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# Novel extraction supports based on immobilised aptamers: Evaluation for the selective extraction of cocaine

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ABSTRACT

A new kind of selective sorbent based on the use of aptamers and dedicated to the selective solid phase extraction was developed. Cocaine aptamer was chosen as model aptamer to demonstrate the feasibility of this material and to provide a complete evaluation of the synthesized sorbent. The effect of different parameters such as the nature of the immobilisation support (silica, agarose), the type of immobilisation (covalent or non-covalent) and the length of the spacer arm ($C_6$ or $C_{12}$) were studied. Therefore, various oligosorbents based on different immobilisation strategies were synthesized and characterised by estimating the extraction recovery and the capacity of cocaine and the binding efficiency of aptamers. Control supports without immobilised aptamers were simultaneously studied in parallel to assess the selectivity brought by the oligosorbents. The oligosorbent based on CNBr-activated sepharose showed the best performances with an extraction recovery for cocaine of 90% while 6% was obtained on the control sorbent. The high selectivity brought by the oligosorbent was then illustrated by applying the oligoextraction followed by LC/MS analysis to a post-mortem blood (cocaine overdose). Results were compared to those resulting from a conventional protein precipitation procedure. The presence of co-extracted interfering compounds was strongly reduced with the treatment by oligoextraction. A limit of quantification of 0.5 ng/mL was obtained that is largely lower than the concentration found after a single intake of cocaine.



## 1. Introduction

LC–ESI-MS is commonly used for the trace analysis of organic compounds in complex matrices such as environmental samples, biological fluids or foodstuffs. However, the principal limitation of this method is its susceptibility to matrix effects [1,2]. Matrix effect is considered to be related to a suppression or enhancement of the analyte response. It often affects the accuracy and precision of the analytical method, leading to less reliable results [3,4]. A comparison between the responses obtained from a standard solution and from a spiked pretreated sample permits to check the absence of matrix effects. Unfortunately, numerous LC/MS methods published in the literature do not deal with this issue increasing the probabilities to obtain false negative or positive results [5]. Several options are available to reduce or eliminate matrix effects. Indeed, an accurate quantitation can be carried out by standard additions or by plotting matrix calibration curves. The use of isotopically labeled internal standards can be also an effective method provided that the standard presents the same matrix effect susceptibility than the target analyte [6]. Unfortunately, a limited number of such standards are available and they are expensive. Another approach consists in eliminating the co-elution responsible of the matrix effect. That can be done by improving the selectivity of the chromatographic separation, often leading to an increase of the analysis duration, or by improving the purification efficiency of the sample pre-treatment [3,7]. Solid phase extraction is commonly used but can be ineffective to remove some interfering compounds. Indeed, since these matrix constituents are co-eluted during the chromatographic separation in reversed phase mode, common hydrophobic SPE sorbents will co-extract them. More selective extraction sorbents are needed to limit the co-extraction of interfering compounds.

Sorbents involving selective retention mechanisms based on molecular recognition of target molecule(s) have been already developed. The first approach consists in the use of immunosorbents (IS) made of antibodies directed against the target analyte immobilised on a solid support [8–10]. Another strategy consists in the use of molecularly imprinted polymers (MIPs), which are sorbents possessing specific cavities complementary to the target analyte in size, shape, and position of the functional groups [11,12]. Recently, a third type of selective SPE sorbents called oligosorbents based on aptamers immobilised on a solid support has been developed [13,14]. Aptamers are oligonucleotides able to bind a specific molecule with an affinity that can be compa-

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rable with those of antibodies. They are identified *in vitro* by an iterative selection process called SELEX [15]. They can be regenerated within minutes whereas antibodies need one or two days to recover their active conformation after denaturation [9]. Moreover, their chemical synthesis allows modifications to improve their stability, detection or immobilisation [16]. The use of aptamers for SPE is in its infancy but aptamer affinity chromatography or DNA affinity chromatography in general has been already developed [17,18]. Aptamer immobilisation for the design of oligosorbents can be adapted from these existing techniques. The choice of the immobilisation strategy depends first on the nature of the terminal functional group that is available on the aptamers. Three terminal modifications are commercially available: amine, thiol and biotin. Immobilisation of biotinylated aptamers on a avidin or streptavidin activated support is one of the most widely used approach [19–21]. The simple and strong association mechanism between biotin and (strept)avidin renders the immobilisation step very easy. The binding efficiency is quite high and only one binding orientation is possible [22]. However, the non-covalent bonds resulting from the use of (strept)avidin can be weakened by organic solvents or chaotropic agents. More robust covalent attachment of amino-modified aptamers can be adapted from similar applications previously developed for the immobilisation of proteins [23]. In a previous study from our group, a cocaine aptamer identified by Stojanovic et al. [24] and already used in several sensors [24–34] had been chosen as model aptamer to demonstrate the feasibility of a selective solid phase extraction based on aptamers [13]. An amino-modified cocaine aptamer was immobilised on a CNBr activated sepharose and applied to the extraction of cocaine from pure media and plasma. To complete this first work, several non-covalent and covalent immobilisation strategies were studied. The specific retention and the capacity on the resulting oligosorbents were evaluated to compare their performances. Covalent immobilisations were carried out by using a CNBr-activated sepharose and glutaraldehyde-activated silica following the procedures previously developed by our group for the immobilisation of antibodies and enzymes [9,35,36]. Another oligosorbent also based on a covalent immobilisation was synthesized by using a thiol activated sepharose. The resulting oligosorbents obtained by covalent bonding were compared in terms of retention and binding efficiency to the oligosorbent resulting from the non-covalent immobilisation of biotinylated aptamers on streptavidin activated agarose. A further evaluation was also carried out for the most promising support by studying the effect of the length of the spacer arm. The great potential of this support was illustrated by applying it to the cocaine extraction from a post-mortem blood corresponding to a case of cocaine overdose. The extraction was followed by a LC/MS analysis. The selectivity brought by the oligosorbent was also evaluated by comparing the obtained results with those obtained after a common protein precipitation procedure.

