

# Sarcosine as a marker in prostate cancer progression: a rapid and simple method for its quantification in human urine by solid-phase microextraction–gas chromatography–triple quadrupole mass spectrometry

Brunella Cavaliere · Barbara Macchione ·  
Marcello Monteleone · Attilio Naccarato ·  
Giovanni Sindona · Antonio Tagarelli

Received: 11 January 2011 / Revised: 18 March 2011 / Accepted: 26 March 2011 / Published online: 15 April 2011  
© Springer-Verlag 2011

**Abstract** Sarcosine is an amino acid derivative of *N*-methylglycine and is involved in the amino acid metabolism and methylation processes that are enriched during prostate cancer progression. It could also serve as a new target to be measured during therapeutic interventions and help in the identification of aggressive tumors for radical treatment. In this study, we present a new urine test that can help early diagnosis of prostate cancer. The method for the quantification of sarcosine in urine consists of a solid-phase microextraction (SPME) step followed by gas chromatography–triple quadrupole mass spectrometry analysis. We used a preliminary derivatization step with ethyl chloroformate/ethanol and the corresponding ester was then extracted by SPME in immersion mode. Several fibers were evaluated and the optimization of the parameters affecting the SPME process was carried out using an experimental design. The optimal values were 20 min extraction time, 10% NaCl, and 270°C using a divinylbenzene/Carboxen/polydimethylsiloxane fiber. The triple quadrupole analyzer acquired data in selected reaction monitoring mode, allowing us to obtain reconstructed chromatograms with well-defined chromatographic peaks. The accuracy and precision of this method were evaluated at concentrations of 70, 250, and 800 ng/ml and were found to be acceptable. Very satisfactory values (0.10 and 0.16 ng/ml, respectively) were also achieved for the limit of detection and the limit of quantification. The proposed protocol represents a rapid, simple, selective, and sensitive

tool to quantify sarcosine in urine samples for prostate cancer diagnosis and for a screening test.

**Keywords** Sarcosine · Prostate cancer · Alkyl chloroformate · Gas chromatography · Solid-phase microextraction · Tandem mass spectrometry

## Introduction

Markers for cancer detection are molecules that may be present in higher than normal concentrations in many body fluids, such as serum and urine, of patients with a tumor. The widespread use of marker tests has led to early diagnosis of diseases such as cancer. In particular, the detection and treatment of prostate cancer was significantly affected by the detection of prostate-specific antigen (PSA) in the blood test. PSA has become an indispensable marker both for diagnosis and for follow-up of patients, particularly after radical prostatectomy. Despite its outstanding performance, the PSA test has significant limitations, such as its lack of specificity, failure to detect a significant number of PSA-negative tumors, and its high levels in both cancerous and healthy tissues, resulting in significant numbers of false-positive cases. Hence, more specific markers are needed for early and reliable diagnosis.

Sreekumar et al. [1] recently applied the metabolic approach for the detection of prognostic biomarkers that enhanced the clinical management of prostate cancer and this was supported by Jamaspishvili et al. [2]. In the original work, the authors profiled 1,126 metabolites across 262 clinical samples (42 tissues, 110 urine samples, and 110 plasma samples). The results showed that the concen-

B. Cavaliere · B. Macchione · M. Monteleone · A. Naccarato ·  
G. Sindona (✉) · A. Tagarelli  
Dipartimento di Chimica, Università della Calabria,  
Via P. Bucci Cubo 12/C,  
87030 Arcavacata di Rende, CS, Italy  
e-mail: sindona@unical.it

tration of sarcosine (*N*-methylglycine) in urine increased greatly during prostate cancer progression to metastasis. This observed trend supported the fact that sarcosine is a potentially important metabolic marker for prostate cancer diagnosis and, above all, could be used successfully for a screening test. Quantitative determination of sarcosine has been performed by gas chromatography (GC)–mass spectrometry (MS) analysis, after a tedious sample preparation procedure and a derivatization step under dried nitrogen using bis(trimethylsilyl)trifluoroacetamide (BSTFA).

Many methods have been developed to determine amino acids in biological fluids. Routine procedures are based on capillary electrophoresis [3–6], high-performance liquid chromatography and ion-exchange chromatography with UV detection [7–10], and mass-spectrometric detection [11–16]. Recently, a method for quantitative determination of sarcosine and related compounds in urinary samples by liquid chromatography–tandem MS (MS/MS) was developed by Jiang et al. [17]. However, unless the rapid direct analysis of fluids by fast desorption methods such as low-temperature-plasma desorption is implemented [18, 19], GC-MS is an analytical approach that will still be widely used even in unspecialized laboratories. 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. One of the most used procedures includes reaction of amino acids with silylating agents such as BSTFA [20–23] and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide [24–26]. Other derivatization procedures have been based on the use of pentafluoropropyl anhydride/2-propanol [27, 28], trifluoroacetic anhydride/2-propanol [29], and pentafluorobenzyl bromide [30–32]. However, these protocols require esterification and acylation steps, which are time-consuming and tedious, compared with the silylation reaction. In any case, all these procedures can be carried out only at high temperature (above 80°C) and, above all, in organic solvents.

Simultaneous esterification and acylation of amino acids can be performed directly in the aqueous phase using alkyl chloroformate [33]. Various reagents, including methyl chloroformate, ethyl chloroformate, propyl chloroformate, and isobutyl chloroformate, have been used for rapid determination of amino acids [34–38]. Moreover, alkyl chloroformate derivatives were analyzed by GC-MS in human urine, plasma, and blood [39–43]. Usually, the *N* (*O,S*)-alkoxycarbonyl alkyl esters were extracted using organic solvents prior to GC analysis. In a few works, alkyl chloroformate derivatives were extracted by solid-phase microextraction (SPME) [44–46]. SPME was introduced in the early 1990s [47, 48] as a simple and effective sample preparation technique which allows preconcentration and extraction to be performed in one

step. A notable feature of SPME is that it eliminates the need for toxic and not environmentally friendly organic solvents.

