

Molecularly Imprinted Polymers for Solid-Phase Extraction of Sarcosine as Prostate Cancer Biomarker from Human Urine

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A highly selective molecularly imprinted polymer (MIP) for sarcosine, a cancer marker, was prepared and its use as solid-phase extraction (SPE) sorbent material was demonstrated. The MIP was prepared by a very simple procedure using methacrylic acid as functional monomer and a mixture acetonitrile/water (4/1, v/v) as porogen, overcoming in this way the problems usually related to the imprinting of biological polar compounds. The MIP was tested in batch experiments in order to evaluate its binding properties and then used as SPE sorbent for the selective clean-up and pre-concentration of sarcosine. The extraction protocol was successfully applied to the direct extraction of sarcosine from spiked human urine indicating that the MIP allowed sarcosine to be pre-concentrated while simultaneously interfering compounds were removed from the matrix.

Key Words : Sarcosine, Molecularly imprinted polymer, Solid-phase extraction, Cancer marker

Introduction

Prostate cancer (PCa) is a major health problem throughout the world, being the most common solid organ malignancy affecting men and a frequent cause of morbidity and mortality. In fact, it is the second leading cause of cancer death in the USA.¹

The serum Prostate-Specific Antigen (PSA) test is still the most important biomarker for the detection and follow-up of prostate cancer. New biomarkers for the detection and staging of prostate cancer are therefore an absolute must, and numerous studies of both serum and urine-based prostate cancer biomarker candidates have been presented the last decade. However, biomarkers for identifying the most aggressive subsets of this malignancy are still missing. Being non-invasive, urine-based tests might be suitable for both clinical and (mass) screening purposes, but also for prediction and to gain prognostic information. Urine-based tests can roughly be divided into three groups; protein-based, DNA-based and RNA-based markers.²

Sarcosine was identified as a differential metabolite that was greatly increased during prostate cancer progression to metastasis and could be detected in urine. Sarcosine levels were also increased in invasive prostate cancer cell lines relative to benign prostate epithelial cells.³ Sarcosine levels seemed to control the invasiveness of the cancer.⁴

For sarcosine determination in biological matrix, its good separation is critical due to its similarity with high-abundant L-alanine. Chromatographic methods in combination with mass spectrometry are most commonly used for sarcosine determination in urine.⁵ Except above-mentioned gas chromatography-mass spectrometry^{6,7} and liquid chromatography with tandem mass spectrometry,⁵ electrospray ionization and tandem differential mobility analysis-mass spectrometry⁸ were used for the same purpose. These methods are

accurate but expensive and analysis can be laborious.

For example, Analysis of amino acids by GC requires derivatization of the carboxylic acid and amino group to increase their volatility, and numerous approaches have been tested. However, these protocols require esterification and acylation steps, which are time-consuming and tedious.^{9,10}

However, for routine sarcosine determination in large sets of clinical samples, simple and inexpensive methods are needed.

Spectrophotometric methods can be used for the determination of amino acids in many samples. The availability of the spectrophotometric apparatus and the simplicity of analytical procedures make the technique very attractive for a wide range of applications. Spectrophotometric determination of sarcosine by ninhydrine is considered, however, it suffers from high detection limits and low selectivity for complicated biological or environmental samples.

Utilizing solid-phase extraction (SPE) with selective reagents in conjunction with spectrophotometric analysis is one way to achieve favorable sensitivity and selectivity. In this work, a very selective molecular imprinting adsorbent for sarcosine was synthesized.

Molecular imprinting is an attractive technique for the synthesis of highly selective polymeric receptors having artificial generated recognition sites, which are able to rebinding specifically a target molecule in preference to other closely related compounds.¹¹⁻¹⁴ These materials are obtained by polymerizing functional and cross-linking monomers around a template molecule, leading to a highly cross-linked three-dimensional polymer network. After polymerization, the template molecules are removed leaving in the polymer network binding sites with shape, size and functionalities complementary to the target analyte. The resulting molecularly imprinted polymers (MIPs) are stable, robust and resistant to a wide range of pH, solvents and temperature.

