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Stir bar sorptive extraction based on restricted access material for the direct extraction of caffeine and metabolites in biological fluids

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Abstract

A biocompatible stir bar sorptive extraction (SBSE) device was prepared using an alkyl-diol-silica (ADS) restricted access material (RAM) as the SBSE coating. The RAM-SBSE bar was able to simultaneously fractionate the protein component from a biological sample, while directly extracting caffeine and its metabolites, overcoming the present disadvantages of direct sampling in biological matrices by SBSE, such as fouling of the extraction coating by proteins. Desorption of the analytes was performed by stirring the bar in a water/ACN mixture (3/1, v/v) and subsequently reconcentrating the sample solution in water to enable HPLC-UV analysis to be performed. The limit of detection, based on a signal to noise ratio of 3, for caffeine was 25 ng/mL in plasma. The method was confirmed to be linear over the range of 0.5–100 μg/mL of caffeine with an average linear coefficient (R^2) value of 0.9981. The injection repeatability and intra-assay precision of the method were evaluated over ten injections, resulting in a %RSD of ∼8%. The RAM-SBSE device was robust (>50 extraction in plasma without significant signal loss) and simple to use, providing many direct extractions and subsequent determination of caffeine and its metabolites in biological fluids. In contrast to existing sample preparation methods for the analysis of caffeine and selected metabolites in biological fluids, this feasibility study using a biocompatible SBSE approach was advantageous in terms of simplifying the sample preparation procedures.

Keywords: Stir bar sorptive extraction (SBSE); Restricted access materials (RAM); Direct extraction; Biofluids; Caffeine; Metabolites

1. Introduction

Stir bar sorptive extraction (SBSE) is a novel extraction technique that was develop by Baltussen et al. [1] in 1999 based on solid-phase microextraction (SPME). The technique utilizes glass stir bars coated with polydimethylsiloxane (PDMS) to extract organic compound from aqueous media. The main advantage of SBSE over SPME is the higher phase ratio that is present in SBSE, producing better recovery and sample capacity [2]. SBSE has been used to analyze volatile organic carbons (VOCs) [1,3], polyaromatic hydrocarbons (PAH) [4], pharmaceutical drugs [5] and pesticides [6] with good results.

SBSE has been used for biological sample analysis in the past [7] but despite succeeding in analyzing a wide variety of biological markers, many problems remained to be solved. Examples of these problems include the potential fouling of the PDMS coating resulting from protein adsorption during extraction and the lack of selectivity of PDMS for more polar compounds. For example, extraction of analytes by SBSE based on PDMS coatings are limited to compounds with high octanol-water partition coefficient ($\log K_{o/w} > 2.7$). Also, the recovery of analytes obtained in biological fluids is typically 50–80% of the recovery in non-biological fluids due to the interaction between the biological matrix with PDMS [10]. Some groups have overcome this difficulty by deproteinization of the biological fluids with an appropriate solvent or acid [8]. However, this additional sample preparation step is more time consuming and can add sample artifacts. Others [9,10] have reduced the bar fouling by using extensive cleaning pro-

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cedures in between extractions but still only a relatively small amounts of extractions (∼30) could be performed by one bar.

Restricted access materials (RAM) are a class of biocompatible absorbent particles enabling the direct extraction of analytes from biological fluid (e.g. plasma and urine) as outlined in a recent review article [11]. In particular, alkyl-diol-silica (ADS) RAM particles are able to fractionate a sample into the protein matrix and the analytes with a controlled pore size that acts as a physical barrier to exclude macromolecules (>15,000 molecular weight). Simultaneously with this size exclusion process, low molecular weight compounds are extracted and enriched, via partition, into the phase's interior [12]. Various extraction phases, such as C_4, C_8, C_{18} and ion exchange [13,14] are available to provide a wide range of selectivity. The exterior of the silica based particles have been modified with diol moieties to prevent irreversible adsorption of proteins and hence acts as a biocompatible surface, enabling direct exposure to biological fluids.