A number of methods using GC for the determination of amino acids have been described; however, these involved the use of MS in single ion monitoring mode. In the present application, sarcosine was derivatized with ethyl chloroformate/ethanol and the corresponding ester was then extracted by SPME in immersion mode. Several fibers were evaluated and the optimization of the SPME variables was carried out by experimental design. Finally, we investigated the capability of MS/MS with selected reaction monitoring (SRM) acquisition for the unambiguous identification and accurate quantification of the biomarker. With respect to known methods, the protocol introduced here has the following advantages: (1) a good chromatographic separation of the *N*(*O,S*)-alkoxycarbonyl derivatives of alanine and sarcosine, prepared directly in the urine sample in a few minutes; (2) the introduction, for the first time, of SPME in the direct detection of sarcosine in urine samples; (3) the known specificity in the assay of sarcosine by the MS/MS method through the selection of appropriate parent–daughter ion couples; (4) higher sensitivity with respect to previous methods [49]; (5) 30 min for a complete analysis, compared with the longer procedure of other methods [1], allowing higher-throughput screening.

## Experimental

### Chemicals and reagents

Sarcosine standard, pyridine, and sodium chloride were purchased from Sigma-Aldrich (Milan, Italy). Sarcosine-*d*<sub>3</sub> was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Ethyl chloroformate and ethanol were obtained from Fluka (Milan, Italy). The SPME fibers tested were purchased from Supelco (Bellefonte, PA, USA) and conditioned as recommended by the manufacturer. Synthetic urine (negative urine control) was obtained from Cerilliant (Round Rock, TE, USA).

### Instrumentation and apparatus

GC-MS analyses for the optimization of SPME variables were performed using a Varian (Walnut Creek, CA, USA) Saturn 2000 GC-MS ion-trap system in electron ionization mode, coupled to a Varian 3400 gas chromatograph equipped with a Varian 8200 autoinjector. The ion trap temperature was set at 210°C with an ionization time of 25 ms, an emission current of 10 μA, and a scan rate of 1,000 ms. The capillary column was a 30 m×0.25-mm inner diameter, 0.25-μm film thickness Zebron GC

ZB-5 ms [95% polydimethylsiloxane (PDMS), 5% polydiphenylsiloxane]. The gas chromatograph oven temperature was initially held at 70°C for 5 min, then ramped at 8°C/min to 210°C, held at this temperature for 2 min, and finally ramped at 60°C/min to 280°C and held at this temperature for 2 min. The carrier gas was helium (purity 99.999%) at a flow rate of 1 ml/min. For SPME analyses, a narrow-bore Supelco 0.8-mm inner diameter gas chromatograph inlet liner was used. Analyses were performed in splitless mode and spectra were acquired in full-scan mode in a mass range of  $m/z$  40–200.

GC-MS analyses for calibration and quantification of real samples were carried out using a TSQ Quantum GC (Thermo Fischer Scientific) system constituted by a Quantum triple quadrupole (QqQ) mass spectrometer and a TRACE GC Ultra equipped with an TriPlus autosampler. The capillary column was a 30 m×0.25-mm inner diameter, 0.25- $\mu$ m film thickness Thermo TR-5MS. The injector temperature was set at 270°C and the gas chromatograph oven temperature was programmed in the same way as for the Saturn 2000 GC-MS system. Helium at a constant flow rate of 1 ml/min was used as the carrier gas; argon at a pressure of about 1.0 mTorr was used as the collision gas. The QqQ mass spectrometer was operated in electron ionization and SRM mode. The transfer line and ionization source temperatures were both set at 250°C. A filament multiplier delay of 16 min was set to prevent instrument damage. The emission current was set at 50  $\mu$ A. The scan width and the scan time were set at 0.1  $m/z$  and 0.1 s. The peak width of the first quadrupole was fixed at 0.7 amu. The transitions  $m/z$  189→ $m/z$  116 for the analyte and  $m/z$  192→ $m/z$  119 for the labeled internal standard were selected for the quantitative assay by MS/MS.

### Samples

The urine samples were taken as aliquots from the 24-h urine specimens collected from ten healthy male volunteers between the ages of 24 and 35 years.

### Analytical procedure

The derivatization was performed according to method used by Vonderheide et al. [44]. A 60- $\mu$ l volume of sarcosine- $d_3$  solution at 10 mg/l was added to 1.44 ml of urine. Afterwards, 750  $\mu$ l of an ethanol and pyridine mixture (3:1 v/v) was added, and the mixture was magnetically

stirred for 10 min. Then, 156  $\mu$ l of ethyl chloroformate was added, and the sample was shaken for 2 min. Finally, 6 ml of NaCl solution at 0.14 g/ml was added. SPME was performed with a 50/30  $\mu$ m divinylbenzene (DVB)/Carboxen (CAR)/PDMS fiber. Equal amounts of urine and derivatization mixture were placed in each septum-closed vial, and the extraction was performed in immersion mode for 20 min. The adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 270°C for 10 min. A blank analysis of the fiber did not display any peak due to the analyte under investigation.

### Calibration procedure

A five-point calibration curve was obtained by spiking a 10% NaCl aqueous solution with known amounts of sarcosine and internal standard to cover a concentration range of 50–1,000 ng/ml with 400 ng/ml of sarcosine- $d_3$  as an internal standard. Each experimental value corresponds to the average of three independent measurements.

