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# Microchip Emitter for Solid-Phase Extraction–Gradient Elution–Mass Spectrometry

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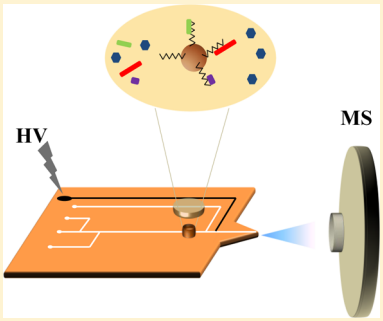
Supporting Information

**ABSTRACT:** A microchip electrospray emitter with a magnetic bead trap has been designed for solid-phase extraction-gradient elution-mass spectrometry (SPE-GEMS). The goal of this method is the detection of analytes at low concentrations and it is here demonstrated using reverse phase coated magnetic beads (Mbs) for the preconcentration and detection of the peptides. The sample is passed through the chip, and the peptides are retained and enriched in the trap. After washing, the peptides are released sequentially by stepwise gradient elution and electrosprayed for mass spectrometry analysis. This approach allows effective sample desalting, enrichment, sequential elution, and MS detection without the introduction of an additional separation step after SPE. Efficient preconcentration of model peptides by SPE and sequential release and analysis of peptides by GEMS were demonstrated for diluted sample solutions within the range of 1 $\mu$M to 10 nM. Fortified human blood serum, protein digest and fractions collected after protein digest OFFGEL separation were analyzed by SPE-GEMS allowing the detection of low abundance peptides usually not observed by direct mass spectrometry analysis. A mathematical model for gradient elution is proposed.




Magnetic beads (Mbs) with different surface functionalizations are widely used as sorbents for solid-phase extraction (SPE), especially in proteomic studies for the purification and fractionation of complex analytes. Traditionally, sample preconcentration and purification with Mbs are performed in an off-line format prior to further separations and analysis, for instance matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[1,2] or high-performance liquid chromatography–mass spectrometry (HPLC-MS).[3] Mbs are usually added to the sample solution, which is then stirred to favor the binding of analytes, and then retained by an external magnetic field while the supernatant is discarded. After a washing step, the adsorbed molecules are eluted and analyzed.[4] Different bead surface modifications have been used for the binding of small organic molecules,[5] vitamins,[6] peptides and proteins of blood serum,[1−3] glycopeptides,[7] phosphopeptides,[8] or allergens.[9] Peptide isolation, desalting and proteome profiling are mainly performed using Mbs with hydrophobic coatings ($C_4$, $C_8$, $C_{18}$, etc.).[10,11] These are commercially available as ready-to-use kits based on well-established off-line protocols for protein or peptide purification, desalting and fractionation prior to MALDI-MS analysis[12,13] with potential process automation.[10,14]

Automation of Mbs based SPE for high throughput analysis and reduction of sample size has been performed online and in-line with HPLC[15] and capillary electrophoresis (CE).[16,17] For further miniaturization, such couplings have been realized in a microfluidic format.[18] Mbs have been used to create SPE concentrator for SPE-HPLC or SPE-CE to replace polymer based monolithic columns[19] or frits and septa for the retention of nonmagnetic particles inside the microchips[20,21] and separation capillaries[22] to form packed-bed columns. Other advantages of Mbs as a sorbent stem from their easy manipulation by application/removal of external magnetic field and hence the possibility of fast sorbent plug renewal simply by system flushing.

In SPE-CE in a microfluidic format, an isocratic elution is typically employed to transfer the sample from the stationary phase to the CE separation capillary.[20,22] As the eluate is separated by electrophoresis, SPE serves only for the enrichment of the analyte and elimination of the impurities. The integration of a multistep elution from SPE sorbents packed inside the separation capillary in CE-MS/MS[23] provides an additional possibility to increase the resolving power of the system. This stepwise gradient approach has been shown to extend the separation efficiency of complex peptide mixtures.[24]

When SPE in a column format is coupled online with HPLC, a gradient elution is normally used to extract the sample from the SPE column to the analytical one for further LC separation.[25] SPE columns can also be treated as small chromatographic columns with application of gradient elution for sample quantitation[26,27] or to define the breakthrough volumes for optimization of SPE-HPLC experiments.[28] However, when using SPE in direct conjunction with UV,[29]

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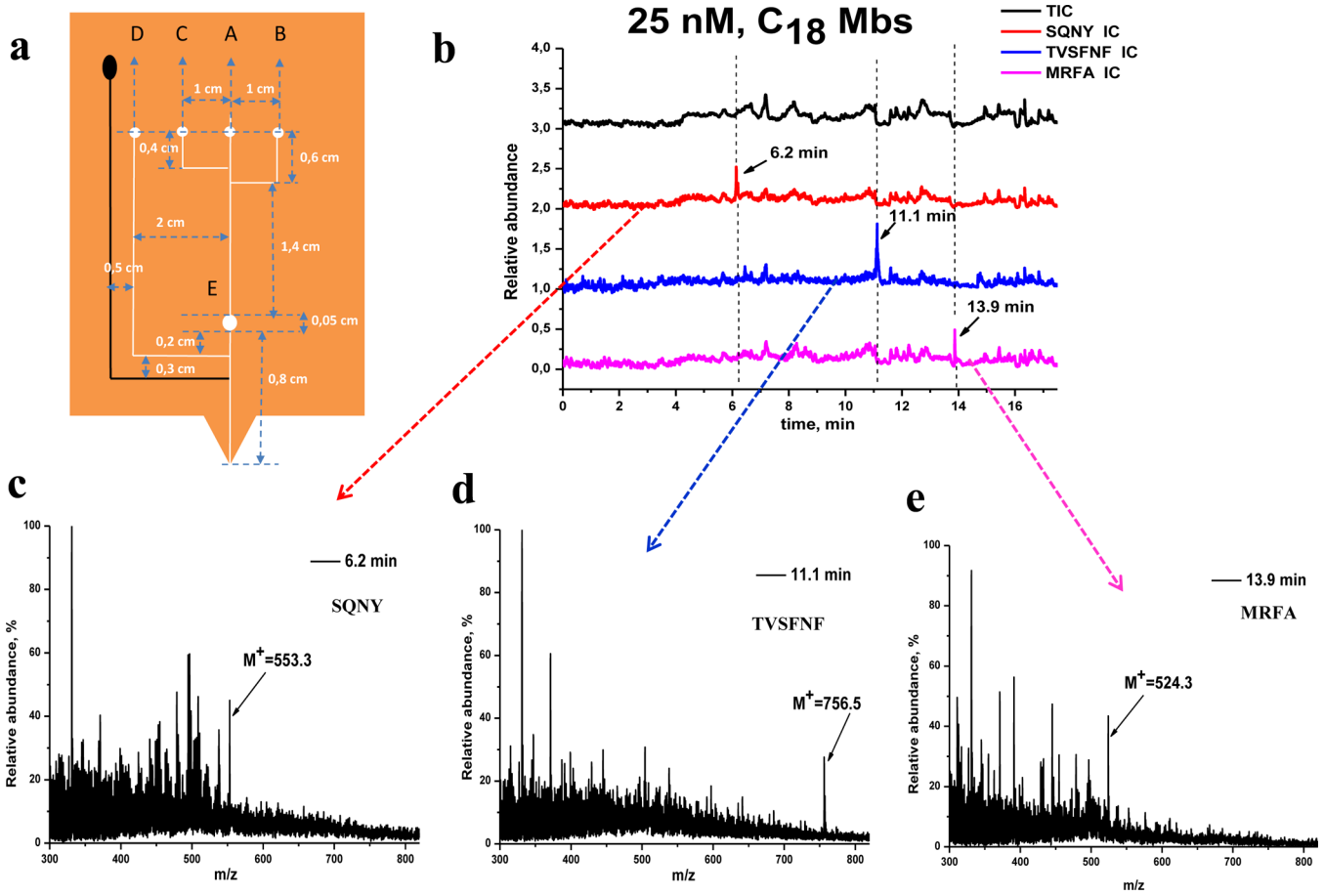


