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Total on-line analysis of a target protein from plasma by immunoextraction, digestion and liquid chromatography–mass spectrometry☆

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ABSTRACT

A total on-line analysis of a target protein from a plasma sample was made using a selective immunoextraction step coupled on-line to an immobilized enzymatic reactor (IMER) for the protein digestion followed by LC–MS/MS analysis. For the development of this device, cytochrome *c* was chosen as model protein due to its well-known sequence. An immunosorbent (IS) based on the covalent immobilization of anti-cytochrome *c* antibodies on a solid support was made and an immunoextraction procedure was carefully developed to assess a selective extraction of the target protein from plasma. For the first time, IS was easily coupled on-line with a laboratory-made IMER based on pepsin. The whole on-line device (IS-IMER-LC-MS/MS) allowed the quantification of cytochrome *c* from 8.5 pmol to 1.7 nmol in buffer medium. Finally, this device was applied to the analysis of only 85 pmol of cytochrome *c* from plasma with a RSD value lower than 10% ($n = 3$).

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1. Introduction

The proteomic community has paid increasing attention to the need for protein biomarkers discovery [1]. Biomarkers are used in a wide range of applications that range from diagnosis and prognosis of diseases to the monitoring of a biological response or therapeutic intervention. So, the analysis of a target protein acting as biomarker from biological samples is nowadays a real challenge for human health.

The proteins identification is commonly carried out by using liquid chromatography coupled with mass spectrometry (LC–MS) [2]. However, ionization by electrospray of large molecules produces a very complex envelope of multiply charged ions that requires sophisticated deconvolution analysis. So, a bottom-up approach is favoured by digesting the proteins to produce smaller peptide fragments, which are then separated and easily identified by LC–MS/MS [2,3]. Moreover, proteins of interest are usually part of a very complex mixture of other proteins and molecules that co-exist in the biological medium. It is estimated that the protein concentrations in biological samples span over 12 orders of magnitude. In plasma, only 22 proteins account for 99% of the plasma protein content and proteins of interest as biomarkers belong to the remaining 1% [4]. Unfortunately, by ionization of a such mixture by electrospray, the identification of the low-abundant proteins is generally hampered by the presence of the more abundant proteins such as albumin, transferrin, haptoglobulin, immunoglobulins, and lipoproteins [5]. Therefore, the aim of this study is the development of an automated method for the analysis of a target protein in a biological sample as plasma that integrates the possibility to remove other proteins which may interfere during the analysis. Immuno-based sample preparation methods are becoming increasingly popular biological tools by exploiting the high affinity of the antigen–antibody interaction. Indeed, immunoaffinity depletion columns containing immobilized antibodies against the high-abundant proteins are commercially available. However, they are very expensive and interfering proteins still remains after this pretreatment [6,7]. Another alternative is the use of immunosorbents (IS) based on the immobilization on a solid support of antibodies developed against a target analyte. Immunoextraction has been largely developed in our department, particularly for the selective trapping of small-size molecules [8–10]. Many examples have been also described for the immunoextraction of larges molecules as antibodies, enzymes, proteins or hormones with antibodies immobilized on various solid supports (agarose, synthetic organic supports, derivatized silica and glass) as reviewed by Hage [11] and Lee and Lee [12]. In most of the reported works, an enzymatic digestion is performed in solution after the selective immunoextraction step. However, the digestion in solution is time consuming [13,14], autoproteolysis of enzymes can occur and manual sample handling can cause a risk of sample contamination. To overcome these drawbacks, the proteolytic enzymes can be immobilized on a solid support and be integrated to the analytical system thus allowing the total automation of the analysis. As recently reviewed, immobilized enzymatic reactors (IMERs) were integrated into the analytical system and dedicated to proteins

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analyses [15,16]. Trypsin is the most widely used enzyme in proteomic field because of its high selectivity of cleavage. Trypsin based IMERs were already successfully developed under various formats, i.e. pre-columns [17–23], membranes [24], capillaries [25–29] and disks [30–32]. Recently, our group highlighted the important effect of the immobilization support on the performances of the resulting IMER [33]. By this work, CNBr activated sepharose showed the best performances as immobilization support for a trypsin based IMER coupled on-line to LC/MS. Indeed, this hydrophilic support prevents non specific hydrophobic interactions with peptides and presents a good chemical stability thus allowing the reusability of this sorbent [33].

The aim of this present study was to combine an immunosorbent for the selective extraction of a target protein with an IMER and to integrate both devices on-line with LC–MS analysis. Until now, few research groups reported the use of an immunosorbent coupled on-line with an enzyme based IMER to ensure a total automated analysis of human hemoglobin proteins from mixture containing several different human plasma proteins [23] and of bovine serum albumin from undiluted human urine samples [34]. All these studies used a commercial trypsin immobilized reactor, Poroszyme® (Applied Biosystems). In addition, the on-line coupling between IS and IMER involved problems of solvent compatibility because protein are eluted at low pH from IS and trypsin cleaves at a slightly basic pH. Consequently, a decrease of the immobilized enzyme activity was unfortunately observed [34]. To overcome this drawback, the same group developed an on-line solution phase digestion [35,36]. The digestion took place in a reaction capillary serving as reaction coil by mixing a proteolytic solution with the LC effluent. However, a decrease of sensitivity of the mass spectrometer can be observed due to the presence of the enzyme and its autodigestion products. More recently, an application for the automated analysis of matrix metalloproteases in urine samples was developed [37]. The on-line procedure consisted of a selective enrichment step of only active form of enzymes by inhibitor affinity chromatography coupled to a digestion on trypsin IMER taking care that the elution buffer does not affect the digestion efficiency and followed by a preconcentration on a trap column and analysis by nano-LC–MS/MS. Taking into account our knowledge concerning the immobilization of biomolecules on solid supports [9], a digestion on immobilized enzymatic reactor was preferred over flow enzyme cleavage to overcome the autodigestion products. In addition, proteolytic enzyme acting under acidic conditions, i.e. pepsin, was chosen. Pepsin cleaves proteins at C-end of hydrophobic amino acids, such as phenylalanine and leucine residues [38]. In contrast to trypsin, only a few studies deal with the use of pepsin based IMER. To our knowledge, no coupling with IS was reported. Most of developed pepsin-based IMERs were followed by a capillary electrophoresis (CE) analysis [39,40]. Pepsin based IMERs were also associated with LC–MS analysis but the study was dedicated to protein dynamics [41,42]. More recently, proteins such as hemoglobin, casein, albumin and myoglobin [43,44] were digested on-line by pepsin IMER followed of a LC–MS/MS analysis. However, proteins were only analyzed in pure samples without selective pretreatment.

