University of South Bohemia
Faculty of Science
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BACHELOR THESIS

DETERMINATION OF SARCOSINE IN URINE OF PATIENTS WITH PROSTATE CANCER DIAGNOSIS

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ANNOTATION

In this thesis, the biochemistry and analysis of Sarcosine, Glycine and Alanine was reviewed. Moreover, a novel method for their determination in urine by means of reversed-phase liquid chromatography was examined and evaluated.

AFFIRMATION

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

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ACKNOWLEDGEMENTS

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ABSTRACT

Sarcosine has been suggested as a potential biomarker for prostate cancer. Its detectability in urine renders it particularly attractive for routine screening, neccesitating the development of a fast and robust method for its determination.

In this thesis, the biochemistry and analysis of small amino acids and their metabolites has been reviewed. Also, LC/MS and GC/MS were examined for quantitatively measuring sarcosine in combination with pre-column chloroformate (ECF)-mediated derivatization. Hereby, the used strategy was a a simple and easy procedure employing ECF/EtOH for a simultaneous derivatization/extraction reaction carried out directly in urine. The determination of sarcosine was successful by both methods, proving that ECF/EtOH may be a convenient and promising reagent for the pre-column derivatization of sarcosine and other related amino acid metabolites in human urine.

CONTENTS

1. INTRODUCTION

Sarcosine is an amino acid naturally occurring in muscles and other body tissues. It is biosynthesized by methylation of glycine and can be detected in human plasma, urine and saliva. Recently, sarcosine has been proposed as a potential biomarker of prostate cancer [1]. Being involved in the amino acid metabolism and methylation processes that are enriched during prostate cancer, urinary levels of sarcosine were shown to be clearly elevated in cancer patients and even enabled to distinguish benign carcinoma from aggressive, rapidly metastasizing ones [1,2]. Thus, investigation of sarcosine and other small acidic metabolites in human fluids seems to be of great interest. Hereby urine is an especially attractive medium, as it can be noninvasively gained and descriptively reflects disease-induced metabolic changes.
This aim of this thesis was to collect latest knowledge about the role of sarcosine and related metabolites in prostate cancer and further to examine LC/MS technology in the combination with a novel pre-column chloroformate-mediated derivatization procedure for the simple and fast measurement of sarcosine and other related amino acid metabolites in human urine.

2. CURRENT STATUS OF KNOWLEDGE

2.1. Biochemistry of Sarcosine, Glycine, Alanine and their Metabolites

2.1.1. Biosynthesis and Functions

Sarcosine is generated either upon the N-methylation of glycine, with *S*-adenosylmethionine as a methyl donor and glycine-*N*-methyl transferase as a catalyst[3] or via a reaction mediated by dimethylglycine dehydrogenase, where dimethylglycine substrate is rapidly demethylated to glycine.

The formation of **glycine** arised either from serine by means of a reaction mediated by glycine hydroxymethyltransferase or from the metabolism of sarcosine, glyoxylate, threonine, serine (choline) or trimethyllysine (carnitine synthesis), most of the precursors enter directly by a diet [4].

Interconversion of Choline and Glycine

Choline and glycine are inter-related through their roles in methyl metabolism (Fig.1). The one-carbon metabolism refers to the folate dependent pathways involved in activating single carbons for protein synthesis, nucleotide synthesis, and DNA methylation. Choline is metabolized to betaine, which donates a methyl group to homocysteine to form methionine, also generating dimethylglycine, which is further metabolized to glycine [4].