**Figure 1.** (a) SPE-GEMS microchip design; microcarbon electrode (black line) and microchannels (white lines): A, main; B, water; C, methanol; D, sheathflow; E, Mbs trapping chamber. SPE-GEMS analysis of peptide mixture 1 on $C_{18}$-coated Mbs. (b) IC chromatograms of peptide ions and total IC during elution. (c–e) ESI-MS spectra obtained during elution of individual peptides from mixture 1. For the analysis 10 $\mu$L of 25 nM sample solution was loaded into the microchip. The time scale for the stepwise gradient elution was the following: 0 min, 5% of methanol in eluent mixture; 3.5 min, 10%; 7 min, 20%; 10.5 min, 30%; 14 min, 40%.

fluorescence,[30] or chemiluminescence[31] detection systems, an isocratic elution is typically employed. This simple elution strategy is also used when direct coupling of SPE to mass spectrometry is performed in high-throughput online SPE-tandem mass spectrometry (HT-SPE-MS),[32] in classical ESI-MS[33,34] or in ESI-MS from a microchip emitter.[35,36] In certain cases, gradient elution is applied for the online coupling of a SPE cartridge to mass spectrometer, e.g. for clenbuterol analysis.[37]

In the current work, Mbs based SPE in a microfluidic format was combined with stepwise gradient elution followed by direct online ESI-MS detection for analysis of peptides in low concentrations. This approach, named thereafter solid-phase extraction–gradient elution–mass spectrometry (SPE-GEMS), allowed effective sample desalting, enrichment, sequential elution and MS detection without the introduction of an additional separation step after SPE. This concept was realized on a microchip fabricated by laser photoablation of a flexible polyimide substrate. Mbs with $C_8$ and $C_{18}$ chromatographic coatings trapped by a pair of permanent magnets inside the microchip were utilized as SPE sorbent. The performance of the SPE-GEMS system was demonstrated on the analysis of model peptide mixtures and was further applied for the analysis of fortified human serum, of myoglobin digest and fractions obtained after OFFGEL separation of the myoglobin digest. Analytical modeling as well as finite element method (FEM) simulations was performed to describe the elution gradient process from the Mbs in this microfluidic system.

## EXPERIMENTAL SECTION

Information about chemicals and materials is presented in Supporting Information SI-1.

**Microchip Fabrication.** An SPE-GEMS microchip designed as shown in Figure 1a was fabricated by scanning laser ablation as described elsewhere.[38] Details about microchip fabrication are presented in Supporting Information SI-2.

**SPE-GEMS Experiment.** In a typical SPE-GEMS experiment, Mbs (5 $\mu$L, 5 mg/mL in 0.1% TFA water solution) were loaded into the microchip chamber. Then, loading of 10 $\mu$L of sample was performed followed by washing with 0.1% TFA water solution. Loading of Mbs and sample solutions was realized via the main microchannel A at a flow rate of 40 $\mu$L/h. Meanwhile, $H_2O$ was injected into microchannels B and C at flow rates of 2 $\mu$L/h per channel. Afterward, stepwise gradient elution was started simultaneously with ESI-MS detection. For all steps of the SPE-GEMS experiment the ESI buffer (50% MeOH, 49% of $H_2O$ and 1% of $CH_3COOH$) was injected as a sheathflow *via* microchannel D at a flow rate of 10 $\mu$L/h.

Two model peptide mixtures were chosen for the preconcentration and separation in SPE-GEMS experiments.


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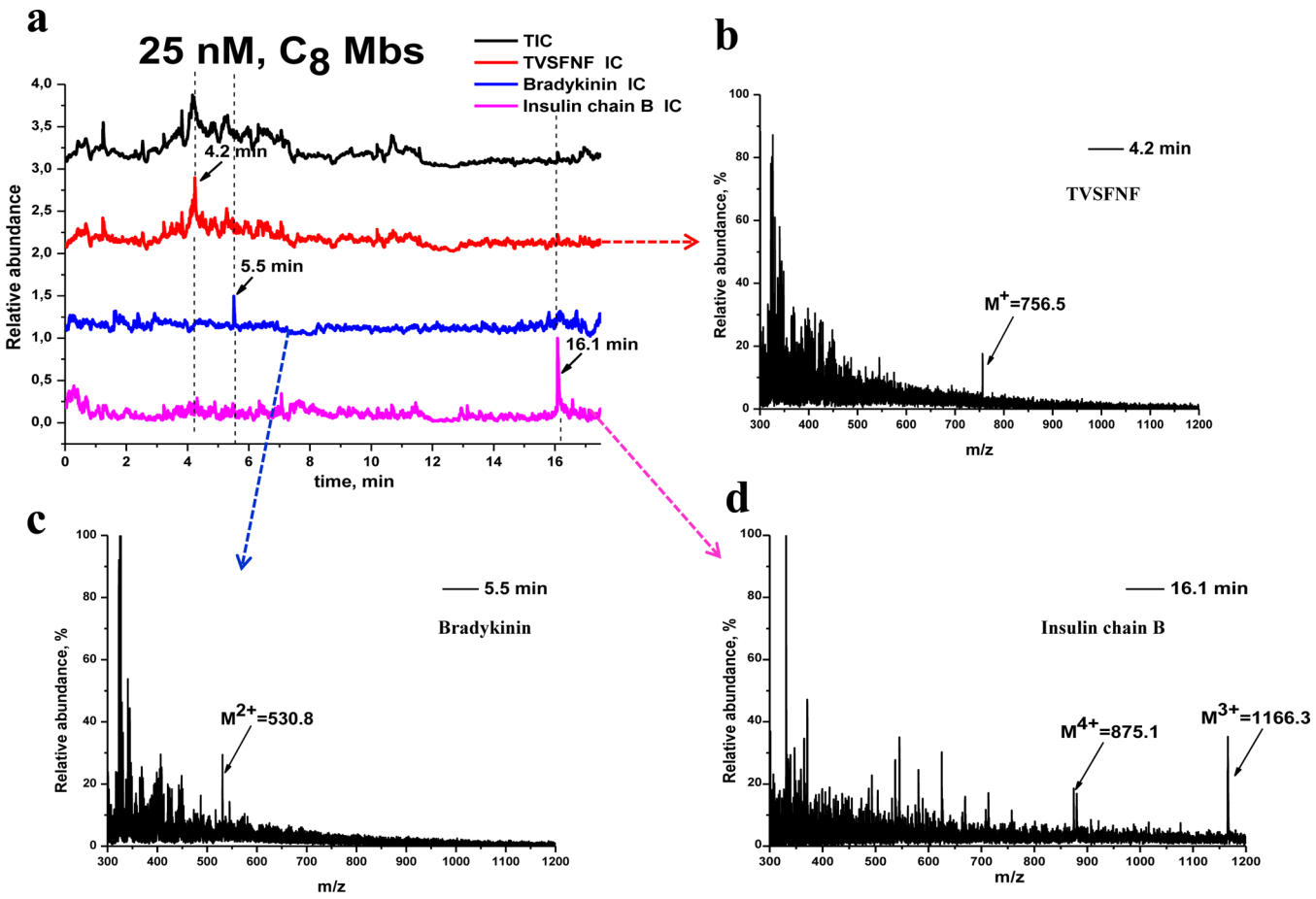