## 2. Materials and methods

### 2.1. Chemical

Cocaine hydrochloride, benzoylecgonine solution, sodium phosphate ($Na_2HPO_4$), trizma hydrochloride, sodium azide, sodium chloride, ethylene glycol, sodium cyanoborohydride ($NaBH_3CN$), iodoacetamide, DL-dithiothreitol (DTT), D-biotin, activated thiol-sepharose (4B) streptavidin activated agarose (CL-4B) and CNBr-activated Sepharose (4B, 90 μm) were from Sigma–Aldrich (Saint-Quentin Fallavier, France). Magnesium chloride, ethanolamine, potassium dihydrogen phosphate ($KH_2PO_4$), hydrochloric acid, and acetic acid were from VWR (Fontenay-sous-bois, France). Sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (Sulfo-SIAB) was from Interchim (Montluçon, France). HPLC-grade acetonitrile and glutaraldehyde-activated silica (40 μm) were from Mallinckrodt Baker (Deventer, The Netherlands). High-purity water was obtained using a Milli-Q purification system (Millipore, Saint-Quentin en Yvelines, France). The 5′-amino-modified with $C_6$ or $C_{12}$ spacer arm and 5′-biotin modified DNA oligonucleotides (sequence 5′-GGGAGACAAGGAAAATCCTTCAATGAAGTGGGTCGACA-3′ for the aptamer and 5′-AAGTGAACAGAAGGCGTCATAGAGCGAAGTACGA-TGTC-3′ for the scrambled oligonucleotide) were synthesized and HPLC-purified by Eurogentec (Angers, France). The sequence of the 5′-amino modified with a $C_6$ spacer arm with a mutation is 5′-GGGAGACAAGGAAAATCCTTCAATGAAGTGGGTGGACA-3′. The selection buffer (SB) contained 20 mM Trizma hydrochloride pH 7.4, 140 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$. The phosphate-buffer solution (PBS) consisted of a 10 mM $Na_2HPO_4$, 1.8 M $KH_2PO_4$, 0.17 M NaCl and 3.3 mM KCl with pH adjusted to 7.4. The potassium-phosphate buffer solution (KPB) consisted of 0.1 M of $K_2HPO_4$ and $KH_2PO_4$ (pH 6).

### 2.2. Apparatus and analytical conditions

An Agilent 1200 series (Agilent Technology, Massy, France) LC system equipped with a binary pump, an autosampler and a diode array detector controlled by Chemstation software was used for the characterisation of the oligosorbents. Cocaine and benzoylecgonine were separated on a Waters SymmetryShield RP18 column (150 mm × 2.1 mm, i.d.; particle size: 3.5 μm, Waters, Saint-Quentin-en-Yvelines, France) maintained at 35 °C with a column oven (Croco-cil, Interchim). The mobile phases used for the separations in isocratic mode were first blended. An isocratic mode was chosen with a mix 5 mM phosphate buffer (pH 6)/acetonitrile (62:32; v/v) at a flow rate of 0.2 mL/min. The detection of cocaine and benzoylecgonine was carried out at 233 nm. The same LC system was used to quantify the aptamers in the solutions resulting from the immobilisation procedure. For this, ion pairing chromatography with 0.1 M triethylammonium acetate (pH 7) as solvent A and acetonitrile as solvent B and using the same column at 50 °C was used. Aptamers were detected by UV absorbance measurements at 260 nm. The gradient consisted in a linear increase of the A/B mixture (93:7; v/v) in 30 min (85.5:14.5; v/v). The equilibration time of the column was 10 min with the A/B mixture (93:7; v/v).

Concerning the treated blood samples analysis, a HPLC Varian system (Varian, Les Ulis, France) made up of a binary high pressure pump (212 LC), a ProStar 410 autosampler and coupled to a tandem quadrupole mass spectrometer (Varian 320) was used. The mass spectrometer was operated in positive electrospray ionisation mode using single ion monitoring mode (SIM). The same analytical column as previously described was used and the detected ion of cocaine was 304.1 $m/z$ with a capillary voltage of 80 V. Separation was carried out in an isocratic mode with a mix of water/acetonitrile both acidified with 0.2% (v/v) formic acid (82:18; v/v) using the same column.

### 2.3. Oligosorbents synthesis

#### 2.3.1. Cyanogen-bromide activated sepharose

The procedure followed to immobilise aptamers on CNBr-sepharose had been previously described [13]. Briefly, prior to immobilisation, the oligonucleotides with a $C_6$ or a $C_{12}$ spacer arm were renatured by heating the aptamer solution (1 g/L in a 200 mM $Na_2HPO_4$ and 5 mM $MgCl_2$, pH 8) at 75 °C for 5 min and leaving it to stand at room temperature for 30 min. Dry CNBr-activated Sepharose (35 mg) was swollen and washed six times with 1 mL of HCl (1 mM). The gel was then rinsed with 1 mL of deionised water and with 175 μL of 200 mM $Na_2HPO_4$, pH 8 and

5 mM $MgCl_2$. Aptamer solution (150 μL) was mixed with the gel overnight at room temperature. The oligosorbent was then packed between two frits in a 1 mL SPE cartridge and washed with 3 mL of 200 mM $Na_2HPO_4$ (pH 8). Remaining active cyanate ester groups were blocked by a 0.1 M Tris solution (pH 8) for 2 h at room temperature. The gel was then washed alternately three times with 2 mL of an aqueous saline buffer (0.1 M acetate and 0.5 M NaCl, pH 4) and 2 mL of a Tris buffer (0.1 M) containing 0.5 M NaCl (pH 8) to remove non-covalently bound aptamers. To evaluate non-specific interactions between cocaine and the sepharose-based sorbent, a control sorbent was prepared following the same procedure but without introducing aptamers. To estimate the risk of non-specific interactions between cocaine and single-stranded DNA, the same immobilisation procedure was followed with a DNA brand having the same base composition as the active aptamer but in a random order, and named scrambled aptamer.