To extend the effectiveness and robustness of SBSE approach to biological fluids, a glass stir bar enclosing a magnet was coated with RAM particles. The novel RAM-SBSE device could be directly stirred in biological fluids for analyte extraction without fouling of the coating from proteins. Its ability to directly extract caffeine and various metabolites from plasma, followed by liquid desorption and HPLC-UV analysis was studied. In contrast to existing methods for caffeine analysis in biological fluids, such as liquid–liquid extraction [15–20], solid-phase extraction (SPE) [21–24], and solid-phase microextraction [25], the main advantage of the novel RAM-SBSE extraction was the ability to perform direct extractions, minimizing long and complicated sample preparation procedures. It was observed that this novel RAM-SBSE device enabled caffeine and selected metabolites to be extracted from spiked plasma with minimum sample preparation.

2. Experimental

2.1. Materials

LiChrospher RP-18 ADS, 25 μm alkyl-diol-silica particles was supplied by Merck (Darmstadt, Germany). The structure of caffeine and its common metabolites is shown in Fig. 1. Caffeine (1,3,7-trimethylxanthine) and was purchased from A&C (Montreal, Canada), 1,7-dimethylxanthine, 1-methyluric acid and 1-methylxanthine was purchase from Sigma-Aldrich (Oakville, Canada). Epo-Tek 353ND 2 part epoxy was used as a binding reagent and obtained from Paisley (Montreal, Canada). Mediatech Dubelco phosphate buffer saline (DPBS) without calcium and magnesium was purchased from VWR (Mississauga, Canada). Magnetic bars of 8 mm × 1.5 mm were purchased from Fisher (Montreal, Canada). All solvents used were HPLC grade or better and were purchased from Fisher. Nano pure water was prepared on site with a Millipore (Milli-Q) system (Nepean, Canada).

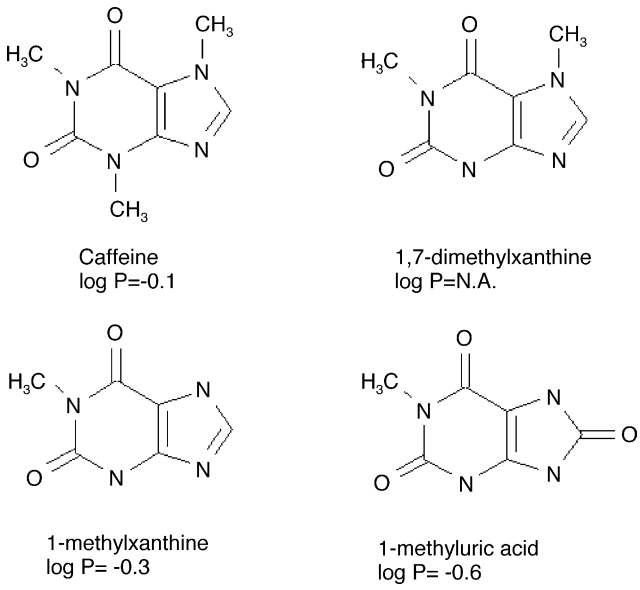

Fig. 1. Chemical structure of caffeine and three of its metabolites.

2.2. Preparation of RAM-SBSE bars

A hollow glass tube with external diameter of ∼2 mm was cut into 17 mm long pieces using a ceramic cutter. One end of the bar was closed using a propane torch. A magnet, after removing the PTFE coating, was inserted in the glass bar and the open side of the glass bar was then sealed with the propane torch. All bars were cleaned with methanol followed by water to remove any surface contamination. A thin and uniform layer of binding agent was applied to the outside surface of the stir bar followed by deposition into a 300 μL HPLC glass insert half filled with the RAM particles (LiChrospher RP-18 ADS). The insert was placed into a 1.5 mL HPLC vial and shaken until complete coating of the bar with RAM particles was observed. The open vial was placed into a 100 °C oven for 15 min for the binding agent to cure.

2.3. Instrumentation and analytical conditions

An Agilent 1100 HPLC system consisting of an autosampler, binary pump, degasser, column thermostat and variable wavelength detector was used to perform the analysis. The column used to analyze the extracted caffeine was an ODS Hypersil (60 mm × 4.6 mm with 3 μm particles) from ThermoHypersil-Keystone (Bellefonte, PA, USA). An isocratic method consisting of 88% deionized water and 12% acetonitrile was used for the optimization of caffeine analysis by RAM-SBSE. The flow rate was 1.5 mL/min, the column temperature was 40 °C, the wavelength recorded was 272 nm and injection volume was 100 μL.