The behavior of MIPs emulates the interactions established by natural receptors to retain selectively a target molecule (*i.e.* antibody-antigen) but without the associated stability limitations. In addition, their preparation is relatively cheap and easy, making them a convenient alternative to the use of natural receptors. For all these reasons, MIPs could be used as selective sorbent materials for the clean-up and pre-concentration of modified nucleosides from a complex matrix, such as urine, before the separation procedures usually used for their determination.

This work describes for the first time the manufacturing of a highly selective MIP for sarcosine, as cancer marker,^{6a} and its use as solid-phase extraction sorbent material.

We propose the manufacturing of a water compatible imprinted polymer having a strong affinity for a polar compound that is sarcosine. It was used as SPE sorbent for the selective clean-up and pre-concentration of sarcosine and the extraction protocol was successfully applied to the direct and selective extraction of sarcosine from spiked human urine.

Experimental

Materials. All materials that were required during the project include, methacrylic acid (MAA), 2,2'-azobisisobutyronitrile (AIBN), ethylene glycol dimethacrylate (EGDMA), ninhydrin, chloroform, methanol, hydrochloric acid, sodium hydroxide, sulfuric acid, ethanol, acetic acid, potassium hydroxide, potassium dihydrogen phosphate, phosphoric acid and acetonitrile were obtained from Merck. In addition, sarcosine, glassine, alanine, valine, and lysine were obtained from Sigma-Aldrich. All chemicals and reagents were of analytical grade and used without any further purification.

Apparatus. UV-Vis spectrophotometer (Cary 100, Varian, Australia) was used for measuring sarcosine in Standard solutions after contact with polymer. Soxhlet extraction apparatus was used for removing the target molecule of the polymer network. A model 744A Metrohm, pH-meter was used for the pH adjustments. The Fourier transform infrared (FTIR) spectra of template molecule sarcosine, non-imprinted and molecularly imprinted polymers were obtained using a 6700 Thermo Nicolet FTIR spectrometer which IR spectra were recorded in the range 400–4000 cm⁻¹.

Synthesis of Polymers. The polymer imprinted with sarcosine (MIP) was prepared as follows: 1 mmol of sarcosine were dissolved in 10 mL of water/acetonitrile (4/1, v/v) in a glass tube and 4 mmol of MAA and 16 mmol of EGDMA were added to the solution. Then, 0.084 mmol of AIBN as initiator was added and the mixture was degassed in a sonicating bath flushing with nitrogen gas for 5 min to remove oxygen, sealed and incubated at 60 °C for 20 h to polymerize.

The resulting polymer was ground in a mortar, sieved and washed several times first with methanol/acetic acid (7/3, v/v), and then with methanol to remove the residual acetic acid. Drying under vacuum afforded particles, which were used for rebinding studies and to prepare SPE cartridges. A

control (non-imprinted) polymer (NIP) was prepared using the same conditions except for the addition of the template (sarcosine).

Determination of Sarcosine by Ninhydrin. To a 5 mL portion of a solution containing up to 0.2 mg sarcosine, 2 mL ninhydrin in acetone 2%(w/v) was added and it was heated in boiling water bath for 10 min until appear of pink color. Then it was cooled and its absorbance was measured at 570 nm against a reagent blank prepared in the same manner.¹⁵

Batch Rebinding Experiments. In 50 mL polyethylene bottles added buffer solution, the sarcosine solution, and immersed imprinted polymer with shaking at 25 °C. At pre-fixed time, an aliquot of the supernatant was separated and the sarcosine was determined spectrophotometrically according of mentioned method by ninhydrin at 570 nm. Also, the adsorbed sarcosine was eluted with water/ethanol (5/5, v/v) and the desorbed sarcosine was measured with mentioned method.

