Lanes III and VI were greatly removed by the QC-FFE; (iii) the elastase and chymotrypsin were also separated from the target trypsin; and (iv) the specific activity of trypsin was >6000 U/mg, being better than the commercial standard of trypsin (http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang = en&N4 = T7409|SIGMA&N5 = SEARCH_CONCAT_PNO|BRAND_KEY&F = SPEC). All of the results in Fig. 4 imply quite good micropreparation of trypsin by the developed QC-FFE.

The enzyme amount of micropreparation via the QC-FFE can well meet the need of further enzyme assay. It was demonstrated in the experiments of QC-FFE that (i) the sample throughput was 3.34 mg/h with the 432 μL volume (collected from Fractions 8 to 11 but not Fraction 7 in Fig. 5), being greatly more than the amount needed for the normal enzyme assay in Figs. 4 and 5 and Supporting Information Table 1-S1–S3; and (ii) during the QC-FFE run, no blockage was caused by protein precipitation, no interruption of background flow was induced by air bubble caused by Joule heating due to effective ice-water cooling, and no break of electric current was caused by the air bubbles in electrode bath, thanks to effective flow of electrode buffer.

However, the recovery and purification factor of trypsin were not as high as those obtained in the previous studies [51, 52]. Table 1 summarizes that the purification factor is about 11.7 mildly higher than that of acidic precipitation (10.7), and the recovery of trypsin from Fractions 8 to 11 is only about 55.2%. The yield was obviously $<68\%$ recovery of trypsin got via affinity binding [51] and 73% obtained via affinity and ion-exchange chromatography [52]. Furthermore, these data (11.7 purification factor and 6228 unit/mg) could not be illuminated by the quite clean band of trypsin in Lanes VIII–XI of Fig. 4, indicating high specific activity and high purification factor. The self-contradiction of data might be caused by the following reason.

It was calculated from Fig. 5B that the losses of enzyme activity of Fractions 6 and 7 were 3.3 and 17.9%, respectively, due to coexistence of elastase and chymotrypsin as well as some impure proteins. The total enzyme loss of Fractions 6 and 7 was up to 21.2%. Hence, the 76.3% total enzyme activity could be traced to the loss of 21.2% (including trypsin, elastase, and chymotrypsin) and the yield of 55.2%. This means that the further optimization on experimental conditions can slightly improve the yield of QC-FFE separation because of coexistence of high-content elastase and chymotrypsin in Fractions 6 and 7, whereas there was 24% loss of enzyme activity which could not be explained quantitatively. This may be due to the following reason. After the micropreparation of trypsin, the inhibitor added into the crude pancreatin by the manufacturer was removed from trypsin and other two pancreatins. As a result, the removal of inhibitor partially activated the self-digestion activity of the three pancreatins even they were kept in acidic aqueous environment. Consequently, the activated self-digestion might lead to the 24% loss of enzyme activity, well explaining the fair recovery (55.2%) and the mild increase of purification factor (11.7) in Table 1 as well as the single trypsin bands of Lanes VIII–XI in Fig. 4.

5 Concluding remarks

A QC-FFE device was successfully constructed, which is reassemblable and rewashable avoiding discard of C-FFE due to the blockage of solute precipitation in chamber. The chamber volume is 108–654 μL slightly higher than that of C-FFE (20–134 μL), supplying the micropreparative ability of QC-FFE. Up to 16 outlets were designed, bestowing fine fraction for sample collection. In contrast to C-FFE and LS-FFE, the device had the following advantages. First, it could be used for the micropreparation of biosample. The results demonstrated that higher than 1 h continuous run could be easily performed via the simple but effective ice-water cooling, and the throughput was 3.34 mg/h with the 432 μL volume, fitting the need of enzyme activity assay in lab. Second, the greater size of QC-FFE in contrast to that of C-FFE effectively eliminates the interference of blockage caused by protein precipitation. Third, the design of deep electrode baths and their buffer circle as well as PAG membrane could completely avoid break of electric current due to air bubble in C-FFE. The experiments on trypsin separation demonstrated the feasibility of micropurification of target enzyme in complex sample matrix via the developed device.