**Figure 2.** SPE-GEMS analysis of peptide mixture 2 on $C_8$-coated Mbs. (a) IC chromatograms of peptide ions and total IC during stepwise gradient elution. (b–d) ESI-MS spectra obtained during elution of individual peptides from mixture 2. Experimental conditions were the same as in Figure 1.

Model mixture 1 processed on $C_{18}$-coated Mbs was composed of peptides Ac-Thr-Val-Ser-Phe-Asn-Phe-OH (TVSFNF, MW = 755.8 Da), Met-Arg-Phe-Ala-OH (MRFA, MW = 523.7 Da), and Ac-Ser-Gln-Asn-Tyr-OH (SQNY, MW = 552.5 Da). Model mixture 2 analyzed on $C_8$-coated Mbs consisted of peptides Ac-Thr-Val-Ser-Phe-Asn-Phe-OH (TVSFNF, MW = 755.8 Da), insulin chain B (MW = 3495.9 Da), and bradykinin (MW = 1060.2 Da). All peptide samples also contained 1 mM NaCl to demonstrate the desalting properties of SPE-GEMS experiments.

The model peptide mixtures were analyzed in concentrations 1 $\mu$M, 100 nM, 25 nM, 10 nM, 1 nM per each component of the mixture. The elution of preconcentrated samples from Mbs was realized using a methanol/water stepwise gradient in the main channel A and by variation of the individual flow rates of water and methanol in channels B and C, respectively, keeping the flow rate of water in channel A at 16 $\mu$L/h. The total flow rates of the solutions in the three channels were set constant at 40 $\mu$L/h. The time scale for the stepwise elution gradient was the following: 0 min, 5% of methanol in eluent mixture; 3.5 min, 10%; 7 min, 20%; 10.5 min, 30%; 14 min, 40%. The experiment was stopped after 17.5 min. A voltage of 3.9 kV was applied to induce the electrospray from the microchip emitter to the source inlet of the Thermo LTQ Velos instrument (Thermo Scientific, San Jose, USA). ESI-MS detection was performed in positive MS mode with normal scan rate for a range of mass-to-charge ratio ($m/z$) of 300–2000.

**Numerical Simulations.** All the details about the FEM numerical simulations performance are presented in the Annex of Supporting Information.

**Human Blood Serum Analysis.** To obtain plasma-derived serum 15 mL of human plasma were clotted overnight at room temperature and then centrifuged during 10 min at 15000*g* to remove the clot. Obtained blood serum was diluted 15 times with 0.1% TFA water solution and fortified either with the peptide TVSFNF or the insulin chain B to a final concentration of 50 nM for each peptide. Fortified serum samples were analyzed by SPE-GEMS experiments using either $C_{18}$- or $C_8$-coated Mbs as SPE sorbent. All experiments were performed in three replicates.

**Protein digest and OFFGEL fractions analysis.** Mixtures of peptides to be analyzed by SPE-GEMS were generated from the tryptic digest of myoglobin and from the OFFGEL separation of obtained myoglobin digest. OFFGEL separation was performed with an Agilent 3100 OFFGEL fractionator (Agilent, Waldbronn, Germany) using an 18-cm IPG strip pH 3–10 (Amersham Biosciences, Otelfingen, Switzerland) which allows the collection of 18 fractions. More details about myoglobin digestion by trypsin and performance of OFFGEL separation are presented in Supporting Information SI-3. For the SPE-GEMS experiments on $C_8$-coated Mbs, myoglobin digest was diluted 500 times with 0.1% TFA water solution to the final concentration of 560 nM, while two chosen OFFGEL fractions with pHs 5.9 and 8.4 were diluted 50 times after collection form OFFGEL setup. In comparison with the SPE-


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Table 1. Enrichment Factors (EF) Obtained for SPE-GEMS Analysis of Model Peptide Mixtures[a]

| | $C_{18}$-coated Mbs | | | $C_8$-coated Mbs | | |
|---|---|---|---|---|---|---|
| peptide | SQNY | TVSFNF | MRFA | TVSFNF | bradykinin | insulin chain B |
| $t_R$, min | 5.6 ± 0.6 | 11 ± 1 | 13.5 ± 0.7 | 4.6 ± 0.5 | 5.2 ± 0.7 | 16.4 ± 1 |
| $EF_{vsESIbuffer}$ | 12 | 23 | 13 | 10 | 10 | 20 |
| $EF_{vs1mMNaCl}$ | 500 | 1065 | 530 | 470 | 400 | 1200 |

[a] $t_R$, average retention time ($n$ = 3); $EF_{vsESIbuffer}$, EF of SPE-GEMS (25 nM) in comparison with a classical ESI-MS detection via a commercial ionization source of a single peptide (250 nM) in the ESI buffer; $EF_{vs1mMNaCl}$, EF of SPE-GEMS (25 nM) in comparison with a classical ESI-MS detection via a commercial ionization source of a peptide solution (10 μM) containing 1 mM NaCl in the ESI buffer.

GEMS analysis of model peptide mixtures the experimental time for these samples was prolonged for 7 min by adding two more steps for the stepwise gradient elution time scale: at 17.5 min, 50% of methanol in eluent mixture; 21 min, 60% of methanol. All experiments were performed in three replicates.

## RESULTS AND DISCUSSION

**SPE-GEMS experiment.** The SPE-GEMS microchip was designed as displayed in Figure 1a to perform an effective sample preconcentration on the Mbs acting as SPE sorbent followed by the stepwise gradient elution with online ESI-MS detection. The chamber for Mbs trapping was made in order to increase the amount of Mbs that could be loaded inside the microchip. For creation of a stepwise gradient of organic solvent inside the main channel A, this channel was connected with two microchannels B and C for injection of water and methanol, respectively. The sheathflow channel D is there to provide a stable electrospray and ion current during the SPE-GEMS experiments. Other details about microchip design and its optimization, adjustment of Mbs quantity, sheathflow parameters, gradient timing, and nonspecific adsorption test are presented in Supporting Information SI-4.

