Microchemical Journal 130 (2017) 153–156

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Studying the interaction between peptides and polymeric nanoparticles used as pseudostationary phase in capillary electrochromatography

Denise A. Grela [a], Valeria Zannoni [b], Nora M. Vizioli [a,c,*]

[a] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Química Analítica y Fisicoquímica, Cátedra de Química Analítica, Buenos Aires, Argentina
[b] Instituto Nacional de Tecnología Industrial (INTI), Centro de Investigación y Desarrollo en Química, Laboratorio de Sistemas de Liberación Controlada, Buenos Aires, Argentina
[c] Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Instituto de Química y Fisicoquímica Biológicas (IQUIFIB), Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 6 April 2016
Received in revised form 29 August 2016
Accepted 30 August 2016
Available online 31 August 2016

Keywords:
Capillary electrochromatography
Capillary electrophoresis
Peptides
Pseudostationary phases
Particles
Interaction

ABSTRACT

In this work, the interaction between synthetic bioactive peptides and polymeric nanoparticles (NPs) used as pseudostationary phase in capillary electrochromatography was studied. NPs were prepared from methacrylic acid and ethylenglycol dimethacrylate with benzoyl peroxide by utilizing a precipitation polymerization technique. The reaction was monitored by infrared spectroscopy. Polymer characterization was performed by dynamic light scattering and transmission electron microscopy. Experimental running conditions were tested, including organic solvent proportion in the background electrolyte, capillary conditioning, applied voltage, sample introduction amount, and how NPs were incorporated into the system. A continuous full filling technique in which the NPs were suspended in the entire electrolyte volume as well as a conventional partial filling technique were used. Results obtained at pH 7.0 suggest that the NPs have a very strong interaction with more basic peptides. The interaction between analytes and NPs was found to be predominantly ionic.

1. Introduction

Nanoparticles (NPs) have been used in separation science in the past decade with great success. Most applications are reported in academic research laboratories, where separation success has been greater than or equivalent to current state-of-the-art chromatographic and electrophoretic methods. In spite of this success, this technology has not penetrated industrial chemistry laboratories (e.g., pharmaceuticals and forensics) [1].

Particularly, the use of nano-sized materials in electro-driven separation systems has received special attention. Even though NPs have demonstrated their utility in separation science, they have not yet found regular application in pharmaceutical and other regulated chemistry environments, because complete understanding of the synthesis process along with size control and complete characterization has not yet been realized, so robustness and reliability are the limiting steps in this technology [1,2].

As we approach the nm range, the molecular physical and chemical properties can vary dramatically and sometimes in an unexpected manner. The potential of NPs seems to have no limits, and the area of separation science has been no exception. As novel stationary phases and dynamic coatings, NPs greatly enhance separation performance in terms of selectivity, efficiency, and resolution. They also fit well with the concept of the past decade, which has seen a major drive towards miniaturization of many analytical systems with the emergence of lab-on-a-chip technologies as the way forward. So far, NPs have found extensive use in capillary electrophoresis (CE), packed capillary electrochromatography (CEC) and open tubular CEC (OT-CEC) formats, and microchip CE (MCE) [3–10]. As stationary phases, NPs display a large surface area- to-volume ratio, which is ideal for low mass-transfer effects in chromatography. This feature is also invaluable in miniaturized electro-migration techniques (e.g., MCE where close electro chromatographic interactions are essential for good resolution and high efficiency) [11].

NPs are relatively easy to synthesize and can be functionalized by a wide range of different chemistries, with amino (NH2), thiol (SH), and carboxyl (COOH) functionalities, being the most popular currently [12–20]. Several kinds of NP and nanostructure have been applied to separation science, including fullerenes, nanotubes, silica, polymers, zeolite, lipids, latex, metaloxides, and silver NPs (AgNPs) and AuNPs [21, 22].