For the first time, an immunosorbent was in this study coupled on-line with a pepsin based IMER to provide a total automated analysis of a target protein from a spiked plasma. A carefully attention was given for the solvent compatibility between these various steps to keep a simple device. For this, all the development was made with a model protein which its sequence is well known, cytochrome *c*. The aim of this work was to show the feasibility of such a method. By the previous results obtained for the development of the trypsin based IMER [33], CNBr activated sepharose was chosen as immobilization support. The resulting IMER was then packed in a pre-column and coupled on-line to the chromatographic system.

Concerning the IS, CNBr activated sepharose was also chosen for the immobilization of the anti-cytochrome *c* antibodies. Firstly, immunosorbent was characterized and a selective extraction procedure was developed. In a second part, IS was integrated to the analytical system including the pepsin based IMER on-line coupled with the chromatographic separation. This automated procedure was evaluated in real medium with a plasma sample.

To provide a detailed evaluation of the IMER and to optimize the on-line coupling, a tool of quantification was used. Here two specific peptide fragments of cytochrome *c* were monitored. The relationship between the peak areas of selected peptides and the amount of the protein was used to quantify the protein and then to measure the recovery yield of the whole procedure including the selective pretreatment.

2. Material and methods

2.1. Reagents

Pepsin from porcine gastric mucosa (EC 3.4.23), myoglobin, bovine serum albumin and horse heart cytochrome *c* were purchased from Sigma (Sigma–Aldrich, Saint Quentin Fallavier, France). Mouse monoclonal anti-horse cytochrome *c* antibodies were purchased from Interchim (Montluçon, France). Human plasma was provided by EFS (Strasbourg, France).

Sodium acetate (CH_3CO_2Na), sodium hydrogen phosphate (Na_2HPO_4), potassium hydrogen phosphate (K_2HPO_4), Trizma hydrochloride ($NH_2C(CH_2OH)_3$, HCl), Trizma base ($NH_2C(CH_2OH)_3$), sodium azide (NaN_3) and sodium chloride (NaCl) were also purchased from Sigma. Potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), acetic acid (CH_3COOH), formic acid (HCOOH), glycine and acetonitrile (MeCN) were purchased from VWR (Fontenay-sous-Bois, France). Ultrapure water was obtained from a MilliQ water purification system and Millex-HV 0.45 μm filters were used for plasma sample preparation (Millipore, St Quentin en Yvelines, France). Cyanogen bromide-activated-Sepharose 4B (Seph-CNBr) used to immobilize pepsin and antibodies was purchased from Sigma.

The phosphate-buffer solution (PBS) consisted of a 0.01 mol L^{-1} phosphate buffer (pH 7.4) containing 0.15 mol L^{-1} of NaCl. The PBS-azide solution is a solution of PBS with 0.02% (w/w) of NaN_3. Myoglobin, albumin and cytochrome *c* solutions were prepared in a saline Tris buffer (Trizma hydrochloride and Trizma base, 50 mmol L^{-1} pH 8 and 10 mmol L^{-1} of $CaCl_2$). The anti-cytochrome *c* antibodies solution was prepared in PBS.

2.2. Apparatus and HPLC-MS analysis

The cytochrome *c* analysis was performed on-line with the system set-up depicted on Fig. 1. This device was composed of four six-port switching valves. The first one was connected to the injection loop (20 μL) and to an isocratic pre-concentration pump (LC-10AS, Shimadzu, Champs sur Marne, France). The second one was connected with the IS anti-cytochrome *c* packed in a pre-column (20 × 2 mm I.D. or 1 mm I.D., CIL, Ste Foy la Grande, France). The third one was connected with the pepsin IMER packed in a pre-column (20 × 2 mm I.D., CIL) and placed in an oven set at 37 °C (Crococil oven, CIL). As ISs and pepsin IMERs are no pressure resistant sorbents, the on-line coupling to the LC analytical column was ensured by an intermediary trapping pre-column containing a polymeric reversed phase (PRP-1, 20 × 2.3 mm I.D., 2 μm, Hamilton, Switzerland) connected with the fourth switching valve. The backpressure generated by this system was not over 5 bars. To perform the chromatographic analysis, this column switching set-up

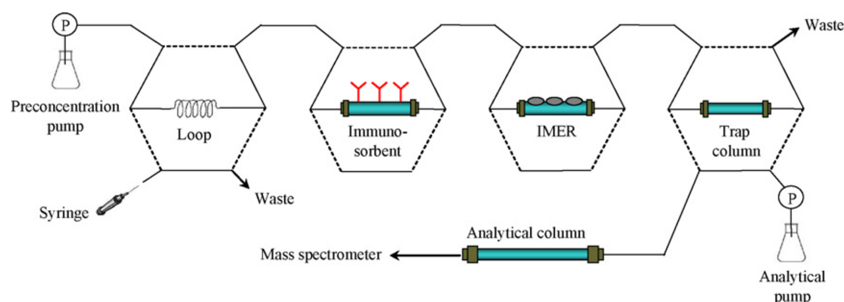

Fig. 1. Chromatographic set-up. Injection volume: 20 μL. Trap column: PRP-1 polymer (20 × 2.3 mm I.D.). Analytical column: Zorbax, Eclipse XDB-C18 (150 × 3 mm I.D., 5 μm).

was coupled to a binary gradient pump series Agilent 1100 series equipped with a diode array detector controlled by Chemstation software and with an ion trap mass spectrometer SL equipped with an electrospray source (ESI) controlled by MSD Trap software (Agilent Technologies, Massy, France). The analytical column was a Zorbax Eclipse XDB-C18 (150 × 3 mm I.D., 5 μm, Agilent Technologies).