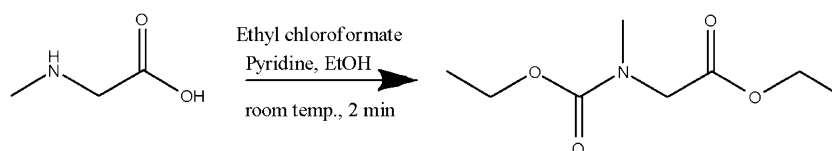
### Optimization of SPME variables

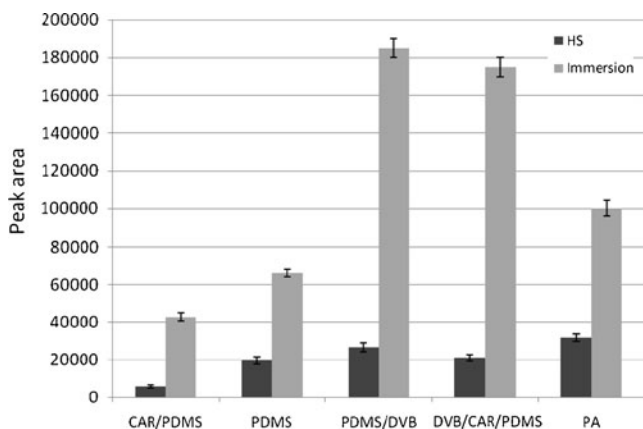
The experimental matrix designs were carried out and evaluated using Statistica 8.0 (2007 edition, StatSoft, Tulsa, USA).

## Results and discussion

Tumor markers are needed to study the etiopathogenesis of the disease; therefore, they can represent an undoubted help in clinical applications. The biomarker currently used for prostate cancer diagnosis is PSA. In over two decades since its introduction, the major limitation of PSA has been its poor specificity in men affected by prostate cancer and its inability to differentiate prostate cancer from other prostate symptoms such as benign prostatic hyperplasia and prostatitis. There is a need, therefore, for a highly specific marker that can reduce the number of false-positive cases and unnecessary biopsies. The determination of sarcosine in urine could potentially be used for noninvasive diagnosis and prognostic evaluation of prostate cancer. Today, an animated debate is in progress concerning the role of sarcosine as a suitable marker to differentiate between patients with and without prostate cancer [49, 50]. If the suitability of sarcosine as a biomarker for prostate cancer progression is confirmed, an enormous number of analyses

**Fig. 1** Reaction between sarcosine and the derivatizing agent





**Fig. 2** Peak areas obtained by performing analyses in headspace (HS) and immersion modes ( $n=3$ ) with five different fibers. CAR Carboxen, PDMS polydimethylsiloxane, DVB divinylbenzene, PA polyacrylate

will have to be carried out. For the sake of comparison, it is estimated that over 25 million PSA tests are performed in the USA alone every year [51]. In light of these considerations, the development of a simple, rapid, and reliable method for the quantification of sarcosine in urine represents an important goal. In particular, it is very important to develop a procedure with the smallest possible quantity of toxic chemicals which are not friendly to the environment. Indeed, analysis by GC has usually been carried out using organic solvents to extract amino acid

derivatives. SPME is a relatively simple, straightforward procedure involving only sorption and desorption. It completely eliminates the use of organic solvents and, in general, permits analyses with satisfactory limits of detection (LODs). Finally, the introduction of autosamplers in the SPME procedure has permitted the entire GC analysis process to be automated.

The reliability of any analytical procedure depends strongly on the possibility of identifying the analytes of interest without any ambiguity, especially when dealing with complex matrices. Since the retention time often does not provide reliable results, GC-MS in conjunction with the MS/MS approach represents a very powerful analytical tool. Thus, MS/MS analysis increases the analytical method sensitivity by significantly diminishing the background without losing its specificity in analyte identification. This feature permits trace analysis of samples derived from complex matrices, above all in the presence of interferences.

#### Optimization of SPME conditions

Conversion of amino acids to the *N*(*O,S*)-ethoxycarbonyl ethyl esters was completed within 1 min upon addition of a few microliters of ethyl chloroformate to a solution of amino acids in aqueous ethanol with pyridine [33]. Therefore, this reaction mixture was used for derivatization

**Table 1** Design matrix in the central composite design for determination of sarcosine by solid-phase microextraction for each fiber

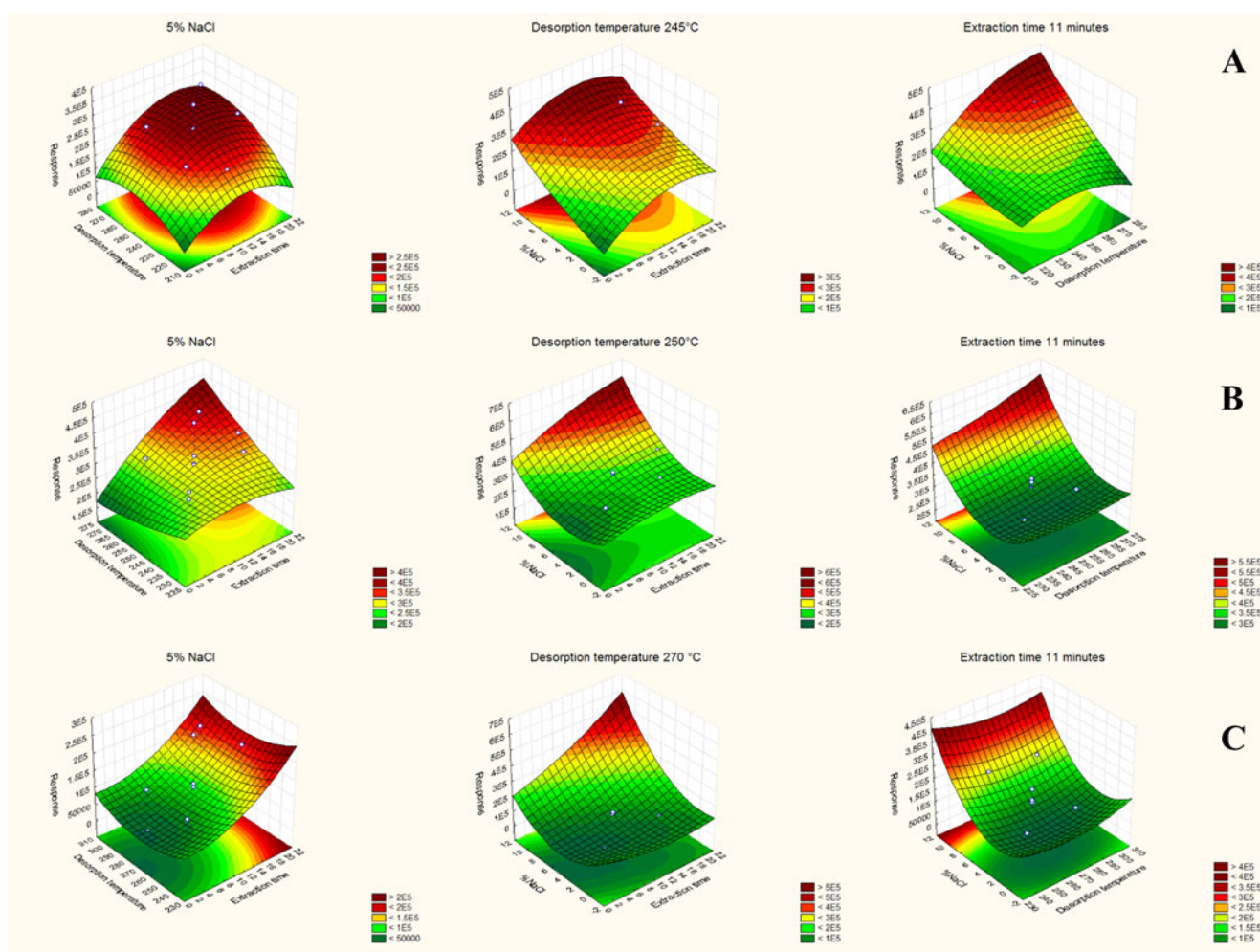
	Extraction time (min)	Desorption temperature (°C)			NaCl (%)
		PDMS/DVB	DVB/CAR/PDMS	PA	
18 (C)	11	245	250	270	5
6	16.4	230	238	252	8
9	2	245	250	270	5
16 (C)	11	245	250	270	5
19 (C)	11	245	250	270	5
13	11	245	250	270	0
3	5.6	260	262	288	2
4	5.6	260	262	288	8
15 (C)	11	245	250	270	5
20 (C)	11	245	250	270	5
11	11	220	230	240	5
14	11	245	250	270	10
8	16.4	260	262	288	8
1	5.6	230	238	252	2
12	11	270	270	300	5
5	16.4	230	238	252	2
10	20	245	250	270	5
17 (C)	11	245	250	270	5
7	16.4	260	262	288	2
2	5.6	230	238	252	8