The NPs can be charged or uncharged to accommodate electroosmotic flow (EOF) in electrophoretic separations, the surface area-to-volume ratio is hugely increased compared to that of the bulk material. At this nm range, quantum-dot (QD) effects can be exploited, and there is huge scope for versatility regarding chemical functionality, while size and shape can be controlled during formation.

* Corresponding author at: Departamento de Química Analítica y Fisicoquímica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, C1113AAD Buenos Aires, Argentina.
E-mail address: nvizioli@ffyb.uba.ar (N.M. Vizioli).

http://dx.doi.org/10.1016/j.microc.2016.08.015
0026-265X/

In summary, the ideal NPs form stable suspensions in a variety of electrolytes, provide analyte selectivity, must be charged when moved concentrated in a plug into the capillary column in order to avoid elution with the EOF, show equal mobility to minimize overall band broadening, give minimal mass transfer effects, do not interfere with detection, and be small and porous with a large surface area. These considerations are important so as to achieve highly efficient separations [23].

NPs have mainly been used in OTCEC and to a limited extent in CE. In the OT formats, NPs are usually fixed on the inner wall of the capillary column [2] or used as pseudostationary phase (PSP)-CEC whereby NPs are added to the buffer [5].

CEC is habitually performed using packed, monolithic or open- tubular columns. However, the use of PSP has become an interesting alternative with the advantage that it does not require frits or packing. It also provides sites for analyte interaction and moves with or against the mobile phase. In this system, the stationary phase is continuously replaced and a renewed column is used for each analysis, which avoids the contamination associated with complex matrix samples and minimizes the need of column change [24]. In some respects, this approach is favored as column-packing technologies have not advanced at the same rate as particle technology, resulting in the absence of a robust method to pack NPs into narrow- bore capillary columns. Capillary packing has been somewhat of a black art in CEC and reproducibility can be quite difficult to achieve [25]. Therefore, more understanding and further advancement are required before there is a robust packing procedure for packing of NP stationary phases. Thus, NPs have found popular use in OTCEC as inner wall coatings or powerful additives to the background electrolyte.

The aim of this work was to evaluate the interaction between synthetic peptides and in-lab synthesized polymer particles acting as PSP in CEC. Different monomer concentrations were tested in order to modify the particle size and, thus, to study their influence on peptide electrochromatographic behavior. Synthesized NPs presented homogeneous size and shape. Experimental running conditions were tested, including organic solvent proportion in the background electrolyte (BGE), capillary conditioning, applied voltage, sample introduction amount, and how NPs were incorporated into the system. Results obtained at pH 7.0 suggest that the NPs have a very strong interaction with basic peptides.

2. Experimental

2.1. Chemicals

The bioactive synthetic peptides bradykinin, angiotensin I, luteinizing hormone releasing hormone (LHRH), oxytocin, methionine-enkephalin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amino acid sequence, relative molecular mass, and isoelectric point of each peptide are listed in Table 1. Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, phosphoric acid, acetonitrile (ACN), and triethanolamine were purchased from Merck (Darmstadt, Germany). Methacrylic acid (MAA), ethylenglycol dimethacrylate (EGDMA), and benzoyl peroxide were also obtained from Sigma-Aldrich. Deionized water was purified with an Easy Pure ™ UltraPure water system from Barnstead-Thermolyne (Dubuque, IA, USA). Nylon membrane filters (0.45 μm) were purchased from Microclar (Tigre, Buenos Aires, Argentina).

Table 1
Analyzed bioactive peptides and their amino acid sequences, relative molecular masses, M_r, and isoelectric points, p*I*.

Peptide	Amino acid sequence	M_r	p*I*
Angiotensin I	DRVYIHPFHL	1296.5	7.91
Oxytocin	CYIQNCPLG	1007.19	7.70
Bradykinin	RPPGFSPFR	1060.2	12.40
LHRH	Pyr-HWSYGLRPG	1182.3	7.30
Methionine-enkephaline	YGGFM	573.7	5.93

2.2. Peptide solutions and running conditions

Stock solutions of synthetic peptides (1 mg/mL) were prepared by dissolving each synthetic peptide in water, fractionated in aliquots, and frozen at −20 °C. Standard solutions were daily prepared (10 μg/mL each) by dilution with water or BGE, when indicated.