The trap column was obviously connected on-line to the analytical column. The LC mobile phase contained a mixture of water and acetonitrile acidified with 1% formic acid. The separation of peptides was achieved by increasing the amount of acidified acetonitrile from 2% to 40% from 0 min to 20 min at 0.4 mL min^{-1}. The peptides were analyzed by LC-ESI-MS/MS. Ionization was performed in positive ion mode with the following source conditions: source voltage was set at 4 kV, the drying temperature at 350 °C, nitrogen drying gas flow at 10 L min^{-1} and nebulizer pressure at 40 psi. In MS/MS analysis, the maximum accumulation time was 150 ms and the collision-induced dissociation (CID) experiments were carried out with a fragmentation voltage of 1.3 V. So, the totality of the acquired MS/MS spectra were exported through MASCOT software (MatrixScience, Inc., Boston, MA) and correlated to the sequences from the database SwissProt for the identification of cytochrome *c*. MASCOT software allows also calculating the sequence coverage by dividing the number of amino acids contained in the identified peptides in the MS/MS experiments by the total number of amino acids of the protein.

2.3. Sample preparation of human plasma

Plasma sample was centrifuged for 3 min at 9000 × *g* (Sigma centrifuge 2K15, Sigma–Aldrich) and 0.8 mL of the supernatant was diluted in 3.2 mL of Tris buffer (pH 8.3). Then, the diluted plasma was filtered through a 0.45 μm membrane filter. Finally, plasma was spiked at 50 ng μL^{-1} of cytochrome *c*.

2.4. On-line digestion by pepsin-based IMER

2.4.1. Preparation of the pepsin IMER

The pepsin-based IMER was prepared according to the procedure described by Kurimoto et al. [45]. Briefly, pepsin (from 0.25 mg to 5 mg) contained in 1 mL of grafting solutions (CH_3CO_2Na, 0.1 mol L^{-1}, pH 5.8, NaCl 0.5 mol L^{-1}) was added to 25 mg of CNBr-sepharose which had been previously swollen in 1 mL of HCl (pH 3) and washed with 1 mL of grafting solution. Then, pepsin was incubated during 16 h at 4 °C. Most of these sorbents were only prepared in order to evaluate the immobilization yields. The sorbents prepared with 1 and 5 mg of pepsin were packed in 20 × 2 mm I.D. pre-columns and named IMERs 1 and 2, respectively. Then, they were connected with a preconcentration pump LC-10AS (Shimadzu) for their washing. At first, the remaining active sites of CNBr sepharose were blocked by percolating a glycine solution (0.2 mol L^{-1}, pH 5.0) at 0.5 mL min^{-1} for 2 h. Then, the pepsin immobilized sorbents were washed successively with 5 mL of the grafting solution followed by 5 mL of acid solution (HCl, pH 3) containing 0.5 mol L^{-1} of NaCl at 0.5 mL min^{-1} to remove non-bounded enzymes. The washing procedure was repeated three times. Finally, the IMERs were stored at 4 °C in a formic acid solution (pH 2).

2.4.2. Evaluation of the amount of immobilized pepsin

As pepsin can be detected in UV, the amount of pepsin immobilized on the support was estimated by measuring the UV absorbance of supernatant before and after the immobilization step. The UV detection was carried out at 280 nm with a spectrophotometer (SpectraMax M2, Molecular Devices, St Gregoire, France).

2.4.3. Digestion procedure by the pepsin IMER

The study of the digestion of cytochrome *c* on the laboratory-made IMERs was carried out with the set-up described in Fig. 1 without connecting the second valve and according to the following protocol. A solution of 5 μg of cytochrome *c* (20 μL) in Tris buffer was transferred on the pepsin IMER with 100 μL of HCOOH solution pH 2 at 0.1 mL min^{-1}. Then, the flow-rate was stopped during 20 min. The resulting peptides were transferred through the trapping PRP-1 pre-column with 500 μL of HCOOH solution pH 2 at 0.1 mL min^{-1}. Finally, the peptides were eluted and transferred from the trapping column to the analytical column by the LC mobile phase to be separated and detected by LC–MS/MS.

2.5. In-solution digestion

Solutions of cytochrome *c* from 20 pmol μL^{-1} to 170 pmol μL^{-1} were digested in 1 mL of HCOOH solution (pH 2) by adding pepsin in an enzyme/substrate ratio of 1/25 (mol/mol) at 37 °C overnight. 20 μL of the solution containing the resulting peptides were injected directly on the analytical column and analyzed by LC–MS/MS.

2.6. Immunoextraction

2.6.1. Preparation of the immunosorbent

The immobilization of the antibodies on Seph-CNBr was achieved according to the procedure already used in our laboratory [46]. Briefly, 35 mg of Seph-CNBr was swollen in 1 mL of an acid solution (HCl, pH 3) during 15 min and washed two times with 1 mL of a mixture of $NaHCO_3$ (0.1 mol.L^{-1}) and NaCl (0.5 mol L^{-1}) at pH 8.3. 100 μg of anti-cytochrome *c* antibodies contained in 100 μL of PBS were then added. Antibodies were incubated during 16 h at 4 °C and the resulting sorbent was packed in a 20 × 2 mm I.D. pre-column. By connecting the resulting pre-column to a pre-concentration pump (LC-10AS, Shimadzu), the remaining uncoupled sites were blocked by percolating a solu-

tion of 0.1 mol L^{-1} Tris buffer (pH 8) at 0.5 mL min^{-1} for 2 h at room temperature. Then, the sorbent was washed alternatively four times with 5 mL of a low-pH buffer (0.1 mol L^{-1} acetate, 0.5 mol L^{-1} NaCl, pH 4) and 5 mL of a high-pH buffer ($NaHCO_3$ 0.1 mol L^{-1}, NaCl 0.5 mol L^{-1}, pH 8.3) at 0.5 mL min^{-1} to remove non-bounded antibodies. Finally, the immunosorbent (IS) was stored at 4 °C in a PBS-azide solution. This IS was named IS A. Another IS was prepared by applying the same immobilization procedure and packed in a smaller pre-column (20 × 1 mm I.D.). For this IS named IS B, the volumes and flow-rates of the washing procedure were divided by a factor 4.