To analyze mixtures of caffeine and metabolites a longer gradient HPLC method was used with an Agilent Eclipse XDB-C_{18} HPLC column (150 mm × 4.6 mm, 3.5 μm particles), and a mobile phase A of 0.1%TFA in water and mobile

phase B of acetonitrile. The gradient was as follows: 0–5 min at 5% B, then 5–25% B in 10 min followed by reconditioning for 5 min at 5% B. The flow rate was 1 mL/min at a column temperature of 40 °C and detector wavelength at 272 nm and injection volume was 100 μL.

2.4. Conditioning of RAM-SBSE bars and extraction of caffeine and its metabolites

The naïve RAM-SBSE bars were initially washed by stirring the bar in deionized water for 30 min, methanol for 30 min and finally PBS:methanol (90:10, v/v) for 30 min. Storage of the bars was maintained in an Eppendorf vial filled with PBS:methanol (90:10, v/v) solution.

The RAM-SBSE bar was placed in the sample solution and stirred for 30 min at approximately 1000 rpm. When the appropriate extraction time had passed, the bar was removed with a steel rod and dipped twice in distilled water and gently dried using a lint-free tissue. The RAM-SBSE bar was then placed into 4 mL of desorption solvent (water:ACN (75:25, v/v)) for 20 min with stirring (1000 rpm). When the desorption was complete the bar was removed with a steel rod and placed into a PBS:methanol (90:10, v/v) solution for 10 min of reconditioning. The sample desorption solvent was evaporated to dryness and then redissolved into 200 μL of water for analysis by HPLC.

Alternatively, the desorption of the analytes from the RAM-SBSE bar could be performed by sonication. This was performed by placing the RAM-SBSE bar into a 300 μL HPLC glass insert containing 200 μL of ACN and sonicating for 20 min. When desorption was complete, the bar was removed with a steel rod and the desorption solvent was ready for HPLC analysis.

2.5. Preparation of biological samples

Rat blank plasma was obtained from Merck Frosst Canada (Montreal, Canada). The plasma was stored at −20 °C until used, once thawed, the plasma was centrifuged at 14,000 rpm for 5 min. Eight hundred microliter of plasma was transferred to a 20 mL scintillation flask and was diluted (to reduce consumption) with 2.7 mL DPBS and 400 μL of methanol. Hundred microliter of concentrated caffeine solution was spiked into the solution. Protein precipitation samples were prepared by vortexing 75 μL of plasma with 75 μL of acetonitrile, followed by 10 min of centrifugation at 14,000 rpm. The supernatant (∼100 μL) was directly injected into the HPLC.

2.6. Scanning electron microscopy (SEM)

A JEOL JSM 5900-LV scanning electron microscope from JEOL (Peabody, USA) was used to image the prepared surface of the glass stir bar and the RAM-SBSE bars. The pictures were acquired under low vacuum, ∼19 Pa, with an accelerator voltage of 15 kV. No sample pretreatment was done to the bars prior to the image acquisition.

3. Results and discussion

3.1. Preparation of RAM-SBSE bar

The coating immobilization procedure was critical to ensure the successful development of the RAM-SBSE device's compatibility with biological fluids. In general, the coating has to withstand the frictional forces associated with high stirring rates during SBSE and provide multiple extractions (>50) in a complex matrix in a reproducible fashion. Among the numerous binding agent tested, the most robust bonding between the particles and the bar was with Epo-Tek 353ND 2 part epoxy. This binding agent was also chosen because it was chemically stable in organic solvent, various pH, broad temperature range (−50 to 250 °C) and it was found to resist the mechanical stress caused by the stirring. Also, this epoxy is biocompatible which ensure a reduced interaction with the biological matrices of the samples.