Selection of the peptide mixtures for the SPE-GEMS analysis was based on the fact that $C_{18}$-coated Mbs are more suitable for small peptides enrichment, while $C_8$-coated Mbs are recommended for larger peptides. All peptide solutions also contained 1 mM NaCl to demonstrate the sample desalting in SPE-GEMS experiments. Examples of ESI-MS spectra and ion current (IC) chromatograms obtained during the SPE-GEMS analysis of model peptide mixtures are presented in Figures 1 and 2 and in Supporting Information SI-5. The signal levels on the MS spectra and IC chromatograms were lower than it could be expected from theoretical considerations. Possible explanations stem from the sample binding capacity of the Mbs, which was lower than awaited and from the dilution of eluted sample caused on the one hand by the sheathflow joining the sample flow, and on the other hand by dilution/mixing processes in the channel after elution. However, effective peptide preconcentration was observed for both types of Mbs.

Peptide enrichment factor (EF = peptide peak signal-to-noise ratio obtained with preconcentration × dilution factor/peptide peak signal-to-noise ratio obtained without preconcentration) was chosen as a characteristic of preconcentration efficiency. The EF values were determined by classical ESI-MS analysis of the sample with a commercial ionization source (with and without 1 mM NaCl). Separate experiments were performed for individual components of the peptide mixtures in order to exclude the influence of mutual ion suppression on signal intensities under infusion mode with a sample flow rate of 50 μL/h and spraying voltage of 3.9 kV. Calculated EF values are presented in Table 1. The peptide solutions were also analyzed via a plain microchip emitter, that is, SPE-GEMS microchip without Mbs loaded into the preconcentration chamber. Corresponding EF values are shown and discussed in Supporting Information SI-6.

For SPE-GEMS analysis on $C_{18}$-coated Mbs, the best preconcentration was obtained for the TVSFNF peptide (Figure 1d). The limit of detection (LOD) defined as signal-to-noise ratio of 3 on mass spectrum for this peptide was 10 nM, while for peptides SQNY and MRFA the LODs were 25 nM. The difference in the EF and LODs values between peptides could arise from their structure and their length. MRFA, the one with the longest retention time, consists of 75% of hydrophobic amino acid residues in comparison with 50% for TVSFNF and 25% for SQNY, but it is composed only of 4 amino acids, so longer peptide TVSFNF displayed higher EF values. The obtained elution order for peptides of the model mixture 1 was confirmed by classical HPLC-MS analysis of this sample (data not shown) using similar water−methanol gradient (from 5% to 40% of methanol in eluent solution). The HPLC separation was performed on 5$C_{18}$-MS II COSMOSIL column (5 μm particle diameter, 2.0 mm i.d. × 150 mm length, Nacalai Tesque, Kyoto, Japan) with Accela HPLC apparatus (Thermo Scientific, San Jose, U.S.A.) and Thermo LTQ Velos instrument (Thermo Scientific, San Jose, U.S.A.). The capacity factors $k'$ obtained for each peptide were the following ($n$ = 3): $k'$(SQNY) = 8.05 ± 0.08, $k'$(TVSFNF) = 9.83 ± 0.09, $k'$(MRFA) = 12.4 ± 0.1.

For $C_8$-coated Mbs possessing a shorter hydrocarbon chain, the peptide TVSFNF was weakly retained and eluted with only 5% of methanol (Figure 2b). Insulin chain B, the longest peptide with the highest number of hydrophobic amino acid residues among the peptides of mixture 2, was eluted with 40% of methanol displaying the highest enrichment level (Figure 2d). The LOD for insulin chain B with the SPE-GEMS microchip was 10 nM in comparison with 25 nM for TVSFNF and bradykinin. In contrast, the typical LODs for ESI-MS detection of the peptide mixtures components were defined as 250 nM for commercial ionization source or 150 nM for the plain microchip emitter.

For salt-containing samples ESI-MS detection using a commercial ionization source gave LODs of 10 μM because the presence of 1 mM NaCl in the analyte solution seriously alters the ESI-MS detection due to the strong ion suppression effect of the salt. Due to the sample preconcentration on Mbs and consequent salt removal, the SPE-GEMS procedure provided an efficient desalting of peptide model mixtures giving EFs up to 1200 for salt containing analyte solutions (comparing with the detection by a commercial ionization source). Typically the ionization and spraying properties of a commercial ionization source and microchip emitters differ significantly, especially in case of the ion suppression caused by the salt presence in the sample. To estimate the desalting efficiency of SPE-GEMS technique independently from the


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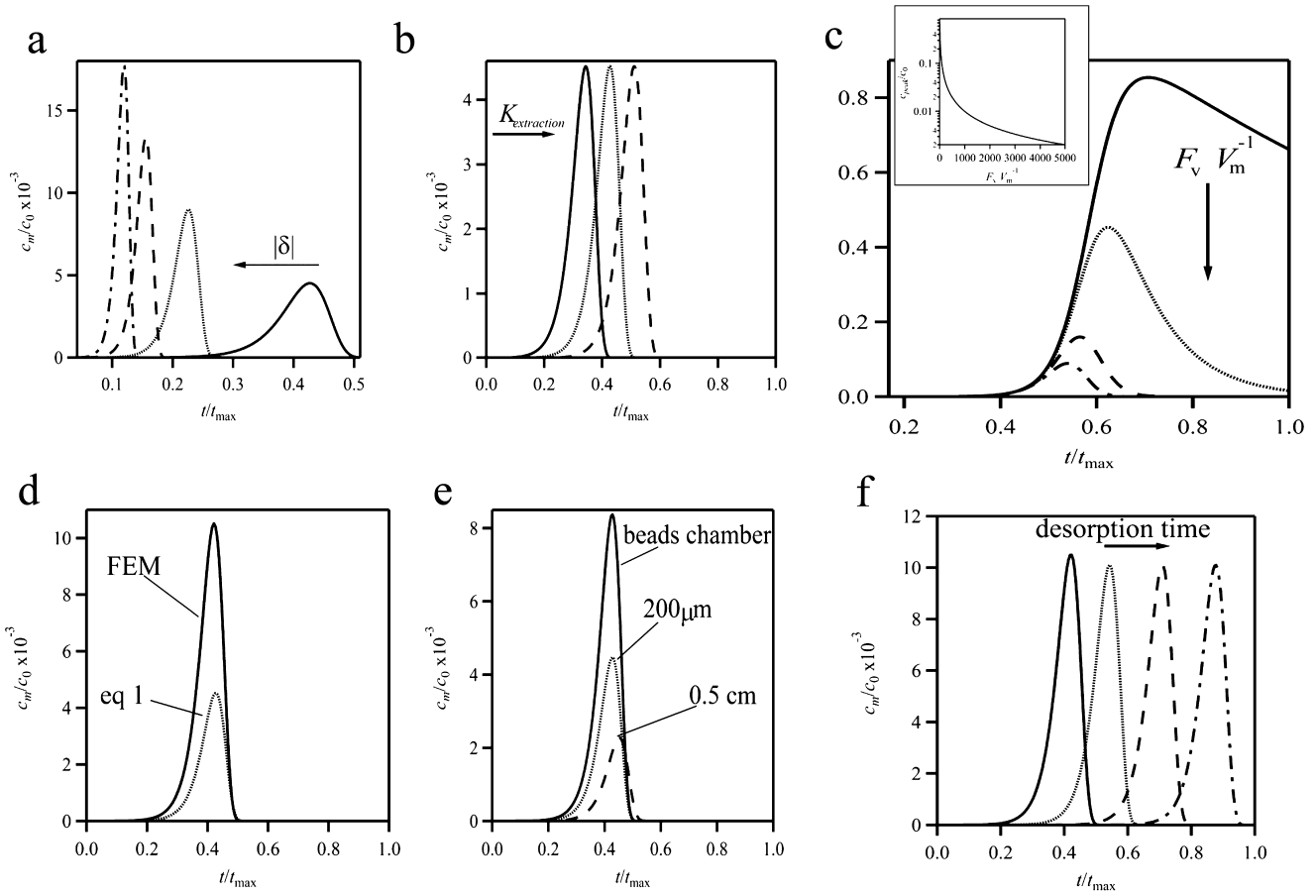