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6 References

[1] Davies, M. I., Lunte, C. E., *Chem. Soc. Rev.* 1997, *26*, 215–222.

[2] Reid, G. E., Rasmussen, R. K., Dorow, D. S., Simpson, R. J., *Electrophoresis* 1998, *19*, 946–955.

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[3] Rusconi, F., Schmitter, J. M., Rossier, J., Maire, M. L., *Anal. Chem.* 1998, *70*, 3046–3052.

[4] Scott, N. E., Bogema, D. R., Connolly, A. M. Falconer, L., Djordjevic, S. P., Cordwell S. J., *J. Proteome Res.* 2009, *8*, 4654–4664.

[5] Natsume, T., Taoka, M., Manki, H., Kume, S., Isobe, T., Mikoshiba, K., *Proteomics* 2002, *2*, 1247–1253.

[6] Posewitz, M. C., Tempst, P., *Anal. Chem.* 1999, *71*, 2883–2892.

[7] Kilar, F., *Electrophoresis* 2003, *24*, 3908–3916.

[8] Otsuka, K., Baba, Y., *J. Chromatogr. A* 2006, *1106*, 118–123.

[9] Zhang, H., Wang, Z., Li, X. F., Le, X. C., *Angew. Chem. Int. Ed.* 2006, *45*, 1576–1580.

[10] Hannig, K., in: Gerritsen, T. (Ed.), *Modern Separation Methods of Macromolecules and Particles*, vol. 2, Wiley, New York 1969.

[11] Hannig, K., Heidrich, H. G., in: Bloemendal, H. (Ed.), *Cell Separation Methods*, Elsevier/North Holland, Amsterdam 1977, Part 4, pp. 45–69 and pp. 92–116.

[12] Hannig, K., *Electrophoresis* 1982, *3*, 235–243.

[13] Kohlheyer, D., Eijkel, J. C. T., Berg, A. V. D., Schasfoort, R. B. M., *Electrophoresis* 2008, *29*, 977–993.

[14] Kasicka, V., *Electrophoresis* 2009, *30*, S40–S52.

[15] Islinger, M., Eckerskorn, C., Volkl, A., *Electrophoresis* 2010, *31*, 1754–1763.

[16] Pamme, N., *Lab Chip* 2007, *7*, 1644–1659.

[17] Lanz, M., Caslavska, J., Thormann, W., *Electrophoresis* 1998, *19*, 1081–1090.

[18] Gratz, S. R., Schneiderman, E., Mertens, T. R., Stalcup, A. M., *Anal. Chem.* 2001, *73*, 3999–4005.

[19] Loseva, O. I., Gavryushkin, A. V., Osipov, V. V., Vanyakin, E. N., *Electrophoresis* 1998, *19*, 1127–1134.

[20] Kasicka, V., Prusik, Z., Sazelova, P., Jiracek, J., Barth, T., *J. Chromatogr. A* 1998, *796*, 211–220.

[21] Malmstrom, J., Lee, H., Nesvizhskii, A. I., Shteynberg, D., Mohanty, S., Brunner, E., Ye, G., Weber, M. L., Eckerskorn, C., Aebersold, R., *J. Proteome Res.* 2006, *5*, 2241–2249.

[22] Zischka, H., Braun, R. J., Marantidis, E. P., Buringer, D., Bornhoud, C., Hauck, S. M., Demmer, O., Gloeckner, C. J., Reichert, A. S., Madeo, F., Ueffing, M., *Mol. Cell. Proteomics* 2006, *5*, 2185–2200.

[23] Raymond, D. E., Manz, A., Widmer, H. M., *Anal. Chem.* 1994, *66*, 2858–2865.

[24] Fonslow, B. R., Barocas, V. H., Bowser, M. T., *Anal. Chem.* 2006, *78*, 5369–5374.

[25] Fonslow, B. R., Bowser, M. T., *Anal. Chem.* 2006, *78*, 8236–8244.

[26] Kostal, V., Fonslow, B. R., Arriaga, E. A., Bowser, M. T., *Anal. Chem.* 2009, *81*, 9267–9273.

[27] Turgeon, R. T., Fonslow, B. R., Jing, M., Bowser, M. T., *Anal. Chem.* 2010, *82*, 3636–3641.