The BGE consisted of 25 mM sodium buffer phosphate, pH 7.0, unless otherwise indicated. Peptide solutions were introduced by 5 s at 0.7 psi, the separation voltage was varied between 5 and 20 kV, normal polarity. At the beginning of daily work, the capillary column was rinsed with 0.1 M NaOH, 2 min at 30 psi, washed with water, 5 min at 30 psi, and BGE, 15 min at 30 psi. Before each run, the capillary was rinsed with 0.1 M NaOH, 2 min at 30 psi, followed by water, 5 min at 30 psi, and BGE, 5 min at 30 psi. All solutions were filtered and degassed before use. When BGE contained ACN, that step included 1 h degassing under vacuum with stirring, alternating with 30 s ultrasonic degassing every 15 min.

2.3. Instrumentation

Separations were performed in a P/ACE MDQ (Beckman Coulter Inc., Brea, CA, USA), equipped with a UV–Vis photodiode array detector. Data were processed by 32 Karat™ software (Beckman Coulter). Fused-silica (FS) capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 50 cm in length (75 μm id × 365 μm od). For all experiments, the CE system temperature was held at 25 °C. In all cases, UV-detection at 214 nm was performed. A stereomicroscope (Riechter, Vienna, Austria) was used for capillary examination.

2.4. Synthesis of NPs

Particles were prepared from MAA and EGDMA monomers (1:2, m/m) by utilizing a precipitation polymerization technique. Different total monomer concentrations were tested, from 2% to 8% v/v in ACN, in order to modify the size of the particles. Benzoyl peroxide was added to the mixture as radical initiator. The solution was carried out at 60 °C in a water bath, during 24 h. Particles were washed by successive centrifugation and re-suspension twice with ACN and three times with 25 mM phosphate buffer solution, pH 8.0 (the last, when necessary to change the solvent).

3. Results and discussion

3.1. NPs characteristics

The synthesis of polymeric NPs was performed by a precipitation polymerization technique from a monomer mixture containing two well-known monomers, MAA and EGDMA. The MAA provides the polymer carboxyl functional groups which can be charged depending on pH of the mobile phase. Thus, MAA gives the polymer an electrically negative character when mobile phase pH is set to a value above 5.0. Additionally, MAA could be chemically modified to couple a wide range of functional groups conferring the system different options for selectivity improvement. The EGDMA, a derivative of methacrylic acid and ethylene glycol that contains two double bounds, is responsible for the crosslinking degree of the polymer.

The utilized precipitation polymerization technique allows NPs to be prepared without the use of stabilizing surfactants, thus simplifying the washing step after NPs generation. A MAA:EGDMA 1:2, m/m, proportion was selected for NPs synthesis. That proportion has been demonstrated to be ideal for obtaining porous and stable particles, which additionally present an optimal surface area-to-volume ratio [17]. The monomer mixture was prepared in ACN at different concentrations in

order to verify how that monomer mixture concentration affects the size and shape of particles. The progress of the polymerization reaction was monitored by infrared spectroscopy checking the signal decrease of the band corresponding to double bounds.

Preparation of NPs yielded spherical beads which showed a quite homogeneous appearance according to the images obtained by transmission electron microscopy (Fig. 1). NPs size was determined by dynamic light scattering. The average diameter values ranged from 120 to 630 nm for NPs synthesized with 2 to 8% monomer mixture in ACN, v/v, respectively.