**Figure 3.** Sample elution process. Analytical model: (a) Effect of the analyte affinity toward eluent ($\delta$ = −68, −136, −204, −272 kJ·mol$^{-1}$ for solid, dotted, dashed, dash-dotted lines, respectively) on the elution chromatogram. (b) Extraction constant effect on analyte elution ($K_{extraction}$= $10^6$, $10^7$, $10^8$ for solid, dotted, dashed lines, respectively). (c) Variation of elution chromatogram upon the value of flow rate factor $F_v \cdot V_m^{-1}$ (1, 10, 50, 100 for solid, dotted, dashed, dash-dotted lines, correspondingly). Inset: Peak concentration behavior depending on $F_v \cdot V_m^{-1}$. FEM model: (d) Comparison of analyte concentrations computed within FEM and analytical models. (e) Mixing inside a liquid flow. Integral concentrations over microchannel cross sections at various distances from stationary phase ("mobile phase" specifies the cross-section position at outer boundary of Mbs phase). (f) Effect of kinetics of partition equilibrium on elution chromatogram ($k_a$ = $10^6$, 1, $10^{-2}$, $10^{-4}$ s$^{-1}$ for solid, dotted, dashed, dash-dotted lines, respectively). Unless specified, $\delta$ = −68 kJ mol$^{-1}$, $K_{extraction}$ = $10^7$, $F_v \cdot V_m^{-1}$ = 2222.2 (corresponds to 40 μL/h), $k_a$ = $10^6$ s$^{-1}$ for all plots.

types of spraying system, its performance was compared with that of a plain microchip emitter. The microchip emitter was found to be more tolerant to the presence of the salt compared to the commercial ionization source providing the LODs of 2.5 μM for peptide solutions containing 1 mM of NaCl. However, the desalting of analyte remains crucial for the sensitivity of the analysis and in this case SPE-GEMS technique provided effective sample purification with the EFs in the range of 100−310. A more detailed discussion about desalting capabilities of the SPE-GEMS approach is presented in Supporting Information SI-6.

The efficiency of the SPE-GEMS system was compared with the commercial protocol for SPE on Mbs.[39,40] It should be acknowledged that these protocols are developed for MALDI-MS detection which is known to be more tolerant to the presence of salts. The ESI-MS analysis yielded LODs of only 1 μM and 5 μM for the model peptide mixture 1 on $C_{18}$-coated Mbs and mixture 2 on $C_8$-coated Mbs, respectively (Supporting Information SI-7). These results demonstrate that the present SPE-GEMS method provides higher enrichment efficiency using a smaller quantity of Mbs and sample. Also the results of performed SPE-GEMS experiments show that the developed technique is suitable for analysis of diluted samples. There is a linear correlation between the initial amount of peptides in a sample and signals obtained for solutions with 10, 25, and 100 nM peptide concentrations, while for 1 μM analyte the signal saturation is encountered due to SPE support saturation. More discussion about this phenomenon is presented in Supporting Information SI-5.

SPE-GEMS analysis ensures not only peptide desalting and enrichment from diluted solutions, but also provides the possibility of fast sample fractionation. Chromatographic parameters were applied to the SPE-GEMS experiment to describe the fractionation process. The average retention times for peptides of model mixtures are presented in Table 1 with reproducibility defined based on three replicates ($n$ = 3 per each sample concentration) in the range of RSD = 5% for MRFA peptide to RSD = 13.4% for bradykinin. The reproducibility of peak relative abundances was in the range of RSD = 9% to RSD = 17%. Resolutions of SPE-GEMS separations of peptide mixtures 1 and 2 were calculated from IC spectra as 21 and 22, respectively. For the chosen model peptide mixtures effective fractionation was achieved. More detailed discussion about the separation properties of SPE-GEMS analysis is presented in Supporting Information SI-5.


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**Mathematical Model.** As shown above, the key advantage of the SPE-GEMS strategy for analysis of peptide samples is the efficient analyte preconcentration on Mbs followed by stepwise gradient elution and MS analysis. The LOD is therefore governed not only by the efficiency of the preconcentration step but also by the separation of target peptides during elution and their identification. To describe the dependence of the sample elution process on the different physicochemical parameters, an analytical model has been developed and FEM simulations were performed.

For analytical modeling of the elution process, partition equilibrium of the analyte between the mobile (solution) and the stationary (Mbs) phases is considered. Detailed development of this model is presented in Supporting Information SI-8. The final equations for the normalized sample concentration in the mobile phase (1) and retention time (2) are

$$\frac{c_m(t)}{c_0} = \frac{\left[1 + \phi K_{extraction} \exp\left(\frac{\beta\delta}{RT}t\right)\right]^{\frac{F_v}{V_m}\frac{RT}{\beta\delta}-1}}{\left[1 + \phi K_{extraction}\right]^{\frac{F_v}{V_m}\frac{RT}{\beta\delta}}} \exp\left[-\frac{F_v}{V_m}t\right] \quad (1)$$

$$t_R = \frac{RT}{\beta\delta}\ln\left(-\frac{F_v}{V_m}\frac{RT}{\beta\delta\phi K_{extraction}}\right) \quad (2)$$

where $K_{extraction}$ is the extraction constant from aqueous into stationary phase, $V_m$ is mobile phase volume in the SPE chamber, $\phi$ is the volume phase ratio between mobile and stationary phases ($V_s/V_m$), $R$ is the gas constant, $T$ is the temperature, $\delta$ is the solvation energy difference between the pure organic solvent and water, $\beta$ is the linear solution composition change rate, and $F_v$ is the volumic flow rate of mobile phase. The time is normalized by the duration of the gradient.