C central point, PDMS polydimethylsiloxane, DVB divinylbenzene, CAR Carboxen, PA polyacrylate

of sarcosine to form the corresponding volatile product (Fig. 1).

SPME was initially performed by immersion of a fiber in a urine sample spiked at 5  $\mu\text{g}/\text{ml}$  and subsequently derivatized. The extraction efficiency of several fibers was evaluated by carrying out experiments with different extraction times. The best results were obtained by utilizing the DVB/CAR/PDMS fiber with an extraction time of 2 min, which was, therefore, chosen for the further analysis. Regrettably, a progressive deterioration of the coating was noted and a complete breakup of the coating was observed after ten analyses. This behavior is similar to what occurred to the CAR/PDMS coating after several extractions of derivatized selenoamino acids [44]. In contrast, in the work of Myung et al. [46] a coating of PDMS and polyacrylate (PA) fibers did not show appreciable degradation as a consequence of about threefold dilution of an aqueous sample with deionized water. Moreover, when an organic

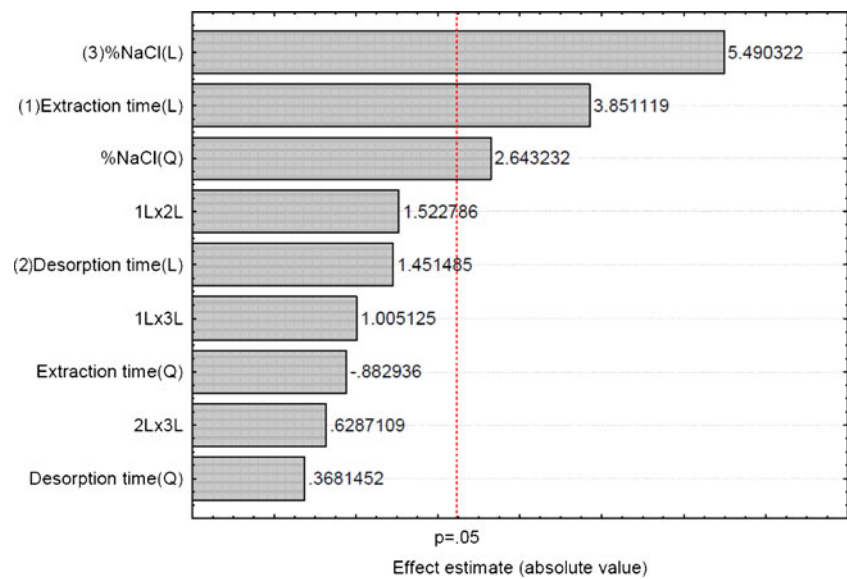
solvent is still present in solution, for example, after a derivatization procedure, it is advisable to perform SPME analysis in headspace sampling to increase the lifetime of the fiber. Therefore, the extraction efficiencies of five fibers (85  $\mu\text{m}$  CAR/PDMS, 85  $\mu\text{m}$  PA, 50/30  $\mu\text{m}$  DVB/CAR/PDMS, 65  $\mu\text{m}$  PDMS/DVB, and 100  $\mu\text{m}$  PDMS) were evaluated by sampling the analyte in headspace mode without sample dilution and in immersion mode after derivatization and addition of 6 ml of water to 1.5 ml of urine. Experiments performed under the same conditions (synthetic urine spiked at 5  $\mu\text{g}/\text{ml}$ , room temperature, extraction time 20 min) showed that more abundant signals were clearly obtained in immersion mode even though a dilution of the sample was required (Fig. 2). Moreover, the highest peak areas were obtained by performing analysis with PA, DVB/CAR/PDMS, and PDMS/DVB fibers and no fiber damage was observed. These fibers were selected for the screening and optimi-