The method utilized for the preparation of the RAM-SBSE bar produced uniform coverage of the bar with the RAM particles. Scanning electron micrograph images were taken (Fig. 2), of the glass bar (blank) and of the RAM-SBSE bar to determine the topography of the bar. As can be seen in Fig. 2a, the initial glass bar had a very smooth surface topography,

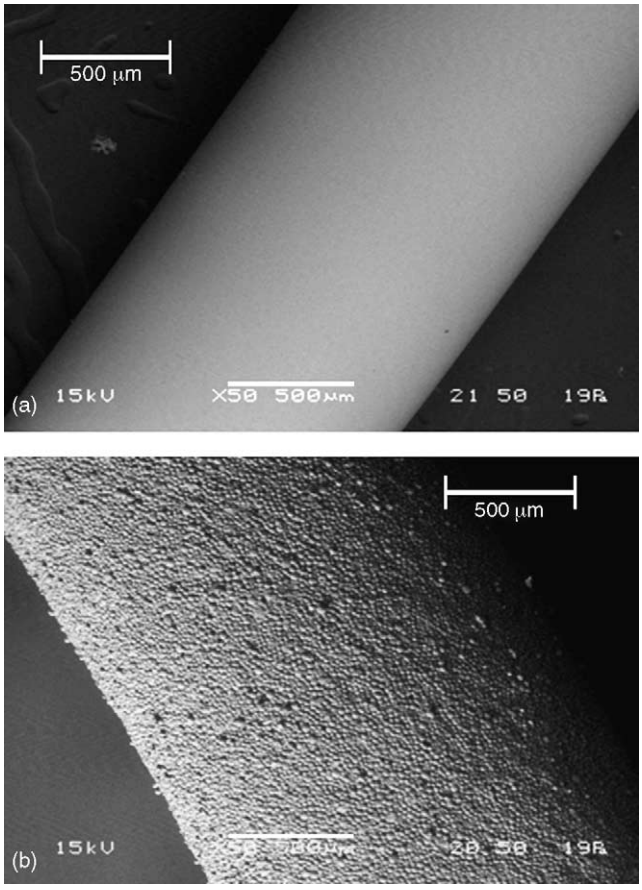

Fig. 2. Scanning electron micrographs of glass stir bar (a) and RAM-SBSE bar coating (b), accelerator voltage 15KV.

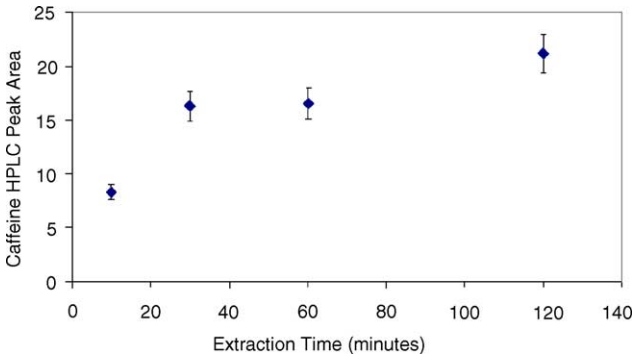

Fig. 3. RAM-SBSE extraction time profile of caffeine spiked in plasma. Experimental conditions: sample = 10 μg/mL caffeine spiked into 4 mL plasma:methanol (90:10, v/v); desorption stir time = 30 min in 4 mL of water/ACN (75:25, v/v). Desorption solvent was evaporated and redissolved in 200 μL of water.

while the RAM-SBSE bar (Fig. 2b), has a fairly uniform coating of RAM particles.

3.2. RAM-SBSE bar characterization

Caffeine was selected as a model analyte to evaluate the extraction performance of the RAM-SBSE bar due to its high polarity. Due to the limitations of PDMS coatings, previous work performed by SBSE has focused on analytes with high $\log K_{o/w}$ (>2.7), while in contrast, the $\log K_{o/w}$ of caffeine is −0.1. An extraction time profile for the RAM-SBSE device was prepared by extracting from caffeine solutions (10 μg/mL in water:MeOH (90:10, v/v)) over a period of time, as shown in Fig. 3. The precision at each timepoint ($N = 3$) was measured and produced a %RSD < 10%. It was observed that after approximately ∼1 h of extraction the RAM-SBSE bar began to reach a plateau, indicating equilibrium. Although equilibrium is not essential for analysis, the time of extraction must be carefully controlled to ensure reproducibility, and the sensitivity of the extraction will be lower if the extraction is stopped prior to equilibrium. The developed method utilized an extraction time of 30 min to limit the overall analysis time, however, improved sensitivity was possible at longer extraction times. To ensure the RAM coating was responsible for the extraction of caffeine, a blank glass and glue coated stir bar was subjected to similar caffeine extraction experiments. In both instances, no caffeine could be detected, confirming the role of the RAM coating for the extraction of caffeine.