Equations 1 and 2 describe the elution process with a linear elution gradient. There are three main parameters controlling analyte elution from the adsorbent. First, the peak shape and intensity depend on the affinity of the analyte to the eluent mixture, $\delta$ (Figure 3a). The higher the solvation power of the eluent, the lower the retention time and the higher the concentration of the solute in the mobile phase. In contrast, the extraction constant $K_{extraction}$ has a minor influence on the peak intensity (Figure 3b), while the retention time is a linear function with respect to log $K_{extraction}$.

Another parameter is the flow rate factor $F_v \cdot V_m^{-1}$ that shows the amount of mobile phase volume passing through the cell in unit time. Its drastic effect on the peak shape and intensity should be taken into consideration, especially in the case of microfluidic flow rates at extremes (Figure 3c). The compromise should be found at intermediate values of fluidic ratio as slow $F_v \cdot V_m^{-1}$ lead to peak smearing while at fast flow rates the peak intensity tends to zero. In SPE-GEMS experiments reported above, a value of $F_v \cdot V_m^{-1}$ equal to 2222.2 was used, corresponding to a flow rate of 40 $\mu$L/h. Theoretically it would be possible to use smaller solution flow rates and smaller values of $F_v \cdot V_m^{-1}$ to obtain better signal intensity, however, in practice these values were limited by the stability of electrospray to be formed, which requires a certain solution flow rate (>30 $\mu$L/h) in microchip channels.

Interestingly, experimental elution profiles obtained for SPE-GEMS analysis of peptide mixtures at 1 $\mu$M concentration (see Supporting Information SI-5, Figures SI-5.1b and SI-5.2b) are in good agreement with theoretically predicted peaks of deformed tailing shape shown in Figure 3b, however corresponding to smaller flow rate factor $F_v \cdot V_m^{-1}$ values taken into account. Most likely, this difference is due to the cylindrical Mbs chamber design implemented in a microchip emitter, which is ignored in the current model.

The analytical model can also be used to estimate the theoretical EF value, for example assuming a maximal surface coverage of the MBs. According to manufacturer specifications, it is 250 fmol of peptide for 25 $\mu$g of magnetic particles,[39] what relates to the case of SPE-GEMS analysis of peptide mixture at 25 nM concentration. As follows from the eqs 1 and 2, the fraction of eluted analyte species at $t_R$ reaches ~$4.5 \times 10^{-3}$ ($\delta$ = −68 kJ· $mol^{-1}$, $K_{extraction} = 10^7$). Similar to the previously presented modeling it is important to consider the flow rate ratio of $F_v \cdot V_m^{-1}$ = 2222.2, which corresponds to 40 $\mu$L/h volumic flow rate for a given geometry of a microchannel (50 $\mu$m height, 100 $\mu$m width and long). Therefore the concentration of analyte inside the mobile phase reaches 4.5 $\mu$M giving the value of EF of 180. This value is based on the assumption of ideal Mbs binding capacity, while in practice it is not the case and considering also the dilution of eluted sample during the experiment the corresponding experimental EF is obtained only as a value of 25. It is worth to mention as well that for more precise theoretical EF estimation 3D FEM simulations should be performed taking into account the geometrical arrangement of the microchip and physicochemical characteristics of the system.

Although the analytical model helps to understand and tune the elution process, it does not take into consideration the arrangement of the microchannel with the respect to Mbs and mixing within the liquid flow as it assumes equilibrium between the mobile and the stationary phases. This could be implemented within FEM simulations considering liquid movement and diffusion/convection of species within certain domain specifying microchannel geometry. Here, the sorbent phase is modeled as a thin layer at the microchannel bottom, whereas in practice it is a fluidized bed inside the microchannel. The simulation of liquid flow inside the microchip is performed using Navier−Stokes equation assuming liquid properties (e.g., density and viscosity of water) with neglect of solvent composition influence on hydrodynamic parameters (see details in Annex to the Supporting Information). The analyte transport within the microchannel is modeled through convection/diffusion partial differential equation (PDE) for solute, that is,

$$\frac{\partial c}{\partial t} + \nabla(-D\nabla c) + \mathbf{u}\nabla c = 0 \quad (3)$$

where $D$ is the diffusion coefficient of species and $\boldsymbol{u}$ is the flow velocity.

The partition constant $K_p$ can be expressed as a function of the sorption $k_a$ and desorption $k_d$ rate constants

$$\Delta G_p^0 = -RT \ln K_p = -RT \ln\left[\frac{k_a}{k_d}\right]$$

Thus, the PDE for species in stationary phase (convection free medium for analyte) is

$$\frac{\partial c}{\partial t} + \nabla(-D_s\nabla c) = -k_a c + k_d c_s \quad (4)$$


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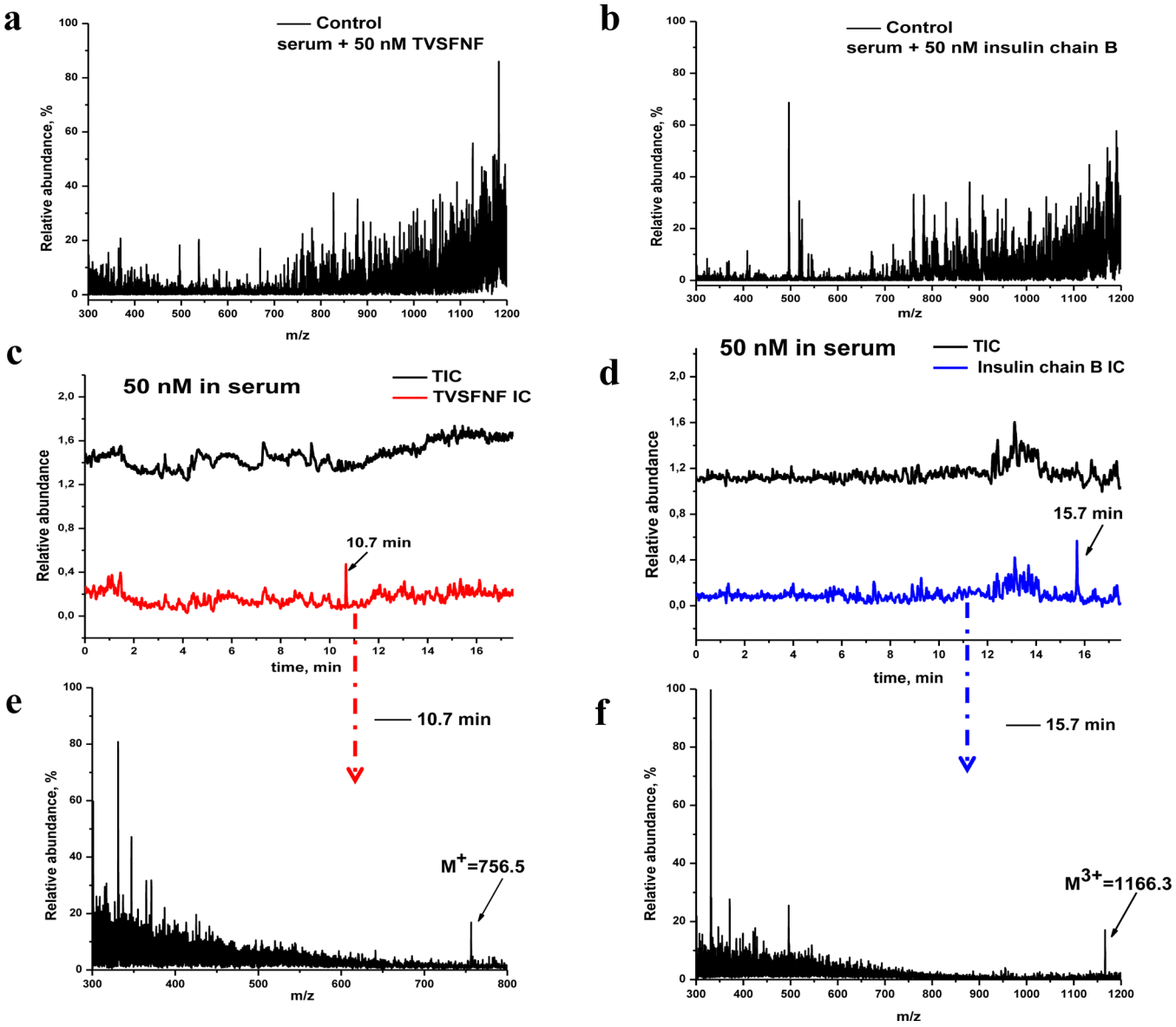