*2.3.2. Thiol-activated Sepharose*

The binding procedure on thiol activated sepharose was adapted from Allerson et al. [37]. Briefly, 5′-amino-modified aptamers were first appended with the bifunctional linker sulfo-SIAB by forming an amide bond between the aptamer's primary amine end and the NHS-ester part of the linker. 24 nmole of 5′ amino-modified aptamers with a $C_6$ spacer arm were treated with 0.65 mg sulfo-SIAB in 288.3 μL sodium phosphate (200 mM, pH 8) for 6 h at room temperature in the dark. The aptamers were then ethanol precipitated, dried, and redissolved in sodium phosphate and 5 mM NaCl (132 μL, 180 mM, pH 8). Aptamers were renatured by heating at 85 °C for 3 min, followed by a rapid cooling on ice. These modified aptamers were then coupled to the thiol-activated sorbent by nucleophilic substitution of the linker's iodine with thiol group of the support producing a thioether linkage. For this, the thiol-activated Sepharose was first washed with 6 mL deionised water and 6 mL of a solution containing 200 mM Tris–HCl buffer (pH 7.5) and 20 mM NaCl following the manufacturer's recommendations. Thiol groups on the sorbent surface were reduced with 3 mL of a solution containing 20 mM DTT, 200 mM Tris–HCl (pH 7.5) and 20 mM NaCl and washed with 4.5 mL of a solution containing 200 mM Tris–HCl (pH 7.5) and 20 mM NaCl both previously deoxygenated. The aptamer solution was then added to the sepharose and incubated overnight (12–16 h) in the dark at room temperature. The resulting oligosorbent was then washed and loaded between two frits in a 1 mL SPE cartridge with at least 4 mL of 200 mM Tris–HCl (pH 7.5). Unreacted thiol groups were blocked in a subsequent treatment of the sorbent with 10 mM iodoacetamide in 200 mM Tris–HCl (pH 7.5). A control support was synthesized in parallel following the same procedure but without aptamers.

*2.3.3. Glutaraldehyde-activated silica*

Immobilisation of aptamers on glutaraldehyde-activated silica was achieved following a procedure adapted from the immobilisation of antibodies and enzymes previously described by our group [9,23,35]. 5′-Amino-modified aptamers with a $C_{12}$ spacer arm were immobilised by reductive amination. The activated silica (30 mg) was successively washed with 2 mL of KPB containing NaCl (1 M) and 2 mL of KPB containing $MgCl_2$ (1 mM). Aptamers (100 μg) were renatured by heating at 90 °C for 10 min and let for 30 min at room temperature and then solubilised in 100 μL of KPB containing $MgCl_2$ (1 mM). This solution was incubated with the silica overnight at room temperature. The supernatant was removed and replaced by 1 mL of KPB containing $MgCl_2$ (1 mM) and a few milligrams of $NaBH_3CN$ powder were added. After one night at 4 °C, the resulting oligosorbent was packed in a 1 mL SPE cartridge and was successively washed with 5 mL of KPB solution, 5 mL of KPB containing NaCl (1 M), 5 mL of a mix KPB/ethylene glycol (90:10; v/v) mixture and with 5 mL of KPB solution. In order to inhibit the unbounded aldehyde functions, an ethanolamine solution (0.2 M) was added for 2 hours. Then, the oligosorbent was washed with 5 mL of KPB solution, 5 mL of KPB containing NaCl (1 M) and stored at 4 °C in a KPB solution containing 0.1% azide. Another synthesis was carried out in the presence of cocaine in two times molar excess by adding 50 μL of a cocaine solution at 89 ng/μL to the immobilisation solution. For each synthesis, a control support was synthesized following the same procedure but without introducing aptamers.

*2.3.4. Streptavidin-activated agarose*

5′ biotinylated aptamers (9 nmole) were dissolved in 3 mL PBS containing EDTA (1 mM) and were then renatured by heating at 75 °C for 5 min and let for 30 min at room temperature. 300 μL of streptavidin–agarose suspension was added to the aptamer solution and incubated overnight at room temperature. The sorbent was then packed between two frits into a 1 mL SPE cartridge and washed with 10 mL of a PBS containing EDTA (1 mM). A control support was synthesized in parallel following the same procedure but replacing biotinylated aptamers by 9 nmole of biotin.

### 2.4. Extraction procedures

Before each extraction procedure, the oligosorbents were conditioned with 5 mL of the selection buffer. The percolation samples consisted of the selection buffer spiked with cocaine. The washing steps were achieved by the percolation of the selection buffer. The elution was achieved using a water/acetonitrile mixture (60:40; v/v) except for the streptavidin activated agarose for which a water/acetonitrile mixture (90:10; v/v) at 60 °C was used. An extraction procedure has been optimized for each support and the corresponding volumes for each step of the extraction procedure are detailed in the caption of Fig. 2.

### 2.5. Blood samples treatments

The post-mortem blood was obtained from the ToxLab Laboratory (Paris) and corresponds to a case where death was attributed to a cocaine overdose. For protein precipitation, 200 μL of whole blood was mixed with 400 μL of acetonitrile. After 1 min of mixing with a vortex, the sample was centrifuged at 6000 × *g* for 5 min. 75 μL of the supernatant was evaporated and resolubilised in 200 μL HCl solution (pH 4). Concerning the oligoextraction of whole blood, 25 μL of blood were mixed with 75 μL of selection buffer and percolated at 4 °C through the oligosorbent. After a washing step consisting of 400 μL selection buffer, cocaine was eluted with 400 μL of a water/acetonitrile mixture (60:40; v/v). This elution fraction was then evaporated, diluted in 200 μL of acidified water (pH 4, HCl) and injected in LC/MS.

## 3. Results and discussion

### 3.1. Choice of the immobilisation support

As for a conventional SPE procedure, an oligoextraction procedure is composed of four steps. After a conditioning step, the sample is percolated through the oligosorbent. Then, the sorbent is washed with a buffer and/or a solvent to remove unwanted compounds that are only slightly retained by the sorbent, without eluting the target analyte strongly retained by the aptamers. Then, the target analyte is eluted with a solution able to disrupt the analyte–aptamer interactions. The extraction recovery corresponds to the percentage of percolated cocaine found in the elution fraction. As explained in a previous work [13], the percolation and washing steps of an oligoextraction procedure can be performed with the selection buffer