SPE Cartridge Experiments. A 200 mg sample of the MIP was packed dry in an empty SPE cartridge between two polyethylene frits. The cartridge was activated by 5 mL of acetonitrile and then conditioned by 5 mL of water. An aliquot (5 mL) of a 1 mg L⁻¹ water solution of sarcosine was loaded on the cartridge at a flow rate of 1 mL min⁻¹. Afterwards, the cartridge was first washed with 1 mL of acetonitrile, in order to elute the unbound compounds, and then sarcosine was eluted with 2 mL of water/ethanol (5/5, v/v). A flow rate of 1 mL min⁻¹ was used in both washing and elution steps. The eluted sarcosine was determined spectrophotometrically by ninhydrine at 570 nm.

The phase distribution ratio (K_d) and adsorption capacity (Q) were calculated using the following equations:

$$K_d = \frac{C_i - C_f}{C_f} \times \frac{V}{W} \quad (1)$$

$$Q = \frac{(C_i - C_f)V}{W} \quad (2)$$

Where Q represents the adsorption capacity (μmol L⁻¹), C_i and C_f represent the initial and equilibrium concentration of sarcosine in the aqueous phase (μmol L⁻¹), W is the weight of the polymer (g) and V is the volume of the aqueous phase (L). The percent extraction, E , was calculated using the following equation:

$$E = \frac{C_i - C_f}{C_i} \times 100$$

Results and Discussion

A highly selective water-compatible MIP for sarcosine was prepared by a very simple procedure using MAA as functional monomer and acetonitrile as porogen. MAA was used as functional monomer to synthesize the MIP since it can form hydrogen bonds with the template molecule in porogen prior to polymerization.

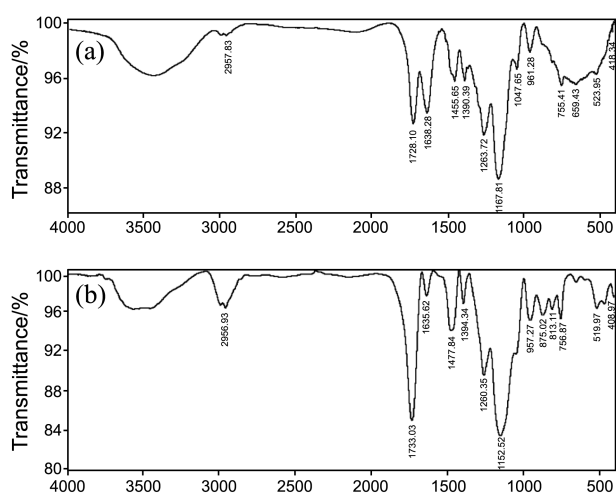


Figure 1. FT-IR spectra of NIP (a), MIP (b) in the 400–4000 cm^{-1} range by KBr pellet method.

FT-IR Spectra. Synthesized molecularly imprinted and control polymers were subjected to characterization by FT-IR. Both polymers have similar IR spectra indicating the similarity in the backbone structure. FT-IR spectra of NIP and MIP were shown in Figure 1.

In the IR spectra, the absorptions due to carboxyl OH stretch (*ca.* 3430 cm^{-1}), carbonyl group stretch (*ca.* 1733 cm^{-1}), C–O stretch (*ca.* 1260 cm^{-1}) and C–H vibrations (*ca.* 730, *ca.* 1380, *ca.* 1460, and *ca.* 2941 cm^{-1}) were observed. In addition to backbone similarity concluding, two important results were also acquired from spectra that are:

(1) The absorbances pertaining to sarcosine, are not observed in the MIP spectrum. This difference proves that imprint molecule has been sufficiently leached from MIP in the soxhlet extraction step.

(2) This feature is clear that absorbances attributed to the C–H stretch of methylene group (*ca.* 2959.15 cm^{-1}), carbonyl group stretch (*ca.* 1733.0 cm^{-1}), C–O stretch (*ca.* 1254.0 cm^{-1}) and C–H bend of CH_2 (*ca.* 1460.0 cm^{-1}) for the molecularly imprinted polymer are relatively stronger than for non-imprinted polymer. From this comparison, it was found that presence of imprint molecule (sarcosine) causes incorporation of ethylene glycol dimethacrylate in the preparation of polymer to be increased.