**Figure 4.** SPE-GEMS analysis of human blood serum samples fortified with 50 nM of TVSFNF peptide on $C_{18}$-coated Mbs and 50 nM of insulin chain B on $C_8$-coated Mbs. (a, b) Control ESI-MS spectra of fortified serum samples obtained using commercial ESI source without any preconcentration. (c, d) IC chromatograms of peptide ions and total IC during stepwise gradient elution. (e, f) ESI-MS spectra obtained during elution of TVSFNF peptide and insulin chain B, respectively. Experimental conditions were the same as in Figure 1.

$$\frac{\partial c_s}{\partial t} + \nabla(-D_s \nabla c_s) = k_a c - k_d c_s \quad (5)$$

where $D_s$ denotes the diffusion coefficient in stationary phase. The above-mentioned PDEs couple mass balance for sorption/desorption given by the reaction rate-like expression with mass transport of analyte (the $c_s$ here denote concentration in mol·L$^{-1}$, not a surface concentration in mol·m$^{-2}$, as the stationary phase is a bulk phase of 1 $\mu$m thickness, not a 2D surface).

The solution of the particular PDE system with proper boundary conditions gives the concentration distribution and fluid velocity map within the microchannel domain. The parabolic flow profile in the microchannel has significant consequences on the elution process evolution. The analyte desorption into eluent occurs just in a thin layer near the stationary phase because the tangential convective mass transport is fast enough in comparison with relatively slow diffusion along the vertical axis of a microchannel (see Annex to the Supporting Information). However, as the local flow rate within this thin region does not reach the average imposed volume velocity, it causes a noticeable discrepancy in the estimation of analyte mobile phase concentration predicted by FEM and analytical models. This effect shows that at given flow rate parameters eq 1 underestimates the amount of desorbed species in the mobile phase compared to FEM modeling, though the retention times are in quite good agreement (Figure 3d). Another important effect is the influence of mixing inside the laminar flow at certain microchannel dimensions on the separation and solutes detection as mixing processes result in a peak broadening and analyte fractions dilution (Figure 3e). Also kinetics of partition equilibrium is of primary importance for retention times. The sluggish rate of partition processes results in an increase of $t_R$ but does not lead to variations of peak intensities (Figure 3f).


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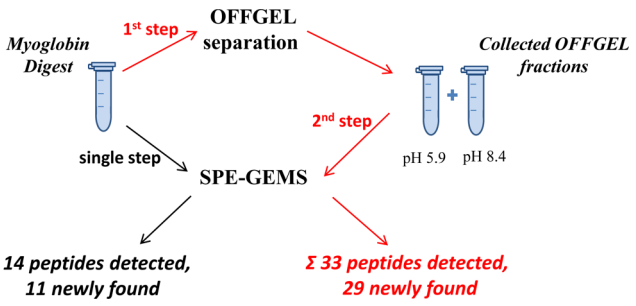
**Fortified Blood Serum Analysis.** To evaluate the performance of the developed microfluidic system on real sample analysis human blood plasma-derived serum fortified with model peptides was submitted to SPE-GEMS analysis. Insulin chain B and TVSFNF peptide were chosen to spike the human blood serum at a concentration of 50 nM as these peptides displayed the most effective enrichment on the appropriate Mbs. ESI-MS spectra and IC chromatograms obtained during fortified serum analysis are presented in Figure 4.

The ESI-MS spectra of the direct serum with a commercial ionization source without any preconcentration did not show any signals for the peptides of interest (Figure 4a and 4b). Meanwhile, on SPE-GEMS spectra TVSFNF and insulin chain B are clearly observed (Figure 4e and 4f). The signal-to noise ratios of the peptide peaks in the SPE-GEMS experiment were smaller than those expected based on the results of the model mixtures analysis: $S/N$ of 5 and 6 ($n$ = 3, RSD = 13% and 11%) for 50 nM concentration in fortified serum in contrast with $S/N$ of 8 and 9 ($n$ = 3, RSD = 10% and 9%) for 25 nM concentration in model water solutions of TVSFNF and insulin chain B, respectively. This is because of the high complexity of blood serum as an object of analysis which contains a lot of hydrophobic peptides competing with the peptides of interest for SPE on Mbs surface. Despite that fact, the two peptides showed the same retention times as in case of the model peptide mixtures analysis within the appropriate error range (average $t_R$ = 10 ± 1 min and $t_R$ = 15.3 ± 1 min for TVSFNF and insulin chain B, respectively, $n$ = 3) and were successfully identified owing to efficient enrichment in SPE-GEMS experiment. Sample preconcentration and purification, which removed part of blood serum components and thus allowed to simplify the complex sample structure, conditioned the breakthrough of peptides under consideration during SPE-GEMS analysis.

**Protein Digest and OFFGEL Fractions Analysis.** As a fractionation and preconcentration technique SPE-GEMS using $C_8$-coated Mbs was tested on the analysis of a myoglobin digest and of two fractions collected after OFFGEL separation of this digest as illustrated in Scheme 1.

**Scheme 1. Schematic Representation of the Experiments Performed with the Myoglobin Digest and Its OFFGEL Fractions**




The two OFFGEL fractions with pH = 5.9 and 8.4 were chosen based on the results of MALDI-MS analysis (data not shown) as these fractions possessed the most dissimilar spectra. Prior to SPE-GEMS experiments, the myoglobin digest (2.5 mg/mL) was diluted 500 times. OFFGEL fractions were diluted only 50 times as typically OFFGEL separation does not provide full sample recovery leading to a decreased analyte concentration in the collected fractions.[41] Prolonged experimental time was applied for these samples to ensure the elution of all peptides enriched on Mbs. The stepwise gradient time scale was the following: 0 min, 5% of methanol in eluent mixture; 3.5 min, 10%; 7 min, 20%; 10.5 min, 30%; 14 min,40%; 17.5 min, 50%; 21 min, 60% of methanol.