**Fig. 3** Response surfaces estimated from the central composite design: peak area of derivatized sarcosine for extraction time versus desorption temperature, extraction time versus concentration of NaCl,

and desorption temperature versus concentration of NaCl. **a** PDMS/DVB fiber, **b** DVB/CAR/PDMS fiber, **c** PA fiber

**Fig. 4** Pareto chart obtained from the central composite design for DVB/CAR/PDMS fiber



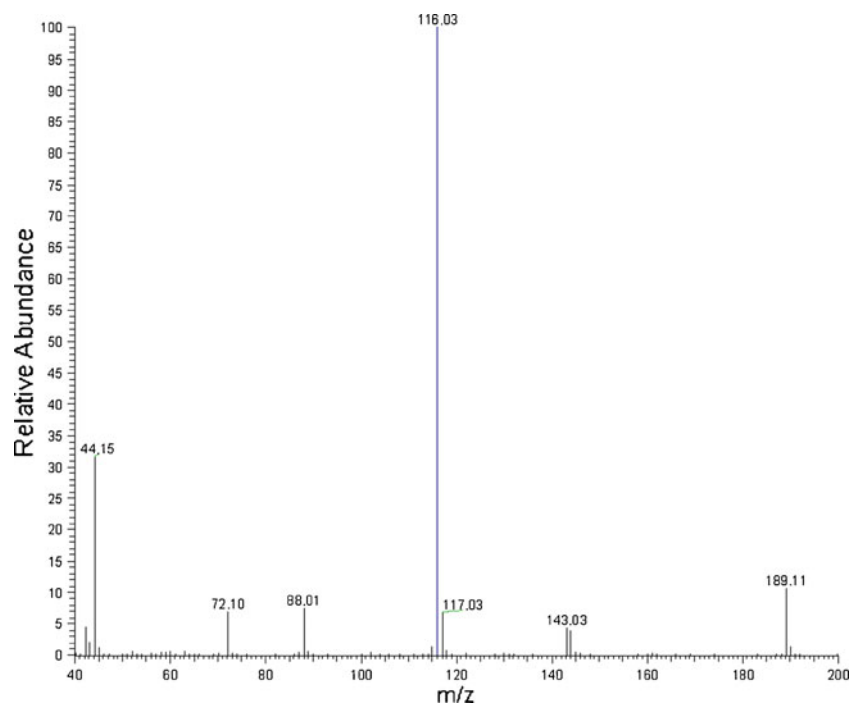
zation designs to obtain the best experimental conditions for each fiber in immersion mode.

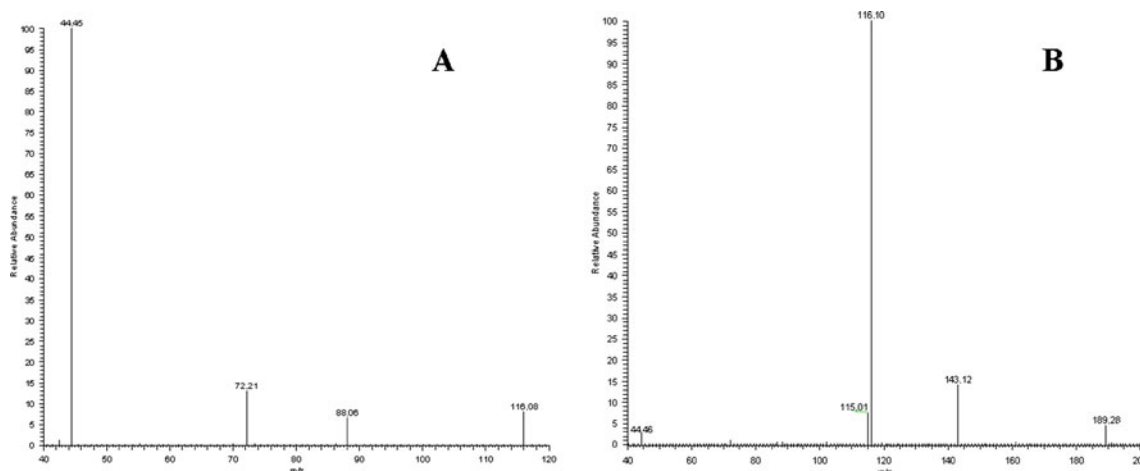
According to the literature [52], several variables can potentially affect the extraction efficiency. For example, sample agitation has an important impact on the kinetics of extraction since it speeds up the equilibrium process, and in these experimental conditions the most intense signals are obtained at the same extraction time [53]. Accordingly, urine samples were stirred during the absorption period. The experimental design was applied to urine samples, without pH adjustments, to set up a fast and simple method, considering the extraction time, the desorption temperature,

and the concentration of sodium chloride added as the only variables. It was decided to include desorption temperature in the optimization process since the sarcosine derivative could be considered a thermally labile compound because of the presence of a carbamate moiety.

A central composite design consisting of a  $2^3$  factorial design with six star points positioned at  $\pm\alpha$  from the center of the experimental domain was performed for each of the fibers. The axial distance  $\alpha$  was chosen as 1.68 to establish the rotatability condition. In this way, the design generates information uniformly in all directions, i.e., a rotation of the design about the origin does not alter the