The impact of NaCl addition was tested to determine if improved extraction efficiencies could be obtained. PBS solutions with NaCl concentration of 100, 250, 500 and 1000 mM NaCl were tested as extraction solvents. The results obtained indicated that the addition of large amounts of NaCl produced only a slight increase in the extraction of caffeine by RAM-SBSE bar (data not shown). The added salt decreases the amount of water molecules available for caffeine solvation resulting in a shift of the equilibrium between the amount of analyte in the coating and the concentration of analytes in solution towards analytes in the coating. However, even if large amounts of salt were added to the extraction solvent, only a small effect was observed on the extraction efficiency of caffeine because caffeine is a polar molecule and is thus capable of electrostatic interactions with the salt ions in solution [26], thus reducing the impact of salt addition. For less polar analytes, salt addition may have a bigger effect. To minimize the requirements of sample preparation, the salt concentration was not adjusted in subsequent sample analysis.

3.3. Desorption method

Two different methods of desorption were tested to ensure effective removal of the extracted analyte from the RAM-SBSE device. Desorption was evaluated by stirring or sonication of the bar into a suitable back extraction solvent. The stir bar was subjected to several desorption steps in fresh solvent to evaluate sample carry-over. Both methods were efficient in preventing any detectable carry-over. Although, the most convenient desorption method was sonication, since this technique allowed the used of very small volume of solvent (200 μL in a HPLC vial insert), sonication caused some degradation of the RAM stir bar coating after 30 desorption cycles. As a result stirring was subsequently used for the desorption step.

The composition of the desorption solvent was also investigated by testing various ratio of water/ACN for the desorption of caffeine from the RAM-SBSE device. The results obtained (see Table 1), show that a mixture of water:ACN (75:25, v/v) is optimal for the desorption of caffeine from the RAM-SBSE device. The desorption of caffeine from the C_{18} extraction coating of the stir bar, required a solvent system that would greatly decrease the absorption binding and partition coefficient between caffeine and the extraction phase. Although an organic solvent would typically be required for a C_{18} extraction phase, the high polarity of caffeine required the presence of water to ensure completed desorption.

The back extraction of analytes by stirring was further optimized by stirring the RAM-SBSE bar for various periods of time in desorption solvent to generate a desorption time profile. As shown in Fig. 4, a plateau was observed after 20 min that corresponds to the complete desorption of caffeine from the RAM-SBSE bar, as no detectable carry-over

Table 1
Effect of desorption solvent composition on the caffeine HPLC peak area[a]

Desorption solvent	Caffeine HPLC peak area
100% Water	21
Water–ACN (75/25, v/v)	36
Water–ACN (50/50, v/v)	20
Water–ACN (25/75, v/v)	22
100% ACN	15

[a] Extractions performed in 4 mL of a 10 μg/mL caffeine solution in PBS:methanol (90:10, v/v) ($N = 1$).

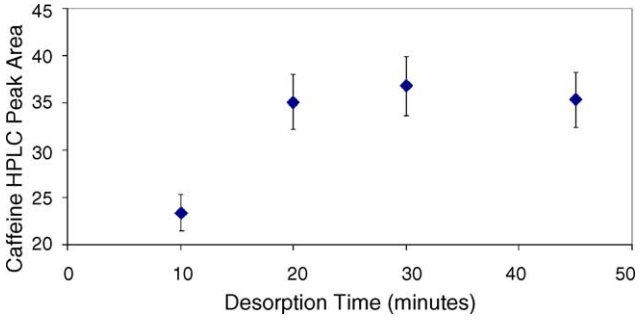

Fig. 4. RAM-SBSE desorption time profile of caffeine spiked in plasma. Experimental conditions: sample = 10 μg/mL caffeine spiked into 4 mL plasma:methanol (90:10, v/v); extraction stir time = 30 min; desorption solvent = 4 mL of water:ACN (75:25, v/v). Desorption solvent was evaporated and redissolved in 200 μL of water.

was observed. Good precision at each timepoint ($N = 3$) was also observed (RSD < 5%).