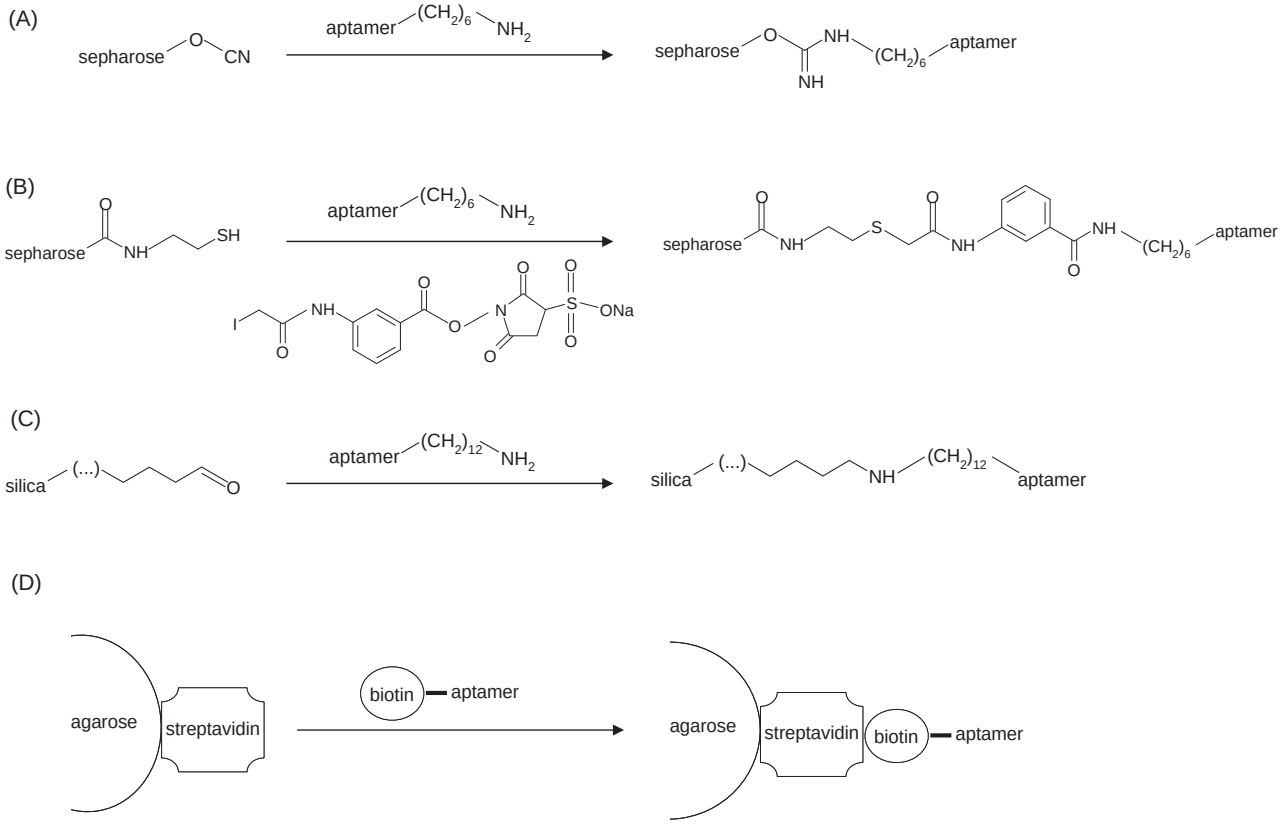


**Fig. 1.** Immobilisation reactions of aptamers on different supports: (A) CNBr activated sepharose, (B) thiol-activated sepharose, (C) glutaraldehyde-activated silica and (D) streptavidin activated agarose.

to maintain the complex cocaine/aptamer as strong as possible. The elution can be achieved using a water/acetonitrile mixture to denature the aptamer and dissociate this complex. This mixture can also be chosen for its compatibility with the HPLC system for a direct injection of the elution fraction.

The first step of this study consisted in evaluating different kinds of supports resulting from various chemical reactions to obtain a stable and efficient binding of aptamers. These supports have to be hydrophilic in order to limit the risk of non-specific retention of unwanted compounds during the percolation of aqueous samples containing cocaine. Moreover, they must present reactive functional groups complementary to the terminal group of the aptamer for an efficient and oriented immobilisation thus favouring a strong retention of the target analyte. The evaluation of the strength of the interaction between the target analyte and the aptamer can be obtained by studying the extraction profile resulting from the determination of the target analyte in the percolation, washing and elution steps. The selectivity of the extraction procedure can then be checked by studying in parallel the retention of the cocaine on a control sorbent. This sorbent is obtained by applying the same immobilisation procedure but without introducing aptamers. A significant difference of retention profiles between the oligosorbent and its control support would indicate a successful immobilisation of active aptamers. Four different immobilisation supports were chosen following the previous criteria: CNBr activated sepharose, thiol-activated sepharose, glutaraldehyde-activated silica and streptavidin activated agarose. The corresponding immobilisation reactions on the different supports are shown in Fig. 1.

### 3.2. CNBr activated sepharose

An oligosorbent based on the use of CNBr activated sepharose support was prepared following the procedure previously described by using an aptamer with a $C_6$ spacer arm [13]. As presented in Fig. 2A, a high retention was developed by the oligosorbent compared to the control support with extraction recoveries of 89% and 6% respectively with a RSD values lower than 9% ($n = 3$). These results are in good agreement with those previously obtained with an oligosorbent resulting from another synthesis and when applying the same extraction procedure: 86% on the oligosorbent and 6% on the control support. Therefore, similar properties of recognition towards cocaine for both oligosorbents resulting of two independent syntheses were obtained. Despite the fact that further experiments would be required to confirm the reproducibility of the synthesis, these results highlight the high potential of the oligosorbent.

### 3.3. Thiol-activated sepharose

The immobilisation procedure used for thiol activated sepharose support was adapted from Allerson et al. [37]. Aptamers were covalently bound via a bridging linker as frequently used. The linker allows a covalent binding of two different chemical groups which otherwise would remain unreactive to each other. The cocaine retention on the resulting oligosorbent and on the control support had been investigated as shown in Fig. 2B. The loss of cocaine on the control support during the percolation and the washing steps show the expected low retention of the compound on the support that does not develop non-specific interactions.