Results obtained are summarized in Table 2. Only peptides observed in all three replicates of each experiment were regarded as identified. The range of the signal-to-noise ratio for peptide peaks of interest in acquired MS spectra was from $S/N$ = 5 to $S/N$ = 30. The lists of the peptides discovered in the whole digest and OFFGEL fractions analysis are presented in Supporting Information SI-9 with the sequences proposed based on the comparison of the observed molecular weights with the theoretical ones from the databases using *FindPept* tool on the ExPASy server (http://web.expasy.org/findpept/).

**Table 2. Results of SPE-GEMS Analysis of the Myoglobin Digest and Two Fractions Collected after Its OFFGEL Separation**

| | | number of peptides identified by SPE-GEMS | | | | |
|---|---|---|---|---|---|---|
| | | matched with ESI-MS control | | newly found | | |
| sample | | common[a] | specific[b] | common | specific | in total |
| digest | | 3 | 0 | 5 | 6 | 14 |
| OFFGEL fractions | pH ~5.9 | 1 | 1 | 8 | 7 | 17 |
| | pH ~8.4 | 2 | 0 | 10 | 4 | 16 |

[a]Peptides observed not only in the SPE-GEMS analysis of the current sample, but also in other samples analysis (e.g., in the analysis of the whole digest or other OFFGEL fraction, or in both cases). [b]Peptides observed only in the SPE-GEMS analysis of the current sample.

As shown in Table 2, among the peptides identified during SPE-GEMS analysis of the myoglobin digest and its OFFGEL fractions, in total only 4 peptides were previously detected in the control ESI-MS experiments with a commercial ionization source (see Supporting Information sections SI-10 and SI-11). One of these peptides was observed as a specific peptide in OFFGEL fraction pH 5.9. The other 3 peptides were observed as common peptides, most probably indicating that they were present in relatively high concentration in the initial samples. $C_8$-coated Mbs are prone to trap long peptides of hydrophobic nature which are presented in low abundance in the initial samples. Especially for the OFFGEL fractions as diluted samples, purification effect from the polymer contamination (see Supporting Information SI-11) and the preconcentration effect of SPE-GEMS technique were significant. Almost all peptides detected in SPE-GEMS experiments (e.g., 15 newly found peptides in fraction pH 5.9) were not observed before on the control ESI-MS spectra. Typically, for each sample half of the newly identified peptides was specific, that means not found in the other samples, demonstrating the ability of SPE-GEMS technique to provide new information about the structure of complex analytes.

Moreover, several long peptides (≥20 amino acid residues) whose theoretical pIs are 2−3 units different from the general pH of the corresponding OFFGEL fractions, were still discovered in SPE-GEMS analysis of the fractions. Similar to the other fractionation techniques, OFFGEL separation possesses a certain resolution and efficiency of peptide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separation depending on the nature of the peptides. Typically, long peptides require more time to migrate to the wells with the pH corresponding to their pIs. Their separation is less efficient than the separation of the short peptides, and they could partially be present in a wide range of OFFGEL wells. However, these long peptides with the pIs unmatched to the pH of the wells are usually present in trace amount in these wells and are masked by highly abundant short peptides with proper pIs. Only the preconcentration in the SPE-GEMS experiment allows the discovery of these long peptides. For instance, three newly discovered peptides IPIKYLEFISDAIIHVLHSK (20 residues, MW = 2336.3, pI = 6.9), HKIPIKYLEFISDAIIHVLHSK (22 residues, MW = 2601.5, pI = 8.5), and HGTVVLTALGGILKKKGHHEAELKPLAQSHATK (33 residues, MW = 3469.8, pI = 9.8) were identified in SPE-GEMS analysis of all three samples: whole myoglobin digest and its two OFFGEL fractions (with average RSD = 13% for peptide retention times between analysis of different samples). A hydrophobic nature in combination with the large number of amino acid residues (≥20 amino acids) enables effective enrichment and identification of these peptides disregarding their initial abundance in the solution. Therefore, data obtained during SPE-GEMS analysis provided additional information about myoglobin digest and the composition of its OFFGEL fractions revealing previously masked peptides.

Furthermore, only few newly identified peptides from OFFGEL fractions pH 5.9 and pH 8.4 were previously detected during the whole myoglobin digest SPE-GEMS analysis. This is due to the fact that the complexity of the digest was first reduced by OFFGEL separation which fractionated peptides according to their pIs and then, was further decreased by the SPE-GEMS analysis which preconcentrated and fractionated peptides based on their length and hydrophobic properties as presented in Scheme 1. The combination of OFFGEL separation with the developed SPE-GEMS technique allowed the discovery of new peptides previously undetectable in the myoglobin digest and permitted sample processing similar to the two-dimensional separation system. Hence the combined technique could be advantageous for complex sample analysis.

## CONCLUSIONS

A SPE-GEMS microchip for solid phase extraction-stepwise gradient elution with direct online MS detection was developed for the analysis of peptides in low concentrations. $C_8$- and $C_{18}$-coated Mbs were used inside the microfluidic device as a SPE sorbent. Performance of the system was shown by the analysis of model peptide mixtures. Effective desalting and enrichment of the diluted samples were achieved. The LODs for peptide TVSFNF on $C_{18}$-coated Mbs and for insulin chain B on $C_8$-coated Mbs were defined as 10 nM. The fractionation capability of the stepwise gradient elution used for direct SPE-MS coupling in the current system was described in terms of a chromatographic separation. Analytical and FEM modeling was performed to characterize the process of sample elution from the Mbs surface and its dependence from the different parameters.

An SPE-GEMS microfluidic device was tested on the analysis of human blood serum fortified with the model peptides. Despite the high complexity of the sample, peptide TVSFNF and insulin chain B were easily identified during the analysis due to the effective preconcentration. Tryptic digest of myoglobin and two fractions collected after OFFGEL separation of this digest were also submitted to SPE-GEMS analysis for further evaluation of this approach. New peptides that were present in the initial samples in low abundance were successfully preconcentrated and detected in SPE-GEMS experiments. Achieved preconcentration and fractionation of previously hidden peptides illustrate the capability of SPE-GEMS analysis to deal with complex samples and to provide new information about their composition. The possibility to change the nature of SPE sorbent simply by loading inside of the microchip Mbs with another surface coating makes the SPE-GEMS technique a versatile tool for desalting, enrichment and fractionation of various types of peptides.

## ASSOCIATED CONTENT

### Supporting Information

Details about microchip fabrication and desing optimization, OFFGEL separation protocol, results of control ESI and MALDI-MS experiments, as well as results of SPE-GEMS experiments and finite element simulations details. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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