3.4. Plasma extraction of caffeine

3.4.1. Caffeine recovery

After performing RAM-SBSE with a caffeine spiked rat plasma sample, it was observed that extraction produced a lower recovery of caffeine relative to caffeine standards in PBS (see Table 2). One possibility for lower recoveries in plasma is the high protein binding of caffeine with plasma proteins [27], which would prevent the absorption of caffeine in the C_{18} phase of the RAM-SBSE bar. To prevent this phenomenon, 10% (v/v) of methanol was added to each sample prior to sample extraction to help disrupt any protein binding. The small addition of methanol did not cause any observable precipitation of the biological material but did ensure a full recovery of caffeine in plasma. As shown in Table 2, a recovery of 102% in plasma:methanol (90:10, v/v) was observed. Blank extractions from plasma were performed to make sure that no interference from the biological matrix extracted by the RAM-SBSE bar co-eluted with caffeine. Fig. 5 shows a typical chromatogram of caffeine extraction from blank and spiked plasma using the RAM-SBSE device. The absence of chromatographic peaks at the elution time for caffeine in the blank plasma sample confirmed the absence of any interference being co-extracted and eluted with caffeine. The impact of plasma dilution was also tested by performing RAM-SBSE extraction from whole plasma (containing 10% methanol, v/v) spiked with 10 μg/mL caffeine. No significant difference in the recovery was observed and no additional matrix components were detected in the HPLC chromatogram, confirming the ability of the RAM-SBSE device to provide a very clean extract in this complex biofluid.

Table 2
Recovery of caffeine with various amount of methanol used in sample solution[a]

% MeOH (v/v)	Average caffeine HPLC peak area		Recovery (%)
	PBS	Rat plasma	
0 ($n = 3$)	43	39	89
5 ($n = 3$)	36	37	103
10 ($n = 8$)	28	29	102

[a] Extractions performed in 4 mL of a 10 μg/mL caffeine solution in PBS or rat plasma sample with various methanol concentrations. Desorption stir time of 30 min in 4 mL of water/ACN (75:25, v/v). Desorption solvent was evaporated and redissolved in 200 μL of water.

3.4.2. Method validation

A calibration curve was prepared for caffeine extraction using the developed RAM-SBSE method from 0.5 to 100 μg/mL caffeine in plasma to yield a linear regression line with a slope of 2.8223 and a y-intercept of 0.3517. The RAM-SBSE method provided good linearity, with a $R^2 = 0.9981$ over a 2 order of magnitude concentration range. The limit of detection and limit of quantitation was experimentally determined using 25 and 75 ng/mL plasma samples, which produced a signal to noise level of 3 and 10, respectively. Future

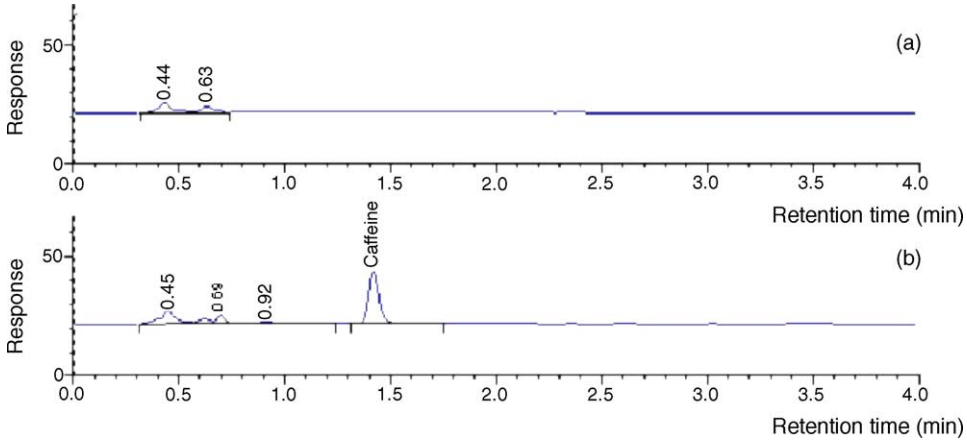

Fig. 5. HPLC RAM-SBSE sample chromatograph for (a) plasma/10%methanol and (b) plasma/10%methanol spiked with 25 μg/mL caffeine. Experimental conditions same as Fig. 4.