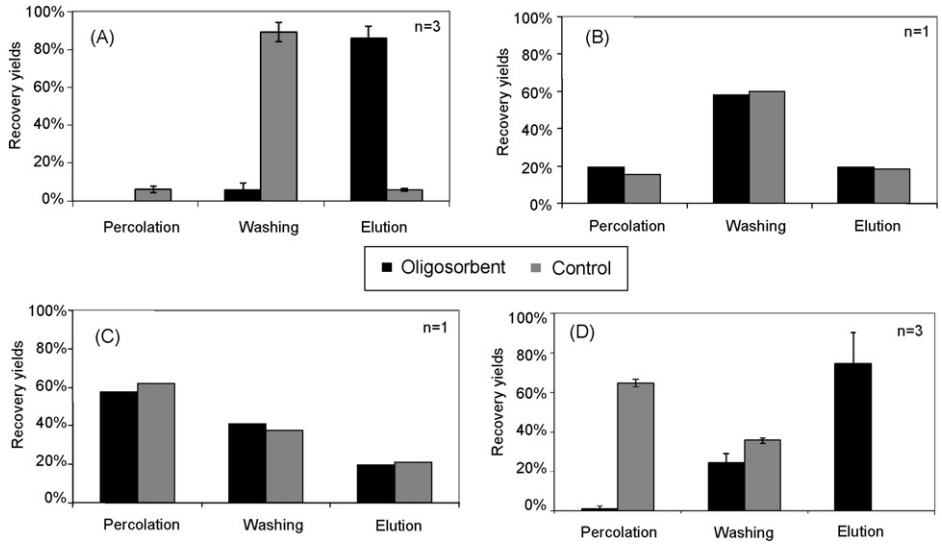


**Fig. 2.** Extraction profiles obtained with different oligosorbents. (A) CNBr-activated sepharose. P: 200 μL spiked SB; W: 300 μL SB; E: 400 μL water/acetonitrile (60:40; v/v); (B) thiol-activated sepharose. P: 500 μL spiked SB; W: 500 μL SB; E: 500 μL water/acetonitrile (60:40; v/v); (C) glutaraldehyde-activated silica. P: 100 μL spiked SB; W: 100 μL SB; E: 100 μL water/acetonitrile (60:40; v/v); (D) streptavidin-activated agarose. P: 500 μL spiked SB; W: 500 μL SB; E: 1000 μL water/acetonitrile (90:10; v/v) at 60 °C. P: percolation; W: washing; E: elution; SB: selection buffer.

However, the same elution profile was obtained using the sorbent that is supposed to contain aptamers. This result means that no active aptamers are available on the sorbent surface. The causes of this ineffective immobilisation may be numerous and may occur in every step of the immobilisation procedure: low efficiency of the binding of the linker because of the rapid hydrolysis of its NHS-ester group before its binding, losses during the ethanol precipitation, non sufficient deoxygenation of the binding solutions, non oriented immobilisation of aptamers through their nucleobases amines, etc. The optimization of this procedure is too long and tedious due to the numerous of steps involved. Therefore, the study of this sorbent was not pursued.

### 3.4. Glutaraldehyde-activated silica

5′-Amino-modified aptamers were immobilised on glutaraldehyde-activated silica, following a procedure adapted from the immobilisation of antibodies and enzymes previously described by our group [9,23,35]. As for previous studied sorbents, a cocaine extraction procedure in pure media was applied to the resulting oligosorbent to evaluate its ability to retain cocaine. As illustrated in Fig. 2C, no difference in retention was observed between the oligosorbent and the control support. Another immobilisation procedure was carried out in presence of cocaine in the immobilisation solution in order to form the aptamer/target complex and then favour the oriented attachment via the terminal amino group thus preventing the attachment of aptamer via another aminogroup of nucleobase involved in the cocaine binding site. However, similar results were obtained. This unsuccessful immobilisation might be due to the presence of a coating of a hydrophilic polymer surrounding the silica beads [35]. This polymer of polyethylenimine is positively charged at the binding solution pH and may probably strongly interact with the polyanionic aptamers rendering their complexation with cocaine impossible. This sorbent is largely used for the immobilisation of others biomolecules (antibodies, enzymes, etc.) because very low non-specific interactions are developed as shown in this study for cocaine. Nevertheless, this sorbent is not suitable for the immobilisation of aptamers.

### 3.5. Streptavidin activated agarose

Biotinylated-aptamers were incubated with streptavidin-activated agarose, this immobilisation mechanism being simply based on the very high affinity developed between biotin and streptavidin. As shown in Fig. 2D, a high difference in retention profiles between the oligosorbent and its control support is observed thus proving an efficient attachment of aptamers. Extraction recoveries of 74% and 0% for the oligosorbent and the control were respectively obtained with a RSD value of 16% ($n = 3$) for recovery value obtained using the oligosorbent. These results show that cocaine is selectively retained on the oligosorbent and that this hydrophilic support does not generate non-specific interactions. Extraction recoveries of $82 \pm 11\%$ ($n = 3$) were obtained on the same cartridge after nine months of storage (less than ten uses during this period) thus also demonstrating the time stability of this oligosorbent despite the non-covalent linkage. These results were obtained after a carefully optimization of the elution conditions. Indeed, the non-covalent attachment of aptamers leads to a more fragile binding and prevents the use of mixtures with high organic solvent content. For previous experiments carried out on a oligosorbent resulting from a covalent immobilisation, the elution of cocaine was ensured by pure water containing up to 40% of MeCN. For this streptavidin based oligosorbent, an efficient elution was expected using ultrapure water at 60 °C because a very low ionic strength, a high temperature and especially the absence of divalent cations can denaturate the aptamer and then elute the target [20,24]. Unfortunately, the elution strength of this solution was not sufficient to elute the whole percolated amount of cocaine: only 72% were eluted with 5 mL of this aqueous solution. A water/acetonitrile mixture (60:40; v/v) at 60 °C was then investigated and permitted the complete elution of cocaine with only 1 mL of this hydro-organic mixture.

In conclusion, these experiments allow identifying two immobilisation supports of choice for obtaining performing oligosorbents: CNBr activated sepharose and streptavidin activated agarose. To achieve the characterisation of these oligosorbents, the capacity of each support was determined.