2.6.2. Immunoextraction procedure

To evaluate their performances, IS A and IS B were coupled directly to a diode array detector (DAD) (valve 2 connected to the DAD instead of valve 3). The development of a selective extraction procedure was made by using a protein mixture containing cytochrome c and two interfering compounds, myoglobin and albumin. The capacity of the IS, i.e. the maximal amount of cytochrome *c* that can be specifically retained by the IS in given conditions was also estimated. For this, increasing amounts of cytochrome *c* ranged from 85 pmol to 1.7 nmol and from 40 pmol to 340 pmol, were percolated through IS A and IS B respectively and the immunoextraction procedures were carried out differently according to the internal diameter of the pre-column of IS. When using IS A, a flow-rate of 200 μL min^{-1} was applied excepted when it is mentioned. After conditioning the IS A with 3 mL of a PBS solution of pH 7.4, samples were injected using a loop of 20 μL by percolating 1 mL of PBS. Then, the pre-column was washed with 1 mL of NaCl 2 mol L^{-1} to remove interferences. For the IS B, after conditioning the pre-column with 0.75 mL of a PBS solution, 20 μL of protein sample were transferred on the IS B by 0.25 mL of PBS and the interferences were removed by percolating 0.25 mL of NaCl 2 mol L^{-1} at 50 μL min^{-1}. During these steps, the removal of proteins (albumin, myoglobin) not specifically retained were controlled by UV detection. Then, the elution of the target protein, i.e. cytochrome *c*, was performed using a formic acid solution (pH 2) at 100 μL min^{-1} for IS A and at 20 μL min^{-1} for IS B. This elution step was followed by UV detection at 400 nm and the peaks obtained were integrated and correlated to the amount of cytochrome *c* specifically retained by the immunosorbent.

2.7. Total automated analysis of cytochrome c by IS/IMER/LC–MS/MS analysis

For the total on-line analysis of cytochrome *c*, IS A and IS B were coupled with IMER 1 according to the set-up described on Fig. 1. By connecting on-line the IMER 1 to the IS, cytochrome *c* was transferred through the pepsin reactor using 150 μL of a formic acid solution (pH 2) at 100 μL min^{-1} for the IS A and 120 μL of the same acid solution at 20 μL min^{-1} for the IS B after the immunoextraction procedure. After the digestion step (stop-flow during 20 min), the valve connected to the trap column was switched and the resulting peptides were transferred through the hydrophobic polymer with 500 μL of formic acid (100 μL min^{-1}). Finally, the peptides were transferred by the LC mobile phase to the analytical column as described in Section 2.4.3.

3. Results and discussion

Considering the complexity degree for the development of a such on-line device, the immunoextraction step and the on-line digestion step were developed and optimized separately keeping in mind the necessity to determine optimal conditions allowing the coupling of both systems.

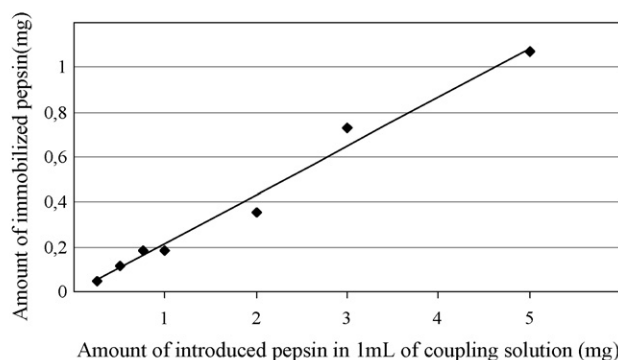

Fig. 2. Amount of pepsin immobilized on 25 mg of CNBr activated sepharose versus amount of introduced pepsin in coupling solution.

3.1. Development of an optimized pepsin IMER

In a previous study related to the evaluation of various sorbents for the covalent immobilization of trypsin, CNBr activated sepharose appeared as the most performing support for trypsin-based IMER [33]. For this reason, the same sorbent was used to immobilize pepsin. In a first step, the immobilization recovery of pepsin was studied.

3.1.1. Evaluation of the immobilization recovery of pepsin

Different amounts of pepsin were introduced in the grafting solution (0.25, 0.5, 0.75, 1, 2, 3 and 5 mg of pepsin) to be immobilized on the solid support (25 mg of CNBr activated sepharose). The amount of pepsin immobilized on the support was estimated by UV measurement according to the procedure described in Section 2.4.2. Fig. 2 presents the amount of immobilized pepsin versus the amount of pepsin initially introduced in the grafting solution.

As expected, the increase of the amount of enzyme in the coupling solution leads to a linear increase of pepsin covalently immobilized on the support. For the whole studied range of pepsin amounts (0.25 to 5 mg) introduced in the grafting solution, a linear part is observed that corresponds to a constant immobilization recovery. By the value of the slope, the immobilization recovery was estimated around 20%.

3.1.2. Quantitative analysis of cytochrome c by LC–MS/MS

The sorbent obtained by introducing 1 mg of pepsin in the grafting solution was packed in a pre-column (20 × 2 mm I.D.). The resulting IMER, named IMER 1 was applied to the on-line digestion of 5 μg of cytochrome *c* (0.43 nmol). After this solid phase digestion of cytochrome *c*, peptides were separated and analyzed by LC–MS/MS as illustration on Fig. 3 showing the resulting total ion chromatogram. After MS/MS experiments, cytochrome *c* has been well identified by MASCOT recognizing until 5 peptides with a sequence coverage of 64%. The complete list of the peptides from digested cytochrome *c* detected by LC–MS/MS and their states of charge are reported on the Table 1. Fig. 3 shows also the total ion chromatogram obtained after injection of a blank sample, i.e. 20 μL of Tris buffer sample. Pepsin was not recognized for this sample proving that there is no leakage form the IMER.

To evaluate the performances of IMERs, two specific peptides of the cytochrome *c* were chosen by considering the best probability of the peptides to belong to heart horse cytochrome *c*, our model protein. For this, a Basic Local Alignment Search Tool (BLAST) provided by the National Center of Biotechnology Information (NCBI, Bethesda, MD, United States) was used to find regions of similarity between biological sequences. The sequences of both peptides are AGIKKKTEREDL (MW 1386.8) and GRKTGQAPGF (MW 1017.5). Reconstructed ion chromatograms were generated for the different

Table 1
Peptides recognized by MASCOT after on-line digestion of 5 μg (0.43 nmol) of cytochrome *c* on pepsin IMER 1.

tR (min)	MW	Peptides sequence	Position	m.c*	*m/z*	Charge
7.3	1386.8	AGIKKKTEREDL	84–95	0	347.7 463.3 694.4 1387.8	4+ 3+ 2+ 1+
9.5	1017.5	GRKTGQAPGF	38–47	0	509.8 1018.4	2+ 1+
10.1	1149.6	IAYLKKATNE	96–105	1	383.9 575.3 1150.6	3+ 2+ 1+
12.8	2111.1	TYTDANKNKGITWKRRTL	48–65	0	704.4 1056.1	3+ 2+
16.3	2201.1	MEYLENPKKYIPGTKMIF	66–83	1	734.4	3+

*Missed cleavage.