Optimization of Adsorption Conditions of Sarcosine on Polymer.

Effect of Time and Flow Rate on Adsorption of Sarcosine: Six portions of standard or sample solutions (25 mL) containing sarcosine (0.125 mg) were transferred into 50 mL beakers. Then exactly 0.2 g of MIP adsorbent were added to each beaker, and the mixtures were shaken vigorously for 30, 60, 90, 120, 150 and 180 min to facilitate adsorption of the sarcosine onto the imprinted polymer particles. After the solutions were centrifuged, the amount of unadsorbed sarcosine in the filtrate solutions was determined spectrophotometrically. Figure 2 shows that an equilibration time of about 150 min was required for 89% sorption. The amount of sarcosine bound to the polymer was calculated by sub-

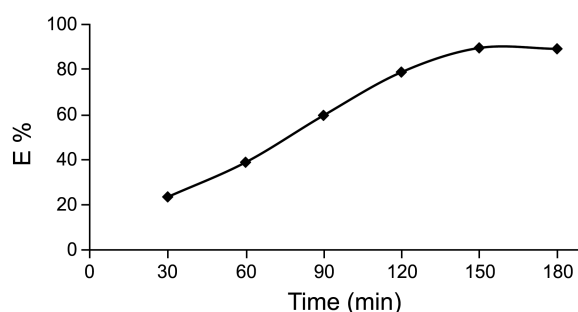


Figure 2. Influence of adsorbing time on the extraction of sarcosine.

tracting the amount of unadsorbed substrate from the initial amount of template.

In addition, effect of flow rate was optimized at flow rate of 0.5–2 mL min^{-1} , in SPE experiment. Obtained results show that maximum adsorption was achieved at flow rates higher than 1 mL min^{-1} , thus flow rate of 1 mL min^{-1} was selected for further experiments.

Effect of Sample pH on Adsorption of Sarcosine: The effect of varying pH values on sarcosine uptake was investigated using the batch procedure. Six portions of standard or sample solutions (25 mL) containing sarcosine (0.125 mg) were transferred into 50 mL beakers, and the pH value was adjusted in range 3–9 with 0.01 mol L^{-1} , HNO_3 or NaOH . Then exactly 0.2 g of MIP adsorbent was added to each beaker, and the mixtures were shaken vigorously for 150 min to facilitate adsorption of the sarcosine onto the imprinted polymer particles. According to the results shown in Figure 3 was found the adsorption quantity of sarcosine increases with the pH value increases and polymer does maximum adsorption at pH = 6.0.

So pH 6.0 is chosen for this experiment and after pH 6.0 the adsorption capacity of the polymer decreases.

Adsorption Capacity of Sarcosine by MIP. Adsorption of sarcosine from sample solution was investigated in batch experiments. At this stage, the effect of sample concentration on the adsorption sarcosine was studied to obtain the best concentration for the sample solution. Solution with 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} mmol L^{-1} concentrations of sarcosine were prepared and the pH value was adjusted to 6.0 with 0.01 mol L^{-1} , HNO_3 or NaOH . Then exactly 0.2 g of MIP adsorbent were added to each beaker, and the mixtures were

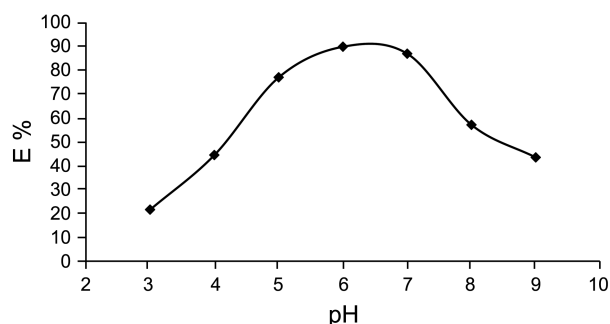


Figure 3. Effect of pH of sample solution on sarcosine uptake.