**Fig. 5** Electron ionization (EI) full-scan spectrum of sarcosine

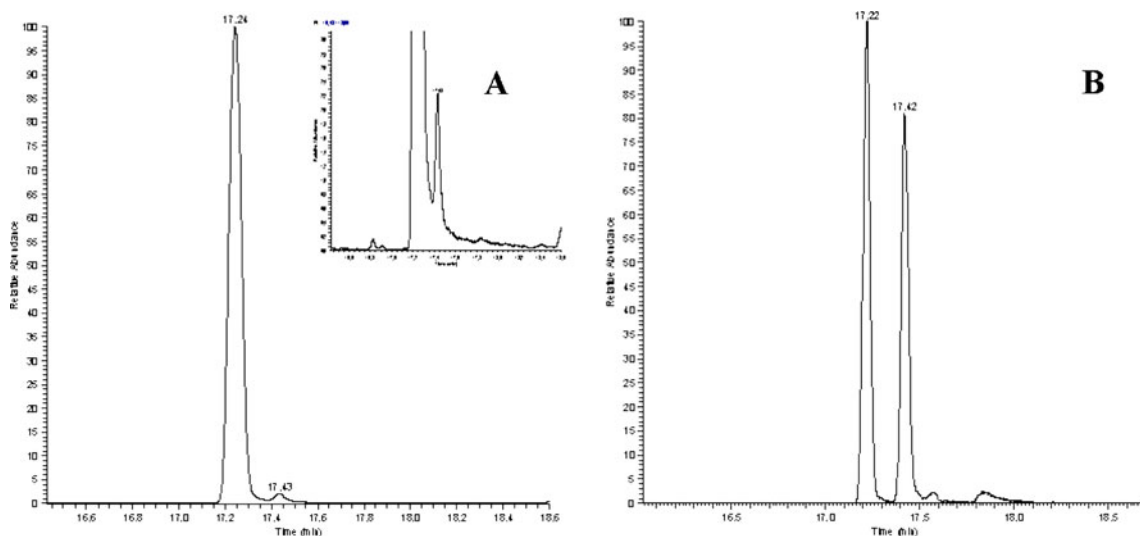




**Fig. 6** EI tandem mass spectrometry (MS/MS) spectra of sarcosine and L-alanine by selecting  $m/z$  116 (a) and  $m/z$  189 (b) as precursor ions

variance contours. In total, the experimental design matrix had 20 runs [ $2^3 + (2 \times 3) + 6$ ], six of them in the central point so that the number of degrees of freedom for the lack-of-fit equals that for replication. The same preliminary tests were carried out to determine the range of the parameters affecting the desorption of the analytes from the SPME fiber. According to these experiments, an extraction time in the range 2–20 min, a concentration of NaCl ranging from 0 to 10%, and a desorption temperature in the range 220–270°C for PDMS/DVB fiber, 230–270°C for DVB/CAR/PDMS fiber, and 240–300°C for PA fiber were selected. All the design experiments were carried out using a synthetic urine sample spiked at 1  $\mu\text{g}$  derivatized sarcosine per liter and the order of the experiments was fully randomized. Table 1 shows the design matrix.

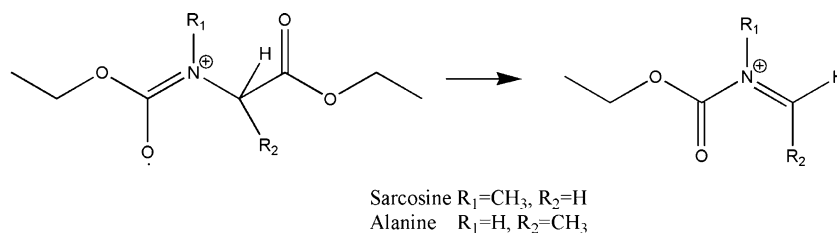
To find the optimal values for the three evaluated variables, all response surfaces were drawn (Fig. 3). It can be observed that for DVB/CAR/PDMS and PA fibers a higher response, regardless of the desorption temperature, is obtained with a higher concentration of NaCl and a longer extraction time. Different results were achieved using PA fiber, for which the best response was obtained at 300°C desorption temperature, 10% NaCl, and 16-min extraction time. In this case, the signal is quadratically dependent on the extraction time, and the amount of derivatized sarcosine within the fiber coating began to decrease after about 16 min. This trend had already been observed by Vonderheide et al. [44] and could be a result of modification of the fiber coatings due to the relatively high amount of pyridine present in the solution. In any case, considering



**Fig. 7** Solid-phase microextraction–gas chromatography–MS/MS (selected reaction monitoring) chromatogram of a real urine sample selecting the **a** transition  $m/z$  116  $\rightarrow$   $m/z$  44 (inset y-axis zoomed

chromatogram) and **b** the transition  $m/z$  189  $\rightarrow$   $m/z$  116. Alanine and sarcosine were detected at 17.22 and 17.42 min, respectively

**Fig. 8** Reaction path taken by sarcosine and alanine in the gas phase



the optimum working conditions for each fiber, the highest response was achieved using DVB/CAR/PDMS fiber at 270°C desorption temperature, 10% NaCl, and 20-min extraction time. Although greater extraction of the analyte can be obtained by choosing a longer extraction time, we decided to select a time of 20 min as a compromise between the minimum analysis time and maximal sensitivity.

The Pareto chart shows the influence that each factor investigated has on the response as well as the possible cross-effect among these factors. The Pareto chart obtained for DVB/CAR/PDMS fiber is shown in Fig. 4.

The bar lengths are proportional to the absolute value of the estimated effects and the vertical line corresponds to the 95% confidence interval. An effect which exceeds this reference line may be considered significant with regard to the response. As can be seen in Fig. 4, the factor which gave a statistically significant positive effect in the response was the concentration of NaCl both for linear and for quadratic coefficients. Moreover, the extraction time was found to be significant and, also in this case, the effect was positive. On the other hand, the signal of sarcosine was not significantly affected by the desorption temperature and interaction terms.

#### GC-QqQ-MS/MS analysis

Sarcosine is an isomer of L-alanine and this amino acid therefore potentially represents an interfering compound in the quantification of sarcosine in real urine samples. The full-scan mass spectra of the two isobaric compounds are similar (Fig. 5) and the MS/MS spectra by selecting  $m/z$  116 and  $m/z$  189 as precursor ions for both isomers show the same characteristic fragments (Fig. 6). Therefore, since there is no characteristic fragment that is isomer-specific, clearly MS/MS alone is not sufficient to discriminate the isomers in a mixture, and a previous satisfactory separation stage is needed.

Several GC oven temperature programs were tested to obtain an acceptable resolution for the sarcosine/alanine pair. A good compromise between resolution ( $R_s=1.8$ ) and analysis time was achieved by using the temperature program described in “Experimental.”