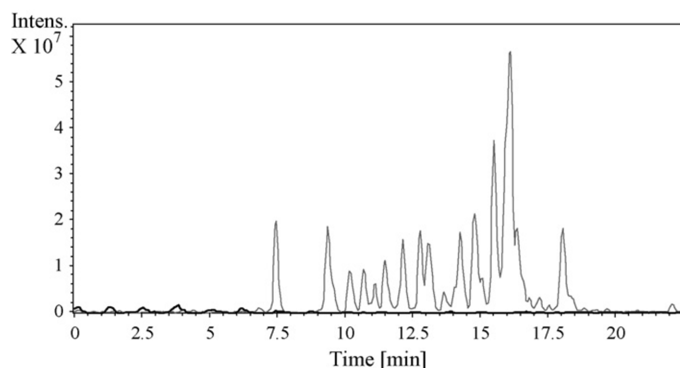

Fig. 3. Total ionic currents of LC–MS/MS analysis after on-line digestion of 0.43 nmol of cytochrome *c* on pepsin IMER 1 (grey) and after injection of a blank sample on pepsin IMER 1 (black).

states of charge of these selected peptides with *m/z* 1386.8 (1 +), 694.4 (2 +), 463.3 (3 +) and 347.7 (4 +) for AGIKKKTEREDL and *m/z* 1018.5 (1 +) and 509.8 (2 +) for GRKTGQAPGF. The peaks areas corresponding to each *m/z* ratios of a given peptide were summed and the resulting values obtained for each peptide were used for the quantitative study of the IMERs.

To check the linearity of the response using this quantitation procedure, different amounts of cytochrome *c* in a range of 8.5 pmol to 3.5 nmol were digested on the pepsin IMER 1 and a calibration curve was established for each peptide. Fig. 4 reports peak areas corresponding to the MW 1386.8 and MW 1017.5 peptides versus the amount of cytochrome *c* loaded on the IMER 1. A linear part is observed for each peptide of cytochrome *c* concentrations for the range from 8.5 pmol to 1.7 nmol with a regression coefficient of $R^2 = 0.9945$ and $R^2 = 0.9881$ for MW 1386.8 and MW 1017.5, respectively. Therefore, a simple quantification of cytochrome *c* becomes feasible in this range. For higher amounts of cytochrome *c*, the curves present a plateau corresponding to the saturation of the digestion sites in given kinetic conditions. This value was estimated at 1.7 nmol of cytochrome *c* (796 μg/g of IMER) for IMER 1. For an amount higher than 1.7 nmol, cytochrome *c* is still digested but with a lower digestion yield. Moreover, it is important to note that the digestion in solution of different amounts of cytochrome *c* (ratio pepsin/protein: 1/25) followed by a LC/MS analysis, give rise to a linear response in the range of the same previous range (8.5 pmol–3.5 nmol) (results not shown). This result confirms that the plateau observed is due to the saturation of the IMER 1 and does not result from signal saturation in MS.

In conclusion, these two specific peptides can be used for the quantification of cytochrome *c* and with this set-up device, cytochrome *c* can be detected and quantified in a range from 8.5 pmol to 1.7 nmol, thus demonstrating the reliability of the quantitative on-line digestion of cytochrome *c* on the pepsin IMER 1.

3.1.3. Effect of the pepsin immobilized amount on the performances of the IMERs

This quantification tool based on both peptides was applied to the comparison of the performances of IMERs prepared with different amounts of pepsin. Another IMER was prepared with 5 mg of pepsin in the grafting solution for 25 mg of CNBr activated sepharose (IMER 2). To compare IMER 1 and IMER 2, 5 μg (0.43 nmol) of cytochrome *c* was digested on each reactor according to the procedure described in Section 2.4.3 in triplicates. The peak areas of both cytochrome *c* specific peptides were then integrated according to the previous quantitation procedure and are reported on Fig. 5. As shown on this figure, the amount of pepsin immobilized on both IMERs (1 and 5 mg of pepsin in the grafting solution) has no significant effect on the digestion recovery because the areas of specific peptides resulting from the use of both IMERs are similar.

By the preliminary study, carried out in Section 3.1.1, it was demonstrated that the pepsin immobilization recovery was constant until 5 mg of introduced pepsin in the grafting solution. Even if the amount of immobilized pepsin increased by a factor five between IMER 1 and IMER 2, the digestion yield is not increased, probably due to a poor accessibility of active sites of the pepsin or to a diffusion limitation by substrate if the enzyme density is

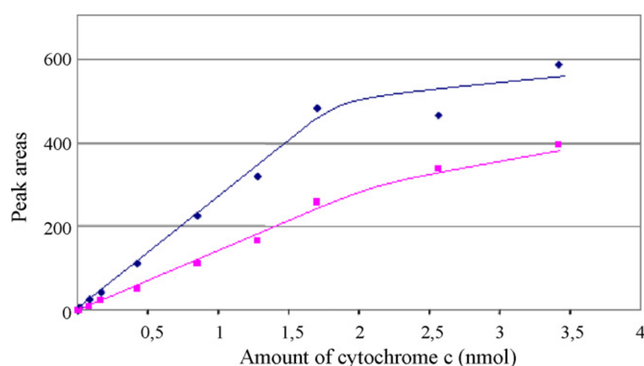

Fig. 4. Peaks areas related to the MW 1386.8 (diamond) and MW 1017.5 (square) peptides versus various amounts of cytochrome *c* after digestion on IMER 1.

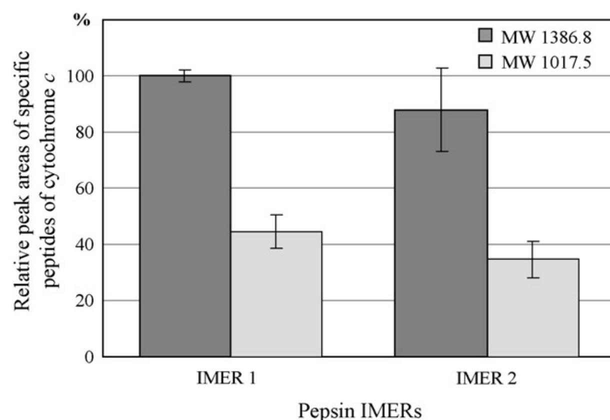

Fig. 5. Peak areas of extracted ion chromatograms of both specific peptides after on-line digestion of 5 μg of cytochrome *c* on the IMERs 1 and 2 (prepared with 1 and 5 mg of pepsin, respectively) and analysis by LC–MS/MS ($n = 3$).

too high. Moreover, by three replicates, RSD were estimated and the best performances were observed for IMER 1. Besides, a RSD inferior to 7% for both peptides was estimated for this IMER, thus demonstrating a good repeatability of the on-line digestion procedure. So, IMER 1 presenting the same performances than IMER 2 in terms of digestion recovery but with a better repeatability, it was chosen to be coupled on-line with the immunosorbent. In addition, IMER 1 presents a very high stability because it has been used continuously during several months without loss of signal.