Since NPs synthesized in our laboratory were going to be used for peptide analysis, it was imperative to evaluate the composition of the BGE before to start PSP-CEC experiments in order to avoid NPs precipitation. Commonly, the utilization of organic polymeric NPs as PSP has been carried out inBGE containing a high (90% or higher) organic solvent content. This fact would be related with polymeric nanoparticle solubility and stability, and retention selectivity of the method depending on the compounds to be separated. Hence, we tested different organic solvent-aqueous solution proportions taking into account the separation would be made at acidic pH assuring the peptides are protonated to perform analyses in a short time. A solution consisting of ACN:25 mmol L^{-1}phosphoric acid- triethanolamine buffer pH 3.0, 90:10, v/v, resulted to be appropriated to maintain stable the NPs without precipitation and was used for the subsequent experiments.

3.2. Peptide analysis

Since the introduction of micelles by Terabe et al. [26] many different species have been used as carriers in electrokinetic systems. These phases are added to introduce selectivity when separation of uncharged solutes or ions with equal mobility is not possible in CE, as well as to improve selectivity when solute resolution is not successful. Separation in these systems is achieved according to the different distribution coefficients of the solutes between the PSP and the background electrolyte. With respect to high performance liquid chromatography (HPLC) and CEC, the application of particles as PSP implies a kind of chromatography based on a one-run column. Therefore, problems related with reconditioning, contamination and ageing of the columns are avoided.

The particles synthesized in our lab were used as PSP to evaluate their effect on peptide separation. In the early experiments, the NPs were suspended into the entire BGE volume, a strategy known as continuous full filling technique [27]. This approach implies a continuous flow of NPs suspension through the capillary column. Once the sample is introduced and voltage is applied, the separation proceeds with NPs suspension in BGE acting as electrolyte. At pH 3.0, NPs are neutral and move towards the cathode by EOF while peptides are positively charged and consequently move faster than NPs on the same direction.

Some technical inconvenient occurred when using the NPs suspension mentioned below as electrolyte. Even though a meticulous conditioning of the capillary column was performed before each run, the current of the system was not stable and often broke down. As a consequence, retention time of peptides did not show good repeatability. Bubble formation was apparently what caused the current instability as it was verified by examining the capillary column under a stereomicroscope. This problem was partially solved by thorough degassing of BGE before the addition of NPs. Despite this exhaustive degassing step, bubble formation during runs could not be totally avoided since tiny gas bubbles could be appreciated inside the column. As a general rule, the BGE must have adequate buffering capacity to neutralize the H^+ and OH^- that are produced at the anode and the cathode, respectively, and electrolyte reservoirs should be replenished regularly for repeatable separations. Although all these precautions had been taken, the problem found in our experiments was not solved. It could be speculated that the presence of the polymeric NPs would favor the formation of gas bubbles through a surface phenomenon given the high surface they confer to the system, causing that negligible amounts of gas the mobile phase concentrate and generate gas bubbles inside the column.

In another attempt to find experimental conditions that allow to study NPs effect on peptide separation, a partial filling technique was used. This approach consisted of introduction of NPs immediately before sample introduction into the capillary column. Once the sample was into the capillary, the separation voltage was applied. Fig. 2 shows the effect of NPs on the separation of the two peptides oxytocin and methionine-enkephalin using a BGE consisting of ACN:25 mmol L^{-1} phosphate-triethanolamine buffer pH 5.0, 90:10, v/v. Since those peptides were positively charged, they moved faster than NPs along the column. Thus, peptides passed through the NPs plug. The presence of NPs allowed resolution between the two peaks to increase. Combining that with an optimal voltage setting, it was possible to obtain a base-line