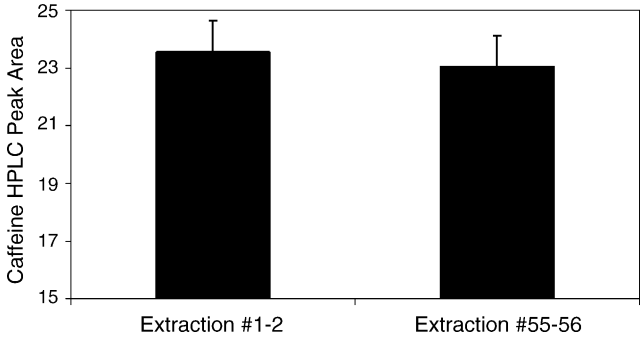

Fig. 6. Reproducibility of caffeine extraction from plasma using single RAM-SBSE device. Experimental conditions same as Fig. 4.

work will examine the adsorption potential at low levels on the RAM-SBSE. Further improvements in the limit of detection could be obtained by switching to a more sensitive detector, such as mass spectrometry. The reproducibility of the extraction method was tested using solution of 10 μg/mL of caffeine in plasma. The %RSD of the method for the repeated extraction of a single samples was determined to be 8.4% ($n = 4$), while the inter-day precision was recorded with multiple samples over several days to yield a %RSD of 8.6% ($n = 14$). Further improvements in the precision of the method would be expected with the addition of an internal standard to the plasma samples; however this was not evaluated in this study.

The robustness of the stir bar was illustrated with over 50 extractions with a minimum loss of extraction efficiency. As shown in Fig. 6, the average HPLC peak area obtained for the initial extractions of caffeine in plasma were very comparable to the average amount recorded after 55–56 extractions. The RAM-SBSE device could withstand repeated and direct exposure to plasma, in addition to considerable handling and manipulation (such as stirring at high RPMs) without decreasing the extraction efficiency and/or reproducibility. To examine the feasibility more, future work will concentrate on developing quality control of the bar to ensure bar to bar extraction reproducibility.

3.5. Analysis of caffeine and metabolites in plasma

The RAM-SBSE bars prepared were used to performed extraction of caffeine and three of its metabolites (see Fig. 1) from rat plasma. Although additional metabolites have been identified for caffeine [21,28], the evaluated metabolites were selected as model compounds based on their polarity. The chromatographic conditions were adjusted to a gradient method for the analysis of the extracts to facilitate better separation of caffeine from metabolites. The results from the RAM-SBSE method were also compared to a standard protein precipitation method where the analytes were extracted from the plasma by the addition 50% (v/v) of acetonitrile for precipitation of proteins followed by centrifugation. Fig. 7 shows the chromatograph for the RAM-SBSE extracted sample (Fig. 7a) and the chromatograph for the manual protein precipitated sample (Fig. 7b). Using both methods, some peaks in addition to the spiked metabolites are observed. The co-extraction of these small organic compounds from the plasma has yet to be elucidated. Regardless, it is quite obvious, that the selectivity of the C_{18} extraction phase in the