[28] Song, Y.-A., Chan, M., Celio, C., Tannenbaum, S. R., Wishnok, J. S., Han, J., *Anal. Chem.* 2010, *82*, 2317–2325.

[29] Fonslow, B. R., Bowser, M. T., *Anal. Chem.* 2008, *80*, 3182–3189.

[30] Raymond, D. E., Manz, A., Widmer, H. M., *Anal. Chem.* 1996, *68*, 2515–2522.

[31] Kuhn, R., Wagner, H., *Electrophoresis* 1989, *10*, 165–172.

[32] Kuhn, R., Wagner, H., *J. Chromatogr.* 1989, *481*, 343–351.

[33] Hoffstetter-Kuhn, S., Wagner, H., *Electrophoresis* 1990, *11*, 451–456.

[34] Hoffstetter-Kuhn, S., Kuhn, R., Wagner, H., *Electrophoresis* 1990, *11*, 304–309.

[35] Hoffstetter-Kuhn, S., Wagner, H., *Electrophoresis* 1990, *11*, 457–462.

[36] Nath, S., Schütte, H., Weber, G., Hustedt, H., Deckwer, W. D., *Electrophoresis* 1990, *11*, 937–941.

[37] Nath, S., Schütte, H., Hustedt, H., Deckwer, W. D., Weber, G.., *Biotechnol. Bioeng.* 1996, *51*, 15–22.

[38] Wagner, H., Kuhn, R., Hoffstetter, S., in: Wagner, H., Blasius, E. (Eds.), *Praxis der elektrophoretischen Trennmethoden*, Springer, Berlin 1989, pp. 223–278.

[39] Turgeon, R., Bowser, M. T., *Anal. Bioanal. Chem.* 2009, *394*, 187–198.

[40] Chen, S., Palmer, J. F., Zhang, W., Shao, J., Li, S., Fan, L. Y., Sun, R., Dong, Y. C., Cao, C. X., *Electrophoresis* 2009, *30*, 1998–2007.

[41] Shao, J., Li, S., Zhang, W., Fan, L. Y., Cao, C. X., Sun, R., Dong, Y. Ca., *J. Chromatogr. A* 2010, *1217*, 2182–2186.

[42] Shao, J., Fan, L. Y., Zhang, W., Guo, C. G., Li, S., Xu, Y. Q., Cao, C. X., *Electrophoresis* 2010, *31*, 3499–3507.

[43] Kohlheyer, D., Eijkel, J. C. T., Schlautmann, S., van den Berg, A., Schasfoort, R. B. M., *Anal. Chem.* 2008, *80*, 4111–4118.

[44] Voytek, P., Gjessing, E. C., *J. Biol. Chem.* 1971, *246*, 508–516.

[45] Keil, B., in: Boyer, P. D. (Ed.), *The Enzymes*, Academic Press, New York 1971, p. 250.

[46] Rick, W., in: Bergmeyer, H. U. (Ed.), *Methods of Enzymatic Analysis*, Academic Press, New York 1965, p. 815.

[47] Dyal, A., Loos, K., Noto, M., Chang, S. W., Spagnoli, C., Shafi, K. V. P. M., Ulman, A., Cowman, M., Gross, R. A., *J. Am. Chem. Soc.* 2003, *125*, 1684–1685.

[48] Fonslow, B. R., Bowser, M. T., *Anal. Chem.* 2005, *77*, 5706–5710.

[49] Altria, K. D., in: Altria, K. D., Wellcom, G. R. (Eds.), *Chromatographia CE Series: Analysis of Pharmaceuticals by Capillary Electrophoresis*, Vieweg und Sohn Verlagsgesellschaft mbH, Braunschweig, Germany, 1998, p. 4.

[50] GIlges, M., KleemIss, M. H., Schomburg, G., *Anal. Chem.* 1994, *66*, 2038–2046.

[51] Powers, J. D., Kilpatrick, P. K., Carbonell, R. G., *Biotechnol. Bioeng.* 1990, *36*, 506–519.

[52] Guyonnet, V., Tluscik, F., Long, P. L., Polanowski, A., Travis, J., *J. Chromatogr. A* 1999, *852*, 217–225.

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