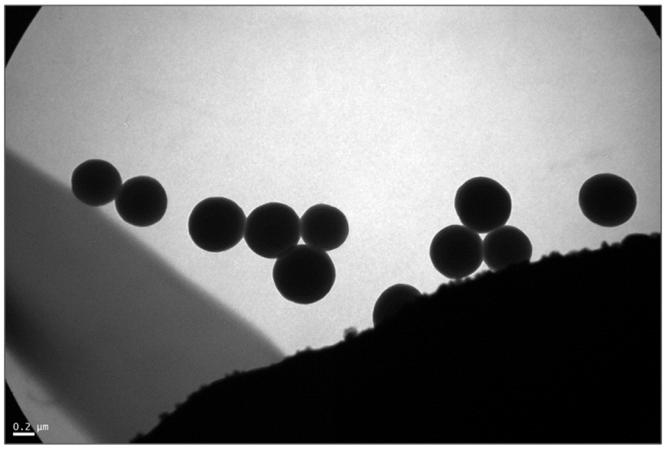

Fig. 1. Transmission electron microscopy image corresponding to NPs synthesized from a 6% monomer mixture (MAA:EGDMA, 1:2, m/m) in ACN, v/v.

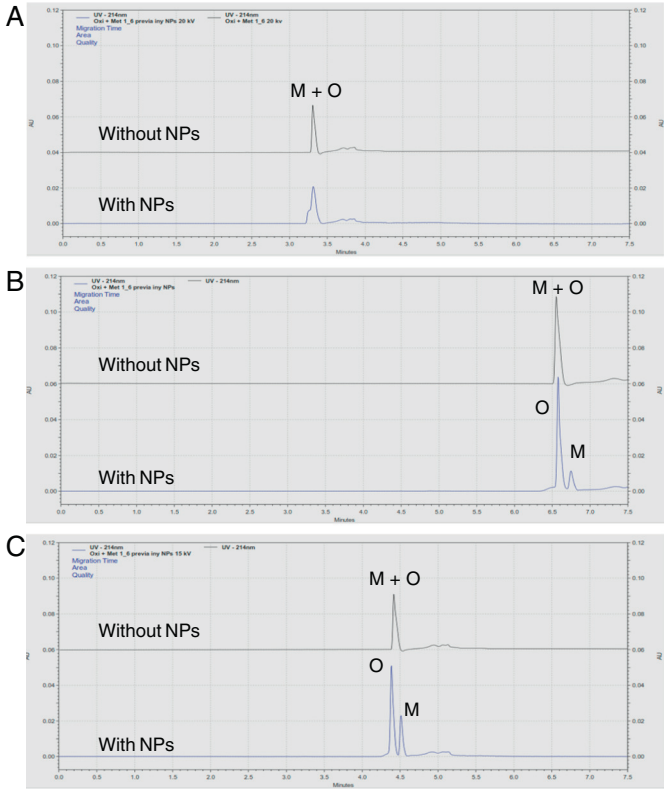

Fig. 2. Effect of applied voltage variation on separation of oxytocin (O) and methionine-enkephalin (M), in the presence and absence of NPs. A) 20 kV; B) 10 kV; C) 15 kV.

resolution for the two peptides (Fig. 2 C)). Since these experiments were performed at pH 5.0, the analytes were positively charged while the NPs were negative. The changes in retention time can be explained by assuming that the interaction was based on an ion-ion interaction between the positive charges of analytes and the negative charges on the NPs.

On the other hand, the high concentration of organic solvent suppressed possible hydrophobic interactions.

Despite the precautions taken during those experiments, current instability could not be totally eliminated and retention time repeatability was not as good as expected (RSD over 5%). Therefore, an aqueous BGE without ACN, adjusted at a pH that NPs formed stable suspensions, was used. A sodium buffer phosphate, pH 7.0, was selected to assure NPs were negatively charged and, thus, they migrated towards the peptide sample. Five synthetic bioactive peptides were assayed. Fig. 3-plot A), shows the electrochromatogram obtained when NPs were not introduced into the capillary column. Curiously, bradykinin and LHRH were not detected in presence of NPs, while, oxytocin and methionin-enkephaline peaks apparently remained without changes under the same conditions (Fig. 3-plot B). That would be indicating the presence of a strong interaction between NPs and peptides bradykinin and LHRH. In order to verify that fact, a solution containing both bradykinin and LHRH was treated with NPs. After centrifugation, the supernatant was analyzed showing that no-detectable amounts of peptides were present (Fig. 4). These findings suggest that particles (with negative net charge) have a strong interaction with basic peptides. Bradykinin and LHRH present higher positive net charge than the other assayed peptides. Besides the basic residues on their amino acids sequences, the two peptides have a serine residue which contributes to the polar character of those peptides. Therefore, they interact with negative charged particles, enabling the particles to completely capture those basic peptides.