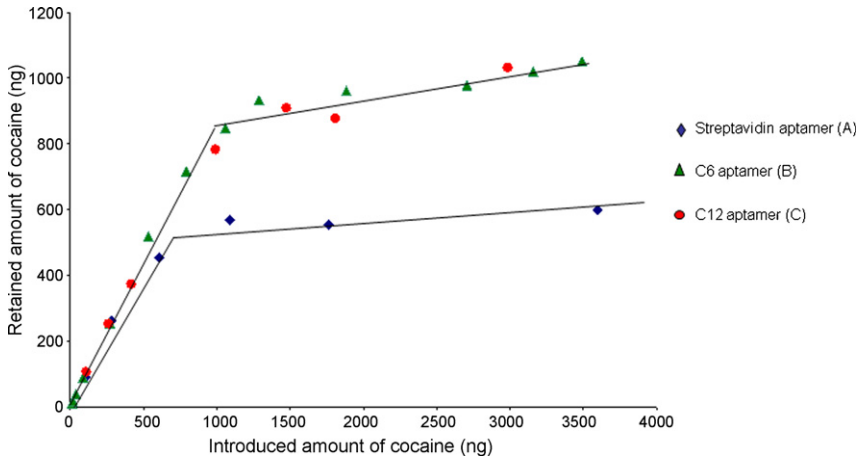


**Fig. 3.** Capacity curves for the oligosorbents based on streptavidin activated agarose support (A) and CNBr activated sepharose with a $C_6$ aptamer (B) and $C_{12}$ aptamer (C).

### 3.6. Capacities of the oligosorbents

The capacity of an oligosorbent corresponds to the maximal amount of cocaine that can be retained by specific interactions. For its determination, increasing amounts of cocaine were percolated on both oligosorbents. The cocaine amounts found in the elution fractions of both oligosorbents for the numerous experiments carried out with different amounts of cocaine are reported in Fig. 3. For both oligosorbents, the resulting curves (A and B) possess two linear parts. First, the retained amount of cocaine proportionally increases with the percolated amount, thus meaning that constant extraction recoveries are obtained. The curve reaches then a plateau corresponding to an overloading of the support. The capacity is reached for an introduced amount of cocaine corresponding to the upper limit of the linear part of the curve. A value close to 690 ng was observed for the oligosorbent based on the streptavidine activated agarose and a value close to 890 ng was observed for the oligosorbent based on the CNBr activated sepharose. Again, this last result is in agreement with the previous study [13] because a value around 730 ng was observed. The higher value obtained for the newly synthesized sorbent can be explained by the use of better adapted vials with a reduced size to manage the low volumes required during the synthesis thus leading to a reduction of the loss of reagents and then an improvement of the amount of immobilised aptamers.

Assuming one molecule of cocaine is retained by one aptamer molecule, this capacity value allows defining the effective binding efficiency that corresponds to the ratio between the amount of immobilised active aptamer and the total aptamer amount introduced in the binding solution. This effective binding efficiency was estimated to 25% for the oligosorbent based on streptavidin activated agarose support and to 24% for the oligosorbent based on CNBr activated sepharose support.

In conclusion, the use of streptavidin activated agarose and CNBr activated sepharose as immobilisation supports led to oligosorbents with similar performances in terms of retention, selectivity and capacity. The easiness of synthesis and the satisfactory specific retention and capacity render the biotin/streptavidin immobilisation strategy very attractive for the development of oligosorbents. However, this non-covalently immobilised support could be fragile when applying elution solutions with high organic content, close to the HPLC mobile phase for direct injection. The more robust covalent linkage formed on CNBr-activated Sepharose was then chosen for further experiments.

### 3.7. Effect of the spacer length on the retention and on the binding efficiency

Aptamers are synthesized by introducing a reactive group at the end of a spacer arm to make easier their immobilisation. The use of a spacer arm should also provide a greater accessibility and conformational freedom to the linked aptamer. Therefore, the length of the spacer can play an important role on binding efficiency and on capacity. In this study, two spacers, hexamethylene $[-(CH_2)_6-]$ $(C_6)$ and dodecamethylene $[-(CH_2)_{12}-]$ $(C_{12})$, were used to immobilise aptamers on the CNBr sepharose support. The resulting oligosorbents were compared in terms of retention and of binding efficiencies.

In a first part, we checked the risk of increasing the non-specific hydrophobic interactions when increasing the spacer length. This non-specific retention is obviously harmful to an efficient purification of real samples. For this, two identical non-specific DNA sequences, one with a $C_6$ spacer and the other with the $C_{12}$ spacer, were also immobilised following the previously described procedure. Actually, this non-specific DNA sequence, named scrambled aptamer, possesses the same bases composition as the cocaine aptamer but in a scrambled order. This scrambled aptamer having no affinity towards cocaine, a difference of retention between both supports would be attributed to the development of non-specific interactions by the spacer. Breakthrough curves were plotted using both supports composed of $C_6$ scrambled aptamer and $C_{12}$ scrambled aptamer by percolating fractions of 100 μL of the selection buffer after the percolation of 200 μL of selection buffer spiked with 20 ng of cocaine. These curves are presented in Fig. 4 and are compared to the breakthrough curve of the control support. A similar behavior was observed between both sorbents constituted by scrambled aptamers proving that the use of a $C_{12}$ spacer compared to a $C_6$ spacer does not involve the development of non-specific hydrophobic interactions with cocaine due to the increase of the carbon ratio. It is also important to notice that the retention was similar to the one obtained with the control sorbent thus confirming that the random DNA sequence is not able to recognize cocaine. Therefore, the recognition of cocaine is only due to the presence of a recognition site in the aptamer conformation. Indeed, the nucleotides constituting the aptamer do not generate non-specific interactions. To complete this study, an oligosorbent has been synthesized by immobilising a cocaine aptamer possessing one mutation compared to the active sequence. The elution profiles corresponding to this oligosorbent is also presented in Fig. 4. It is interesting to note that its behavior is very close to