To carry out analysis in SRM mode, the precursor ion was chosen to achieve more sensitivity (ion with higher abundance) and more specificity (ion with higher  $m/z$  ratio). Initially,

the transition  $m/z$  116  $\rightarrow$   $m/z$  44 was chosen and the collision energy and scan time were optimized. Nevertheless, an SPME-GC-MS/MS analysis of a real urine sample from a healthy individual showed unsatisfactory separation between L-alanine and sarcosine as a consequence of a high concentration of alanine (Fig. 7a).

A careful comparison between the full-scan mass spectra of alanine and sarcosine indicated that the ion current at  $m/z$  189 for alanine is significantly lower than the corresponding ion current of sarcosine. The reaction paths taken by sarcosine and alanine in the gas phase are identical (Fig. 8). The only observed difference is in the energetics of the unimolecular dissociations. In the reconstructed chromatogram obtained by GC-MS, an equimolar amount of sarcosine and alanine showed a ratio of 21.4 for the ion current of  $m/z$  189. A similar ratio was obtained from the SRM scan (transition  $m/z$  189  $\rightarrow$   $m/z$  116). Therefore, the different reactivity of sarcosine and alanine can be associated with the different energy content ( $\Delta G_0$ ) of the reacting species, with sarcosine being more stable by the donor effect of the methyl group on the nitrogen atom. In light of these considerations, the transition  $m/z$  189  $\rightarrow$   $m/z$  116 was considered for the assay of sarcosine and, after optimization of the collision energy, the same urine sample was analyzed in SRM mode. In this condition, a very satisfactory separation was achieved because the response factor of alanine is smaller than that of sarcosine (Fig. 7b).

#### Analytical performances

The linearity of the calibration curve was studied by spiking a 10% NaCl aqueous solution with known amounts of sarcosine and 400 ng/ml of sarcosine- $d_3$  as an internal standard to cover a concentration range of 50–1,000 ng/ml. The response function ( $y=1.095x+0.007$ ) was found to be

**Table 2** Summary of mean accuracies (%) and relative standard deviations (RSD;  $n=5$ )

Spiked value (ng/ml)	Found value (ng/ml)	RSD (%)	Accuracy (%)
70	78.0 $\pm$ 0.6	0.74	111.4
250	259 $\pm$ 1.5	0.59	103.6
800	792.0 $\pm$ 1.0	0.13	99.0



linear, with a correlation coefficient ( $R^2$ ) of 0.9999 in the range considered.

Accuracy and precision were evaluated at three concentrations (70, 250, and 800 ng/ml) in synthetic urine by analyzing a spiked sample five times. The accuracy values (111.4%, 103.6%, and 99.0% for the three concentrations, respectively) and the relative standard deviations (0.74%, 0.59%, and 0.13%) obtained can be considered satisfactory (Table 2).

The LOD and the limit of quantitation (LOQ) were calculated following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry, that is, as follows:

$$S_{\text{LOD}} = S_{\text{RB}} + 3\sigma_{\text{RB}},$$

$$S_{\text{LOQ}} = S_{\text{RB}} + 3\sigma_{\text{RB}},$$

where  $S_{\text{LOD}}$  is the signal at the LOD,  $S_{\text{LOQ}}$  is the signal at the LOQ,  $S_{\text{RB}}$  is the signal of the reagent blank, and  $\sigma_{\text{RB}}$  is the standard deviation for the reagent blank. The concentrations were calculated from the standard curve. Also for these analytical parameters, the proposed method allows one to achieve very satisfactory values: LOD=0.10 ng/ml, LOQ=0.16 ng/ml.

#### Application to real samples

The method developed was applied to the analysis of ten urine samples from healthy individuals. Each sample was analyzed in triplicate in accordance with the procedure described in "Experimental." The mean value of the concentration of sarcosine was 211 ng/ml and the minimum and maximum values were 73.4 and 470 ng/ml, respectively. The data obtained are compatible with the range of urinary sarcosine concentrations observed in urine samples collected by the INTERMAP Study and analyzed by GC-MS [54].

#### Conclusions

In the present work, a rapid and easy method for the quantification of sarcosine in urine samples was developed for noninvasive diagnosis and prognostic evaluation of prostate cancer. Sarcosine was derivatized with ethyl chloroformate/ethanol and the corresponding ester was then extracted by SPME in immersion mode. Several fibers were evaluated and the optimization of the parameters affecting the SPME process was readily achieved using a central composite design. The combined use of chloroformate as a derivatizing agent and SPME as an extraction technique allows one to minimize the presence of organic solvents in the final protocol. The capability of QqQ-MS to minimize

matrix interference and improve the signal-to-noise ratio was confirmed. Indeed, this feature allowed us to gather reconstructed chromatograms with well-defined chromatographic peaks and then more easily to integrate them. Moreover, an appropriate choice of the SRM transition ( $m/z$  189  $\rightarrow$   $m/z$  116) together with a suitable gas chromatograph oven temperature program permitted a very satisfactory separation between L-alanine and sarcosine. Finally, the excellent performances in terms of linearity, accuracy, precision, and LOD and LOQ values make the method developed suitable to be adopted for quantification of sarcosine in urine samples as a potentially important metabolic marker for prostate cancer diagnosis and for a screening test.