Angiotensin deserves a special comment since it appears to be affected by the presence of NPs but not in the same extent than bradykinin and LHRH. Considering its amino acid sequence, it can be observed the presence of three basic amino acids and one acidic residue while it has not any other polar residue. This fact could explain the different effect in comparison with oxytocin and methionine-enkephaline, where the interaction between peptides and NPs was imperceptible in the experimental conditions here applied.

4. Conclusion

NPs synthesized from MAA and EGDMA studied in this work showed a very strong interaction with more basic peptides. That interaction between peptides and NPs was found to be predominantly ionic. Regarding experimental conditions, variation of those parameters related to running conditions greatly affected NPs behavior and, as a consequence,

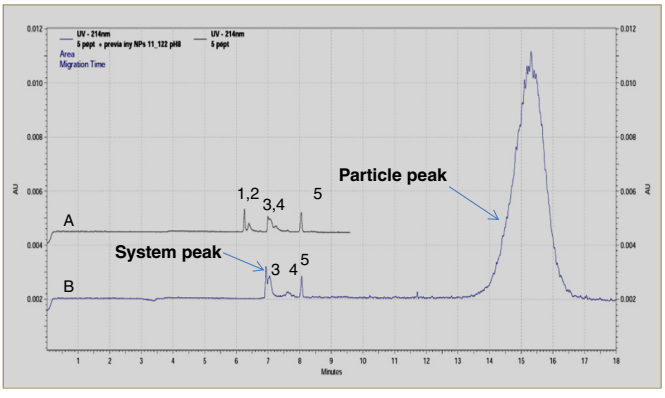

Fig. 3. Analysis of bradykinin (1), LHRH (2), oxytocin (3), angiotensin I (4) and methionine-enkephaline (5) in absence (A) and in presence (B) of particles. Sample and NPs introduction: 0.3 psi, 3 s.

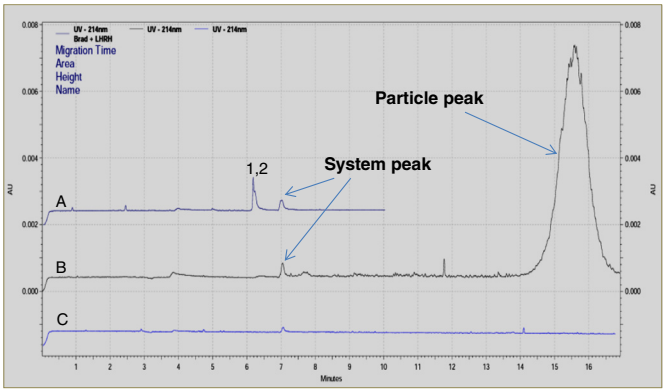

Fig. 4. Analysis of (A) bradykinin (1) and LHRH (2); (B) bradykinin and LHRH after introduction of NPs; (C) supernatant resulting from centrifugation of a 10 μg/mL bradykinin and LHRH solution treated with particles. Sample and particle introduction: 0.3 psi, 3 s.

peptide separation. The application of the polymeric NPs studied in this work as PSP resulted a useful tool to improve the separation of peptides by CEC. However, it is necessary to emphasize the importance of detailed knowledge and control of running conditions to ensure successful results. Additional experiments are in progress to explore the technique performance in the analysis of complex protein samples.