3.2. Immunoextraction of cytochrome c

For the preparation of immunosorbents, anti-cytochrome *c* antibodies were covalently bonded to CNBr activated sepharose according to the procedure described in Section 2.6.1. Briefly, 100 μg of anti-cytochrome *c* antibodies was immobilized on 25 mg on CNBr activated sepharose and packed in pre-column 20 × 2 mm I.D. (IS A). The first study consisted to develop a selective procedure for the extraction of cytochrome *c* on immunosorbent. In a second part, immunosorbent was characterized. All theses experiments were carried out by directly connecting IS to an UV detector.

3.2.1. Selective immunoextraction procedure

An immunoextraction procedure as solid phase extraction procedure on conventional sorbent involves three basic steps. The first step consists of the loading of the sample through the sorbent called the percolation step. Then, the sorbent is washed with a buffer and/or a solvent to remove interfering compounds that are only slightly retained by the sorbent, without eluting the strongly retained target analyte. Then, the target analyte is eluted with a solution that is able to disrupt the analyte-antibody interactions. For this elution step, the most common strategy consists of the use of an acid solution or to increase the ionic strength.

To assess the selectivity of the extraction procedure of cytochrome *c* on the immunosorbent (IS), albumin and myoglobin were added as potential interfering proteins to the aqueous sample. They were spiked with cytochrome *c* and percolated through the IS. Taking into account the high concentration of albumin in plasma sample, this most abundant interfering protein was spiked at 2.5 mg mL^{-1} while cytochrome *c* was spiked only at 50 μg mL^{-1}. Myoglobin was also introduced in a higher concentration of cytochrome *c* at 0.1 mg L^{-1}. The immunoextraction procedure was firstly developed on IS packed in a pre-column of 2 mm I.D. (IS A). This IS was placed on a switching valve and directly connected to UV detection. The removal of interfering proteins (albumin and myoglobin) and the elution of the target analyte cytochrome *c* were followed at 280 nm for albumin and at 400 nm for myoglobin and cytochrome *c*. As shown on the UV chromatogram presented on Fig. 6a, the total elution of myoglobin from IS A is observed during the percolation step of 1 mL of the spiked sample, thus indicating that this protein is not retained by the IS. Concerning the washing step, 1 mL of a solution of NaCl 2 mol L^{-1} allows the complete removal of the albumin as shown in Fig. 6b. This washing step is then necessary to ensure a high selectivity during the extraction procedure because it allows the removal of albumin and remaining the retention of cytochrome *c* on IS. The stronger retention of albumin compared to the myoglobin can be explained by its high molecular mass (65 kDa). Indeed, albumin possesses 580 amino acids against 153 for the myoglobin that can involve more non specific interactions with the immunosorbent. At last, an acid solution of formic acid (pH 2) was used for the complete elution of cytochrome *c*, this solution allowing the disruption of the selective antigen–antibody interactions. As shown on Fig. 6c, cytochrome *c* is eluted from IS A with about 320 μL of formic acid (pH 2) at 100 μL min^{-1}. This

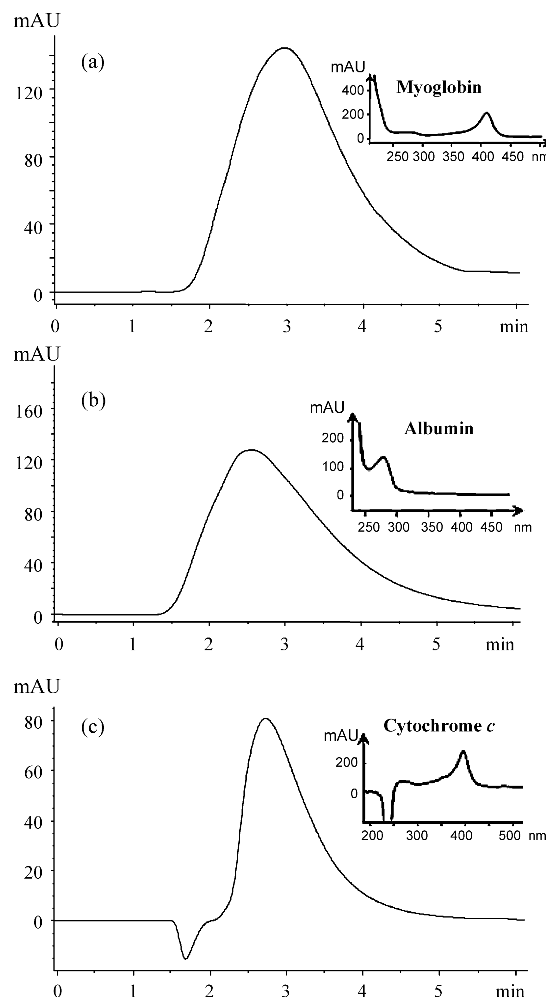

Fig. 6. UV analysis of the effluent of the IS A after the percolation of 20 μL of a PBS solution spiked with 1 μg of cytochrome *c*, 2 μg of myoglobin and 20 μg of albumin. (a) Effluent corresponding to the percolation step (PBS solution at 0.2 mL/min). (b) Effluent corresponding to the washing step (solution of NaCl percolated at 0.2 mL/min). (c) Effluent corresponding to the elution step (formic acid solution pH 2 at 0.1 mL/min). UV detection at 280 nm for albumin and at 400 nm for myoglobin and cytochrome *c*.

volume was obtained by front flush elution and it was verified that the use of back flush does not allow the reduction of this volume.