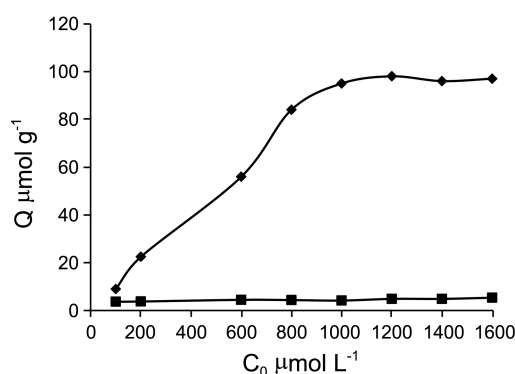


Figure 4. The effect of sarcosine initial concentration on the adsorption quantity of synthesized polymer (MIP and NIP). Other conditions: 0.2 g of synthesized polymer, pH 6.0, shaking time 150 min, temperature 25 °C.

shaken vigorously for 150 min to facilitate adsorption of the sarcosine onto the imprinted polymer particles. In order to reach the Saturation the initial sarcosine concentrations were increased until the plateau values (adsorption capacity values) were obtained. For NIP similar experiment was performed.

The data were shown in Figure 3. The average maximum adsorption capacity was 97 and 5 $\mu\text{mol g}^{-1}$ for MIP and NIP respectively.

Comparison of MIP and NIP Adsorption. Two solutions were prepared at a concentration of 10^{-4} mol L^{-1} of sarcosine and the pH value was adjusted to 6.0 with 0.01 mol L^{-1} , HNO_3 or NaOH . Then were added 0.2 g of MIP to a solution and to another 0.2 g NIP and the mixtures were shaken vigorously for 150 min to facilitate adsorption of the sarcosine onto the imprinted polymer particles. Then both filtrate and sarcosine concentration was measured in solutions. The results in Table 1 show that MIP performs better adsorption than the NIP and this confirms the accuracy of the molecular format.

Efficient Eluent: In order to choose a proper eluent for the retained sarcosine, after the extraction of 0.025 mmol sarcosine from 25 mL of aqueous sample solution, the sarcosine was stripped with 5 mL of various concentrations of different organic and mineral acids. In order to choose the most efficient eluent, different organic solvents and various concentrations of different acids in organic solvents were tested. As it is shown in Table 2, polar eluents are more effective for stripping of sarcosine from polymer.

From the data given in Table 2, it is obvious that 5 mL of water/ethanol (5/5, v/v) can strip the retained sarcosine almost quantitatively. Thus, this eluting solvent was chosen

Table 1. Comparison of MIP and NIP

Extraction %	K_d	Final concentration/ $\mu\text{mol L}^{-1}$	Initial concentration/ $\mu\text{mol L}^{-1}$	Polymer Type
91.5	1.34	85	1000	MIP
4	0.005	960	1000	NIP

Table 2. Effect of type of eluent on extraction efficiency (n = 3)

Recovery%	Eluent
60.6 ± 0.4	Ethanol
87.5 ± 0.9	Water/Ethanol (3/7, v/v)
93.2 ± 0.6	Water/Ethanol (5/5, v/v)
81.32 ± 1.2	Water/Ethanol (7/3, v/v)
53.4 ± 1.9	2 M Acetic acid
23.6 ± 3.2	Acetonitrile
43.8 ± 2.7	Acetonitrile/Acetic acid

for further studies.