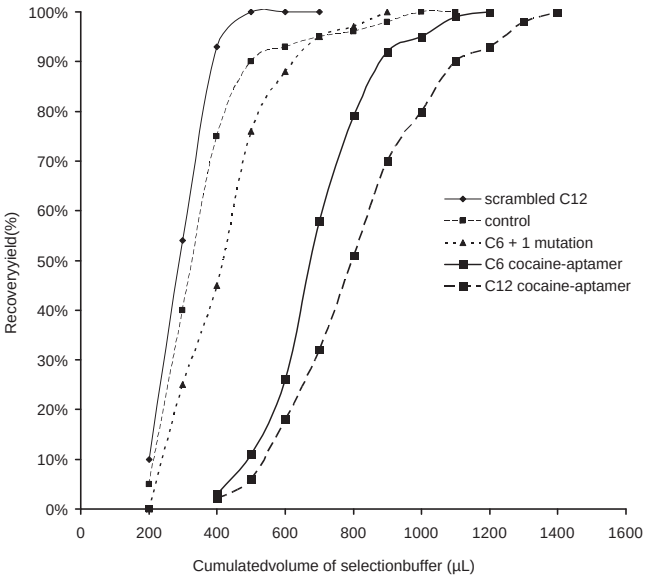


**Fig. 4.** Breakthrough curves obtained for the CNBr-sepharose-based oligosorbents (prepared with anti-cocaine aptamers, scrambled aptamers and the anti-cocaine aptamer containing one mutation and with $C_6$, $C_{12}$ as spacers) and the control sorbent when percolating several fractions of the selection buffer after the percolation of 200 μL of selection buffer spiked with 20 ng of cocaine.

the control sorbent or to the "scrambled" aptamers based sorbents. This result highlights the power of the SELEX process to determine an aptamer sequence of high affinity towards the target molecule. Again, it demonstrates that the recognition of cocaine is carried out by the development of selective interactions between cocaine and the recognition site.

In a second part, the specific retention, on the $C_6$ oligosorbent and on the $C_{12}$ oligosorbent prepared with the cocaine aptamers was compared. Both breakthrough curves are presented in Fig. 4. The complete elution came slightly earliest for the $C_6$ oligosorbent. However, the breakthrough volume that is a key parameter when developing an extraction procedure was quite similar for both sorbents, about 400 μL. The extraction protocol previously developed on the $C_6$ oligosorbent can therefore be applied to both sorbents because both oligosorbents present the same performances in terms of retention.

To evaluate the effect of the length of the spacer on the binding efficiency, this parameter that corresponds to the ratio of immobilised aptamers amount on the introduced aptamer amount in the binding solution, were then evaluated by quantifying the $C_6$ and the $C_{12}$ aptamers by ion pair chromatography and UV detection. Once the oligosorbent is packed in the SPE cartridge, washings are carried out to remove aptamers that are simply adsorbed on the support. Therefore, the supernatant and the washing solutions were all analyzed to determine the amount of immobilised aptamers. These experiments showed that 50% of aptamers were removed during the washing steps. Therefore, the determination of non-bonded aptamers by analyzing them only in the supernatant leads to an important error in the estimation of the real amount of immobilised aptamers. Indeed, an accurate estimation of the total binding efficiencies requires the determination of aptamers in the supernatant and in the washing solutions. Values of $37 \pm 8\%$ and $67 \pm 15\%$ ($n = 3$) were observed for $C_6$ and $C_{12}$ oligosorbents respectively. This high difference can be explained by an effect of the spacer length on the accessibility of the reactive amino group. However, this experiment only allows estimating the total amount of immobilised aptamers without any information about the ratio of active aptamers, i.e. aptamers that are able to selectively trap cocaine. Indeed, some of them can be immobilised with a wrong orientation, or fixed to support at several points preventing them to adopt the adequate conformation for the binding of cocaine. Capacity measurements were then carried out to evaluate the amount of active immobilised aptamers. The capacity of the $C_{12}$ oligosorbent was then studied and compared with the capacity of the $C_6$ oligosorbent in Fig. 3 (curves C and B respectively). Values of 890 ng and 940 ng were observed for the $C_6$ and the $C_{12}$ oligosorbent respectively for 35 mg of dry support. The effective binding efficiencies deduced from these capacity measurements are 24% and 25% for the $C_6$ and the $C_{12}$ oligosorbent respectively. Considering the percentage of total binding previously mentioned (37% for $C_6$ oligosorbent and 67% for $C_{12}$ oligosorbent), a more significant difference was expected. Although the $C_{12}$ spacer led to binding yields twice higher than the $C_6$ spacer, the capacity values were quite close thus proving that a high percentage of aptamers bound with the $C_{12}$ spacer arm are inactive. The immobilisation of $C_6$ aptamers seems to be less efficient in terms of total binding efficiency but leads to a higher rate of active aptamers. Finally, both oligosorbents present similar performance in terms of retention and capacity.

### 3.8. Evaluation of the selective retention of a cocaine metabolite

The oligosorbent based on CNBr-activated sepharose with a $C_6$ spacer arm was applied to the extraction of benzoylecgonine, the main urinary cocaine metabolite. Both structures only differ by the lack of a methyl group on benzoylecgonine that possesses a carboxylic acid group ($pK_a = 3.15$ [38]) thus resulting from the cocaine ester group hydrolysis. The extraction procedure previously developed for cocaine and described in Fig. 2 was applied to a sample spiked with benzoylecgonine (200 ng). The retention profiles obtained on the oligosorbent and on the control sorbent were similar demonstrating that benzoylecgonine is not recognized by the aptamers (data not shown). Considering the acid properties of benzoylecgonine, the compound possesses a negative charge in the aqueous buffer at pH 7.4 that can lead to an electrostatic repulsion with the highly negatively charged aptamer. Therefore, a similar experiment was carried out by percolating an acidified and spiked sample. A compromise between a high ratio of protonated benzoylecgonine without causing the hydrolysis DNA was found for a pH value of 3. The obtained profile was similar to the one obtained at pH 7.4. This oligosorbent is therefore able to bind cocaine with a very high affinity and also have high levels of molecular discrimination against benzoylecgonine.