Keeping in mind the development of the on-line coupling between the IS and the IMER, this elution volume is largely higher to the IMER void volume which was estimated about 45 μL. Therefore, only a part of the eluted protein could be digested on pepsin IMER during the 20 min of "stop-flow". To overcome this drawback, another alternative consists of the decrease of this elution volume by using a lower amount of immunosorbent. For this reason, another functionalized support prepared using the same immobilization procedure as for IS A (see Section 2.6.1) was obtained and packed into a 20 × 1 mm I.D pre-column (IS B) leading to a reduction of the IS volume by a factor 4. The selective extraction procedure developed with IS A was easily adapted to IS B by decreasing the percolated volumes and the flow-rates of the different solutions percolated through the IS (see Section 2.6.2). The elution volume of 1 μg of cytochrome *c* from IS B was 140 μL of acid solution instead of 320 μL for IS A. The reduction of the amount of immunosorbent effectively allows the reduction of the elution volume, thus favouring the association of the IS with the IMER.

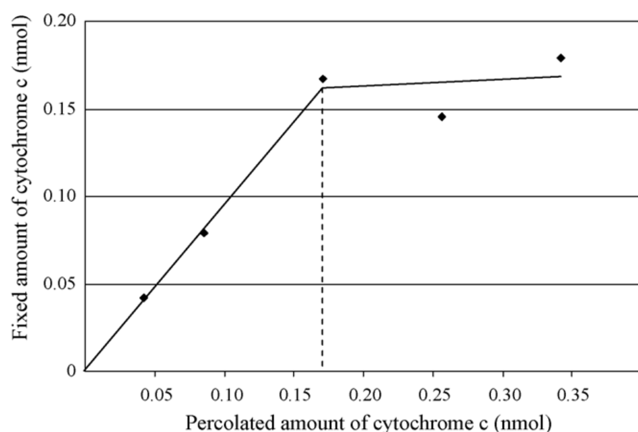

Fig. 7. Retained amount of cytochrome *c* versus the amount of cytochrome *c* percolated through the IS B.

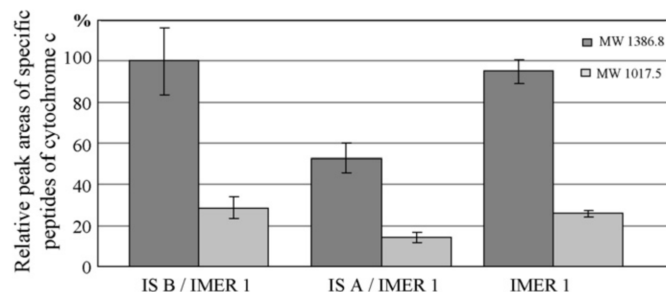

Fig. 8. Relative peak areas of the two specific peptides of cytochrome *c* after the direct on-line digestion of 1 μg of cytochrome *c* on the IMER 1 or after a preliminary immunoextraction step on IS A and IS B ($n = 3$).

3.2.2. Capacity of IS

During the percolation step, it is important to prevent the overloading of the IS capacity that could lead to a partial loss of the target analyte. Indeed, the capacity of an IS is defined as the maximal amount of the analyte that can be selectively retained onto the sorbent during the extraction procedure described previously. In these conditions, the capacity depends on various parameters such as the amount of antibodies used for the immobilization, the bonding density and the random orientation of the antibodies during the immobilization process [9]. For these reasons, the real capacity of IS B was experimentally measured. For this, increasing amounts of cytochrome *c* from 0.04 nmol to 0.34 nmol were percolated with 250 μL of PBS through the IS B directly connected to the UV detector. Fig. 7 reports the resulting curve that corresponds to the retained amount of cytochrome *c* versus percolated amounts of cytochrome *c* when applying the selective extraction procedure previously developed (see Section 2.6.2). This curve presents a linear part defining the amounts of protein than can be quantitatively and selectively extracted by the IS. By the value of the slope of this linear part, the extraction recovery was estimated at 95% that clearly demonstrates the high potential of this immunosorbent for the selective on-line sample pretreatment of a proteins mixture. For higher percolated amounts of protein, the curve reaches a plateau corresponding to the maximum amount of analyte that can be retained on the IS B. The capacity can be easily estimated at the upper limit of the linear part. For this IS, the capacity was evaluated to 0.17 nmol of cytochrome *c*. Taking into account the amount of antibodies immobilized on the sorbent (25 μg), the presence of two antigen-binding sites by antibody and the fact that it has been largely admitted that the binding rate of antibodies during the immobilization procedure and the resulting accessibility of the recognition sites for the antigen can reduce the real capacity by a factor 2 [9], the value of 0.17 nmol was expected. This value is largely above the amount of proteins of interest analyzed in real samples. Therefore, with IS B, the total analysis of a target protein will not be limited by the capacity.

The binding capacity was also determined for IS A by percolation of cytochrome *c* from 0.085 nmol to 1.7 nmol and a capacity of 0.65 nmol of cytochrome *c* (data not shown) was founded. As expected, the capacity of IS B is approximately four times smaller compared to IS A due to the reduction by a factor 2 of the internal diameter of the pre-column. This result illustrates the good repeatability of the immobilization procedure.

After developing the immunoextraction and on-line digestion procedures, immunosorbent (IS B) and pepsin IMER (IMER 1) were coupled and totally integrated to the analytical system.

3.3. Automated analysis of cytochrome c in human plasma sample

3.3.1. Optimization of the on-line coupling

As previously mentioned (see Section 3.2.1), the elution volume required for IS B seems better adapted to the coupling between the immunosorbent and the IMER than IS A. Indeed, by reducing the volume of IS, the elution volume required for IS B is effectively reduced compared to thus required for IS A. However, both IS were coupled on-line with IMER. The transfer volume between IS and IMER was optimized taking into account the void volumes of different columns (IS A, IS B and IMER) and the volume of connections tubing. The digestion of 1 μg (85 pmol) of cytochrome *c* were carried out in triplicates on pepsin IMER 1 hyphenated either with IS A or with IS B according to the procedure described in Section 2.7. To asses the performances of the on-line coupling, the results were compared to the on-line digestion on pepsin IMER 1 without a preliminary immunoextraction step. The peaks areas of the two specific peptides of cytochrome *c* obtained after on-line analyses are presented on Fig. 8.