Interference Effects. After the evaluation of MIP efficiency, the selectivity of the MIP was investigated in cartridge experiments. In particular, the performance of the MIP as sorbent for SPE of sarcosine was evaluated and compared with the corresponding NIP material for SPE. Typically, 2 mL of a 10 mg mL^{-1} water solution of each amino acids, which were glassine, alanine, valine, and lysine, was loaded on the MIP-SPE and NIP-SPE cartridge. Then, the cartridge was washed with 1 mL of acetonitrile in order to elute the unbound compounds and to increase the selective interaction. Finally, amino acid were eluted with 2 mL of water/ethanol (5/5, v/v). The collected amino acids were carefully analyzed spectrophotometrically by ninhydrin. In addition, 2 mL of a 10 mg mL^{-1} water solution of a binary mixture of these amino acids and sarcosine were loaded on the MIP-SPE cartridge.

Table 3 shows the recovery yields in the elution solution after the extraction of the amino acids using MIP and NIP cartridge. The extraction recovery yields were 87.2% for the MIP and only 4.1% for the NIP. The control polymer (NIP) under the same conditions allowed most the sarcosine to elute in the loading and washing steps.

Also, Table 4 shows the elution profile obtained with the

Table 3. The recovery yields in the elution solution after the extraction of the amino acids using MIP and NIP cartridge (n = 3)

Amino acid	Recovery	
	MIP-SPE	NIP-SPE
Sarcosine	87.2 ± 1.2	4.1 ± 2.1
Glassine	17.6 ± 3.3	3.5 ± 1.7
Alanine	15.8 ± 2.1	5.1 ± 2.6
Valine	6.8 ± 3.6	4.6 ± 2.7
Lysine	8.4 ± 2.9	4.7 ± 1.9

Table 4. Recovery yields in the elution solution after the extraction of the binary mixture using MIP cartridge (n = 3)

Amino acid	Sarcosine Recovery
Glassine-Sarcosine	76.5 ± 3.3
Alanine-Sarcosine	74.2 ± 4.1
Valine-Sarcosine	85.4 ± 2.1
Lysine-Sarcosine	83.1 ± 2.7

Table 5. Determination of sarcosine in human urine (n = 3)

	Spiked mg L ⁻¹	Measured mg L ⁻¹	Recovery %
	0	ND	-
Human urine	5	4.2 ± 0.9	84 ± 2.1
	10	8.1 ± 1.3	81 ± 2.7

MIP-SPE cartridge when the binary mixture was loaded. As can be seen, sarcosine was eluted during the elution step with a recovery yield of 85.4-74-2%. These data confirmed the possibility of washing interfering compounds from the MIP while retaining the analyte and emphasized the selectivity of the MIP for sarcosine.

Extraction of Sarcosine from Spiked Human Urine.

The MIP-SPE cartridge prepared as described above was activated by 5 mL of acetonitrile and then conditioned by 5 mL of water.

An aliquot (2 mL) of human urine and 0.5 mL of phosphate buffer spiked with sarcosine (5 and 10 mg L⁻¹) was loaded on the cartridge at a flow rate of 1 mL min⁻¹. Successively, the cartridge was first washed with 1 mL of acetonitrile and then sarcosine was eluted with 2 mL of water/ethanol (5/5, v/v). A flow rate of 1 mL min⁻¹ was used in both washing and elution steps. All the fractions eluted were collected and the eluted sarcosine was determined spectrophotometrically by ninhydrin at 570 nm.

As shown, in Table 5 sarcosine was strongly and selectively extracted (thus retained) by the MIP indicating that the MIP allowed sarcosine (the analyte) to be pre-concentrated while simultaneously interfering compounds were removed from the matrix.

Conclusion

This study described the synthesis of a highly cross-linked nonpolar polymer having a strong affinity for polar compounds such as amino acids. The use of a MIP for SPE as an alternative method to other techniques of the separation and pre-concentration of sarcosine offered several advantages including low cost, high capacity with high recovery, very good extraction efficiency and this method provides selective,

simple and practical strategy for the determination of sarcosine. This work represents the first application of a sarcosine-imprinted polymer in SPE and demonstrates the feasibility of using this MIP for the selective clean up and pre-concentration of sarcosine from real urine samples.

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