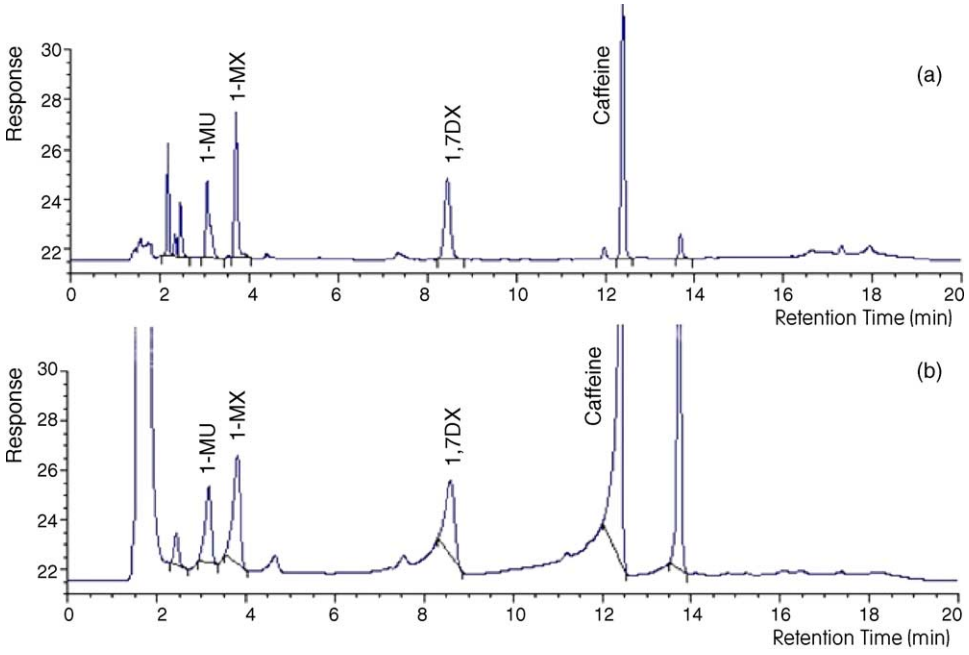

Fig. 7. HPLC sample chromatograph of 10 μg/mL caffeine, 1,7-dimethylxanthine, 1-methylxanthine and 1-methyluric acid in plasma with 10% (v/v) MeOH by (a) RAM-SBSE extraction and (b) protein precipitated plasma sample (10 μL injection). RAM-SBSE experimental conditions same as Fig. 4.

RAM-SBSE device provided a much cleaner extract from these biological fluids as indicated by the reduced number and size of peaks in the chromatographic baseline. This effect was very pronounced in the initial part of the chromatogram, which is a significant region since many metabolites (polar compounds) will have an early elution time on commonly used C_{18} analytical columns. Protein precipitated samples, on the other hand, extracted considerable more matrix components from the plasma matrix, thereby adding more potential interference. Also, under these method conditions, the four compounds analyzed from the protein precipitation sample illustrated show peak fronting as a result of the high organic content (50% acetonitrile, v/v) of the sample injected. No such problems were observed for the samples extracted with the RAM-SBSE device.

The results obtained for the developed HPLC RAM-SBSE method for the determination of caffeine and metabolites shown very comparable or improved limits of detection and recoveries. The main difference lies in the ability of the RAM-SBSE device to allow the repeated and direct extraction of small organic materials from macromolecules like proteins in biological matrices. This leads to cleaner chromatography than existing published methods using HPLC [15–17,20,21,23,24]. Also, the microscale RAM-SBSE extraction procedure reduced the requirement of toxic solvents, such as chloroform or methanol used in liquid–liquid and solid-phase extraction, and thus reduced the cost associated with the disposal of these waste and safety concerns of exposure to high volumes of solvents.

4. Conclusions

A novel RAM-SBSE bar was developed for the direct extraction and desorption of caffeine and three of its metabolites in biological samples. There was no requirement to precipitate proteins from the sample prior to extraction, therefore minimizing sample preparation time and eliminating potential sample preparation artifacts. The binding capacity, extraction efficiency and reproducibility of extraction were suitable for UV determination over a wide range of caffeine concentrations in plasma and urine. The RAM-SBSE device was robust to withstand the frictional forces associated with stirring at high RPM and could be re-used for over 50 times with minimum loss in extraction efficiency.

The utilization of the RAM material for many classes of drugs ensures the potential versatility and usefulness of this approach. More fundamentally, the extraction phase located inside the pores of the coating, can be designed towards the class of compounds under analysis. For example, phases with C_4, C_8 or ion exchange functional groups will enable the extraction of analytes over a wide range of polarities.

The simplicity and compatibility of SBSE with many analytical systems such as HPLC and GC will also extend the versatility of the RAM-SBSE device for bioanalysis. To enabled a more automated procedure, extractions by RAM-SBSE bar coupled to thermal desorption GC/MS are considered for future work in our lab. Additionally adsorption potential at low levels will also be investigated in addition to examining the bar to bar reproducibility of the extraction.

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