### 3.9. Cocaine extraction from blood in a post-mortem case

To illustrate the high potential of the oligosorbent for the cocaine selective extraction from complex matrices, oligoextraction of cocaine from a post-mortem blood was carried out and compared to a protein precipitation procedure that is commonly used for the pre-treatment of such complex and viscous matrices [39,40]. Treated samples were analyzed by liquid chromatography coupled to mass spectrometry. The very high sensitivity of the MS detector allows decreasing the required enrichment factor of the oligoextraction procedure. Thus, only 100 μL sample were percolated instead of 200 μL thus allowing increasing the washing volume from 300 μL to 400 μL. The total volume of percolation and washing solutions being constant and composed of the same solution (spiked and non spiked selection buffer respectively), the extraction recovery should be unchanged. Although whole blood and selection buffer compositions are very close in terms of pH, ionic strength and calcium and magnesium contents, the blood sample was diluted four times by the selection buffer before its percolation. The role of this dilution is to correct the blood composition variability and also to make easier its percolation through the cartridge by decreasing its viscosity. Therefore, 25 μL of whole blood

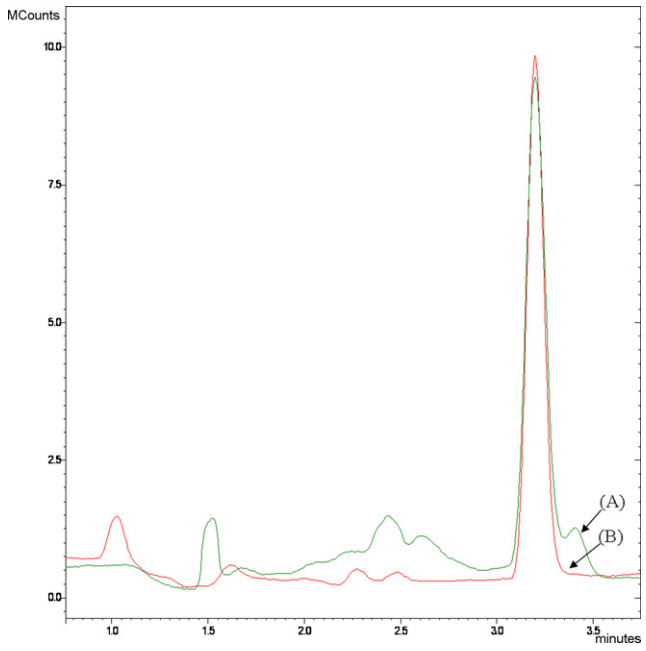


**Fig. 5.** Chromatograms corresponding to the injection of a post-mortem blood sample treated by protein precipitation (A) and by oligoextraction (aptamers immobilised via a $C_6$ spacer arm on CNBr activated sepharose) (B).

was mixed with 75 μL of selection buffer and percolated at 4 °C through the oligosorbent. After a washing step with 400 μL selection buffer, cocaine was eluted with 400 μL of a water/acetonitrile mixture (60:40; v/v). This elution fraction was then evaporated, diluted with 200 μL of acidified water (pH 4, HCl) and injected in LC/MS. Chromatograms corresponding to the injection of blood treated by protein precipitation and by oligoextraction are presented in Fig. 5. Despite the high selectivity of the SIM mode, several compounds with the same *m*/*z* ratios as cocaine are detected due to the very high complexity of the matrix after its treatment by protein precipitation (Fig. 5A). An interfering compound is even co-eluted with cocaine disturbing its quantification. The oligoextraction gave a cleaner chromatogram and no compound possessing the same *m*/*z* ratio is co-eluted with cocaine (Fig. 5B). Cocaine concentration in this post-mortem blood sample was estimated by standard addition method to 26 ng/mL with a limit of quantification of 0.5 ng/mL. The slope of the standard addition plot was compared to the slope of the capacity plot previously presented in Fig. 5. In both cases, a slope around 0.85 was estimated. As the slope corresponds to the extraction recovery, this experiment shows that there is no matrix effect disturbing the oligoextraction. As shown in the figure, a similar peak area was obtained for the protein precipitation. Therefore, the precipitation recovery can be considered around 85% that is in good agreement for with others studies using the treatment by protein precipitation [39,40]. In addition, a better sensitivity was obtained by oligoextraction with a limit of quantification of only 0.5 ng/mL. Obviously, a detection in MRM mode would allow an easy quantification for both treatment techniques but the very high degree of purification brought by the oligosorbent can be very helpful if matrix effects are encountered. Indeed, if co-eluted compounds causing peak suppression or peak enhancement are present, both SIM and MRM modes are affected. Therefore, the oligoextraction appears as an easy way to circumvent matrix effects. Further experiments were carried out by reusing the oligosorbent after its use for the treatment of the post-mortem blood. Indeed, the presence of deoxyribonuclease (DNase) in the biological fluids could damage the aptamer. By this way, the oligosorbent applied to the extraction of the whole blood sample was then further applied to the extraction of cocaine from a spiked selection buffer sample. The same extraction recovery was obtained (85%, data not shown) that demonstrates the good reusability of the oligosorbent. This good stability is probably due to the fact that aptamers are immobilised on a solid support and therefore they are less sensitive to the presence of DNase.

## 4. Conclusion

Several activated supports were evaluated for the immobilisation of aptamers and their use as a selective tool in a SPE procedure. A successful immobilisation occurred for two of them, streptavidin activated agarose and cyanogen-bromide activated sepharose. Based on a covalent binding, the last one offers a higher flexibility concerning the choice of the elution conditions and a good stability in biological samples. The CNBr sepharose based sorbent was therefore preferred for further investigations. The spacer arm length effect was then studied by immobilising aptamers with different spacer arm lengths: hexamethylene ($C_6$) and dodecamethylene ($C_{12}$). Specific and non-specific retention were quite similar whatever the spacer arm used. Concerning the binding efficiencies, the $C_{12}$ spacer led to a binding yield twice higher than the $C_6$ spacer but the part of active aptamer was lower with the $C_{12}$ spacer arm then with the $C_6$ spacer arm thus giving rise to sorbents with similar capacity for cocaine. Considering the high binding efficiency of $C_{12}$ aptamers, an improvement of the active/bound aptamer ratio can be expected in optimizing the binding procedure. The $C_6$ oligosorbent was successfully applied to the cocaine extraction from post-mortem blood and led to a better purification than a protein precipitation treatment commonly used for whole blood analysis. The direct loading of such complex sample on the oligosorbent was possible and the elution fraction was injected to the chromatographic system without any other treatment. The high selectivity of the aptamers allowed the efficient suppression of interfering compounds. Therefore, the oligosorbents represent tremendous tools for the selective extraction of a target analyte from complex matrices. In the future, others oligosorbents made with aptamers directed against others molecules will be evaluated to check if this synthesis procedure give the same results independently of the aptamer nature.

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