As shown on Fig. 8, the total analysis using IS B give higher peak areas compared to the analysis using IS A, thus demonstrating that the on-line coupling between IS A and IMER 1 is not optimal. These results can be explicated by the transfer between IS and IMER. Indeed, the volume estimated at 150 μL required to displace the protein from the IS A to the IMER by integrating the whole void volumes of the device (void volume of the IMER and inner volume of connecting tubing) is not sufficient for the complete elution of cytochrome *c*, thus explaining the lower intensity signal of the on-line coupling with IS A. In addition, the peak areas of both specific peptides obtained after the total analysis using IS B and those obtained without immunoextraction step are very similar, thus demonstrating the good feasibility of the coupling between IS B and IMER. Finally, these results highlight the good repeatability of the total analysis with a RSD lower than 17% with IS A and IS B for both peptides.

The improvement in selectivity of the whole device was also checked. For this, an on-line analysis 20 μL of a mixture containing 2 μg of cytochrome *c*, 20 μg of albumin and 2 μg of myoglobin was carried out by using IS B and IMER 1. Only cytochrome *c* was recognized by MASCOT thus confirming the results obtained when developing the selective immunoextraction procedure and highlighting the high degree of selectivity of the whole device.

In conclusion, the device integrating IS B and IMER 1 presents the best performance and was apply to a plasma sample.

3.3.2. Application to spiked plasma

To assess the performances of the whole device, a plasma sample was applied. Indeed, as previously mentioned, human plasma presents numerous highly abundant proteins but also other low-abundant proteins that are necessary to remove when focusing on only one target protein. A human plasma sample was then

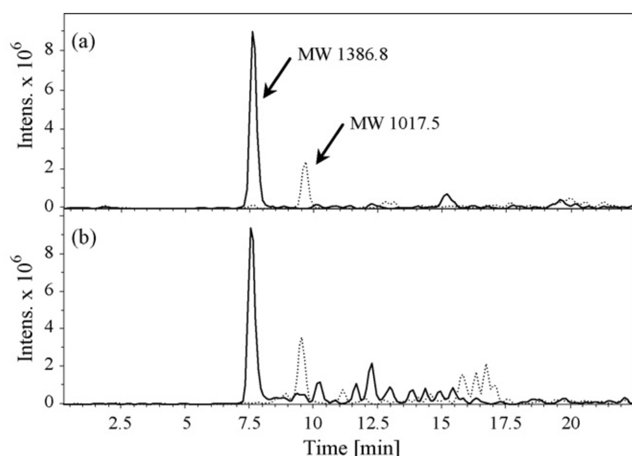

Fig. 9. Extracted ions chromatograms of LC/MS analysis of the two specific peptides of cytochrome *c* after total determination of 85 pmol of cytochrome *c* spiked in pure aqueous sample (a) and in plasma sample (b) by IS B/IMER 1/LCMS. The peptide MW 1386 and 1017.5 are represented by black and dotted line, respectively.

centrifuged, diluted (1:5 v/v plasma-Tris), filtered and spiked at 50 ng μL^{-1} with cytochrome *c*. A fraction of this sample (20 μL) containing 85 pmol of cytochrome *c* was directly percolated through the IS B, the immunoextraction procedure was then applied and followed by the on-line digestion by IMER 1 and by the LC–MS/MS analysis. This sample analysis was carried out in triplicates. First of all, no interfering proteins, in particular the high abundant proteins of plasma as albumin, were detected by MASCOT database thus confirming the high selectivity of the IS in real medium. In addition, the total analysis of 85 pmol of cytochrome *c* in plasma sample was compared with the response obtained for a pure sample spiked in the same amount of cytochrome *c*. Fig. 9 shows the extracted ion chromatograms of both specific peptides of cytochrome *c* (MW 1386.8 at 7.5 min and MW 1017.5 at 9.6 min) obtained in pure sample (Fig. 9a) and in plasma sample (Fig. 9b). As shown on the figure, the peak areas of both specific peptides obtained for the analysis of the plasma are very similar compared to the peak areas obtained for the aqueous sample. Indeed, the peak intensities for the peptide MW 1017.5 differ from only 20% while the peak intensities for the most abundant peptide (MW 1386.8) are identical. A good repeatability was also obtained for the total analysis in plasma because a RSD < 10% was obtained for the high abundant specific peptide MW 1386.8 and a RSD < 20% for the second peptide. Therefore, a quantitative analysis from 85 pmol of cytochrome *c* can be easily performed in a complex biological fluid as plasma. Furthermore, the signal-to-noise ratio observed for this amount of cytochrome *c* (85 pmol) was estimated at 60 for the peptides MW 1386.8. By this way, we can estimate a limit of quantification close to 14 pmol of cytochrome *c*. By this application, satisfactory repeatability and sensitivity were clearly showed. In addition, no matrix effect was observed, thus demonstrating the feasibility of this on-line device for the analysis of a target protein.

4. Conclusion

A total automated analytical system for the analysis of cytochrome *c* was developed in this study including an immunoextraction step coupled on-line with a solid phase digestion step followed by a LC–MS/MS analysis. The same functionalized support, CNBr activated sepharose, was used to develop an immunosorbent using immobilized anti-cytochrome *c* antibodies and a pepsin based reactor. After the development of a high selective extraction procedure giving rise to 100% of extraction recovery, the immunosorbent was characterized in terms of capacity. The pepsin based IMER was also characterized and allowed a quantitative digestion of cytochrome *c* in a range from 8.5 pmol to 1.7 nmol. Then, the on-line coupling between the immunosorbent and the IMER was carried out and optimized owing to the use of a quantification method based on the exploitation of the signal of two specific peptides. The hyphenation was successfully performed by analysing 85 pmol of cytochrome *c* spiked in an aqueous solution with a good repeatability. The potential of the on-line procedure was also evaluated in real media by applying a plasma sample. Once again, 85 pmol of cytochrome *c* was easily detected with the same response of the two specific peptides than in pure aqueous sample thus clearly demonstrating the performance of this method.

The easy and rapid setting-up of this technique makes it very useful for the analysis of target proteins in complex biological fluids. In addition, this method allows a high sensitivity compared to conventional approaches and a real decrease of the analysis time.

Our future works will be oriented to the miniaturization in nano-LC/MS of the whole procedure, in particular the development of immunosorbent and enzymatic reactor in capillary. Indeed, the development of miniaturized analytical devices is currently one of the most popular research topics in analytical chemistry, since it allows not only increasing the sensitivity, but also reducing the sample volume. In this way, a high improvement in sensitivity below nM is expected. Then, this miniaturized device will be evaluated with others proteins as biomarkers from real samples.

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