**Acknowledgements** This project was funded by the Calabrian APQ-RAC network, QUASIORA, and the University of Calabria.

#### References

- Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J et al (2009) *Nature* 457:910–914
- Jamaspishvili T, Kral M, Khomeriki I, Student V, Kolar Bouchal Z (2010) *Prostate Cancer Prostatic Dis* 13:12–19
- Smith JT (1999) *Electrophoresis* 20:3078–3083
- Oguri S, Yokoi K, Motohase Y (1997) *J Chromatogr A* 787:253–260
- Kolch W, Neusüß C, Pelzing M, Mischak H (2005) *Mass Spectrom Rev* 24:959–977
- Williams BJ, Cameron CJ, Workman R, Broeckling CD, Sumner LW, Smith JT (2007) *Electrophoresis* 28:1371–1379
- Le Boucher J, Charret C, Coudray-Lucas C, Giboudeau J, Cynober L (1997) *Clin Chem* 43:1421–1428
- Feste AS (1992) *J Chromatogr B Biomed Appl* 574:23–34
- Biggs HG, Gentilcore LJ (1984) *Clin Chem* 30:851–855
- Gatti R, Gioia MG (2008) *Biomed Chromatogr* 22:207–213
- Qu J, Wang Y, Luo G, Wu Z, Yang C (2002) *Anal Chem* 74:2034–2040
- Piraud M, Vianey-Saban C, Petritis K, Elfakir C, Steghens JP, Morla A, Bouchu D (2003) *Rapid Commun Mass Spectrom* 17:1297–1311
- Rashed MS, Bucknall MP, Little D, Awad A, Jacob M, Alamoudi M, Alwattar M, Ozand PT (1997) *Clin Chem* 43:1129–1141
- Casetta B, Tagliacozzi D, Shushan B, Federici G (2000) *Clin Chem Lab Med* 38:391–401
- Chace DH, Hillman SL, Millington DS, Kahler SG, Roe CR, Naylor EW (1995) *Clin Chem* 41:62–68
- Matsumoto I, Kuhara T (1996) *Mass Spectrom Rev* 15:43–57
- Jiang Y, Cheng X, Wang C, Ma Y (2010) *Anal Chem* 82:9022–9027
- Harper JD, Charipar NA, Mulligan CC, Zhang X, Cooks RG, Ouyang Z (2008) *Anal Chem* 80:9097–9104
- García-Reyes JF, Mazzotti F, Harper JD, Charipar NA, Oradu S, Ouyang Z, Sindona G, Cooks RG (2009) *Rapid Commun Mass Spectrom* 23:3057–3062
- Gehrke CW, Nakamoto H, Zumwalt RW (1969) *J Chromatogr A* 45:24–51
- Duncan MW, Poljak A (1998) *Anal Chem* 70:890–896
- Woo KL, Lee DS (1995) *J Chromatogr B Biomed Appl* 665:15–25

23. Shen X, Deng C, Wang B, Dong L (2006) *Anal Bioanal Chem* 384:931–938
24. Simek P, Heydova A, Jegorov A (1994) *J High Resolut Chromatogr* 17:145–152
25. Chaves das Neves HJ, Vasconcelos AMP (1987) *J Chromatogr* 392:249–258
26. Starke I, Kleinpeter E, Kamm B (2001) *Anal Bioanal Chem* 371:380–384
27. Pätzold R, Brückner H (2006) *Amino Acids* 31:63–72
28. Davis BA, Durden DA (1987) *Biomed Environ Mass Spectrom* 14:197–206
29. Zumwalt RW, Roach D, Gehrke CW (1970) *J Chromatogr* 53:171–194
30. Fiamegos YC, Stalikas CD (2006) *J Chromatogr A* 1110:66–72
31. Blount BC, Duncan MW (1997) *Anal Biochem* 244:270–276
32. Guidetti P, Schwarcz R (2003) *Mol Brain Res* 118:132–139
33. Hušek P (1998) *J Chromatogr B* 717:57–91
34. Sobolevsky TG, Revelsky AI, Miller B, Oriedo V, Chernetsova ES, Revelsky IA (2003) *J Sep Sci* 26:1474–1478
35. Sobolevsky TG, Revelsky AI, Revelsky IA, Miller B, Oriedo V (2004) *J Chromatogr B Anal Technol Biomed Life Sci* 800:101–107
36. Huang ZH, Wang J, Gage DA, Watson JT, Sweeley CC, Husek P (2003) *J Chromatogr* 635:271–281
37. Haberhauer-Troyer C, Álvarez-Llamas G, Zitting E, Rodríguez-González P, Rosenberg E, Sanz-Medel A (2003) *J Chromatogr A* 1015:1–10
38. Vincenti M, Minero C, Lago S, Rovida C (1995) *J High Resolut Chromatogr* 18:359–362
39. Husek P (1995) *J Chromatogr B Biomed Appl* 669:352–357
40. Namera A, Yashiki M, Nishida M, Kojima T (2002) *J Chromatogr B Anal Technol Biomed Life Sci* 776:49–55
41. Villas-Bôas SG, Delicado DG, Åkesson M, Nielsen J (2003) *Anal Biochem* 322:134–138
42. Tao X, Liu Y, Wang Y, Qiu Y, Lin J, Zhao A, Su M, Jia W (2008) *Anal Bioanal Chem* 391:2881–2889
43. Kaspar H, Dettmer K, Gronwald W, Oefner PJ (2008) *J Chromatogr B* 870:222–232
44. Vonderheide AP, Montes-Bayon M, Caruso JA (2002) *Analyst* 127:49–53
45. Deng C, Li N, Zhang X (2004) *Rapid Commun Mass Spectrom* 18:2558–2564
46. Myung SW, Kim M, Min HK, Yoo EA, Kim KR (1999) *J Chromatogr B* 727:1–8
47. Arthur CL, Pawliszyn J (1990) *Anal Chem* 62:2145–2148
48. Arthur CL, Killam LM, Buchholz KD, Pawliszyn J, Berg JR (1992) *Anal Chem* 64:1960–1966
49. Jentzmik F, Stephan C, Miller K, Schrader M, Erbersdobler A, Kristiansen G, Lein M, Jung K (2010) *Eur Urol* 58:12–18
50. Pavlou M, Diamandis EP (2009) *Clin Chem* 55:1277–1279
51. Constantinou J, Feneley MR (2006) *Prostate Cancer Prostatic Dis* 9:6–13
52. Lord H, Pawliszyn J (2000) *J Chromatogr A* 885:153–193
53. Llompard M, Li K, Fingas M (1998) *Anal Chem* 70:2510–2515
54. Kaspar H, Dettmer K, Chan Q, Daniels S, Nimkar S, Daviglus ML, Stamler J, Elliott P, Oefner PJ (2009) *J Chromatogr B* 877:1838–1846