Acknowledgments

Financial support of the following institutions is deeply appreciated: Universidad de Buenos Aires (grant no. 20020090200687), Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, grant no. 1122009010007601, and Agencia Nacional de Promoción Científica y Tecnológica, ANPCyT, grant no. PICT 2013-1541.

The authors have declared no conflict of interest.

References

[1] E. Guihen, Nanoparticles in modern separation science, Trends Anal. Chem. 46 (2013) 1–14.
[2] Y. Moliner-Martínez, S. Cárdenas, B.M. Simonet, M. Valcárcel, Electrophoresis 30 (2009) 169–175.
[3] M.R. Ivanov, A.J. Haes, Analyst 136 (2011) 54–63.
[4] C. Nilsson, S. Birnbaum, S. Nilsson, J. Chromatogr. A. 1168 (2007) 212–224.
[5] Z. Zhang, B. Yan, Y. Liao, H. Liu, Anal. Bioanal. Chem. 391 (2008) 925–927.
[6] V. Kostal, J. Katzenmeyer, E.A. Arriaga, Anal. Chem. 80 (2008) 4533–4550.
[7] E.J. Song, S.M. Babar, E. Oh, M.N. Hasan, H.M. Hong, Y.S. Yoo, Electrophoresis 29 (2008) 129–142.
[8] V. Kašička, Electrophoresis 37 (2016) 162–188.
[9] F. Priego-Capote, L. Ye, S. Shakil, S.A. Shamsi, S. Nilsson, Anal. Chem. 80 (2008) 2881–2887.
[10] P. Spégel, L. Schweitz, S. Nilsson, Anal. Chem. 75 (2003) 6608–6613.
[11] Y. Zhu, C. Zhou, S. Qin, Z. Ren, L. Zhang, H. Fu, W. Zhang, Electrophoresis 33 (2012) 340.
[12] C. Nilsson, I. Harwigsson, S. Birnbaum, S. Nilsson, Electrophoresis 31 (2010) 1773.
[13] F.-K. Liu, J. Chromatogr., A 1216 (2009) 9034.
[14] L. Yang, C. Chen, X. Liu, J. Shi, G. Wang, L. Zhu, L. Guo, J.D. Glennon, N.M. Scully, B.E. Doherty, Electrophoresis 31 (2010) 1697.
[15] Q. Qu, X. Zhang, M. Shen, Y. Liu, X. Hu, G. Yang, C. Wang, Y. Zhang, C. Yan, Electrophoresis 29 (2008) 901.
[16] A.-H. Duan, S.-M. Xie, L.-M. Yuan, Trends Anal. Chem. 30 (2011) 484.
[17] C. Nilsson, S. Birnbaum, S. Nilsson, Electrophoresis 32 (2011) 1141.
[18] C.K. Lo, M.C. Paau, D. Xiao, M.M.F. Choi, Electrophoresis 29 (2008) 2330.
[19] Y. Wang, W.R.G. Baeyens, C. Huang, G. Fei, L. He, J. Quyang, Talanta 77 (2009) 1667.
[20] H. Li, G.-S. Ding, J. Chen, A.-N. Tang, J. Chromatogr., A 1217 (2010) 7448.
[21] U. Pyell, Electrophoresis 31 (2010) 814.
[22] C.P. Palmer, E.F. Hilder, J.P. Quirino, P.R. Haddad, Anal. Chem. 82 (2010) 4046.
[23] B. Gottlicher, K. Bachmann, J. Chromatogr., A 780 (1997) 63–78.
[24] C. Nilsson, S. Birnbaum, S. Nilsson, Electrophoresis 32 (2011) (2011) 1142–1147.
[25] B.C. Iacob, E. Bodoki, R. Oprean, Electrophoresis 35 (2014) (2014) 2722–2732.
[26] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111–113.
[27] P. Viberg, P. Spégel, J. Carlstedt, M. Jörntén-Karlsson, P. Petersson, J. Chromatogr. A. 1154 (2007) 386–389.