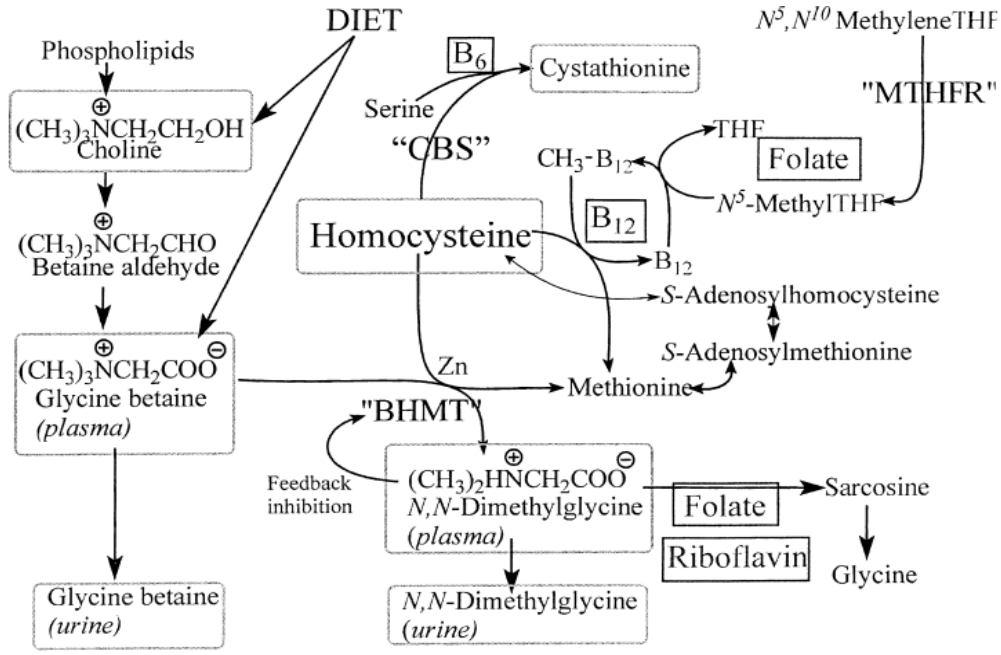


Fig. 1: Choline-Glycine pathway[16]

Glycine is present mainly in proteins although most proteins incorporate only small quantities of glycine. A notable exception is collagen, which contains about 35% glycine[5]. Proteinogenesis however accounts for about 50% of the metabolism of administered glycine, since the extent of conversion to protein serine is about four times that of direct incorporation into protein glycine[6]

Glycine serves as a precursor not only for serine, but also for a number of other nitrogenous metabolites (Fig.2). It provides the central C_2N subunit of all purines and enables biosynthesis of heme, choline, creatinine and glutathion. As a gluceogenic amino acid, glycine may also be used to gain pyruvate and finally glucose. Upon its catabolism, glycine also assists synthetic reactions via the formation of a one-carbon tetrahydrofolate compound.[5]

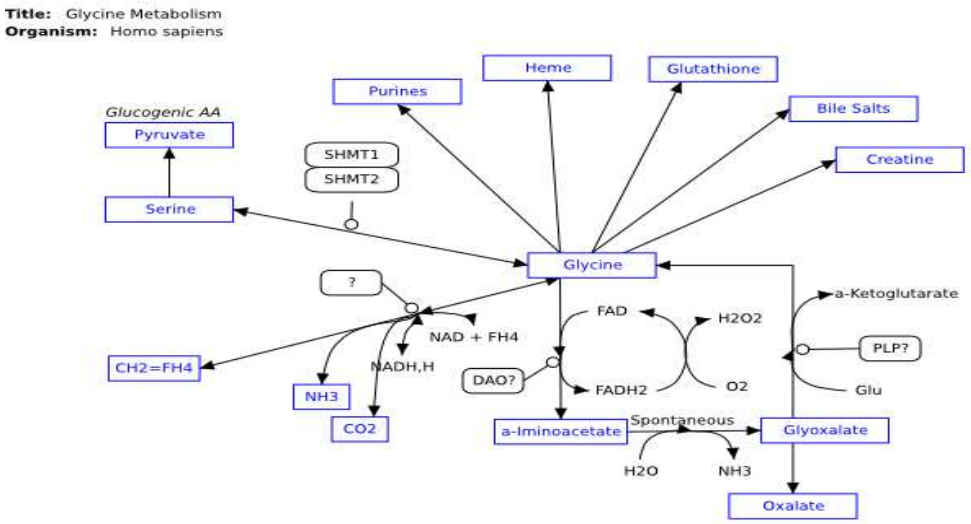


Fig.2: Glycine-related metabolite network

Moreover, glycine further functions as an inhibitory neurotransmitter in the central nervous system, especially in the spinal cord, brainstem, and retina, where it participates in a variety of motoric and sensoric functions. Glycine is also present in the forebrain, where it has been shown to function as a coagonist at the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor. Glycine promotes the glutamate action, a major excitatory neurotransmitter. Thus, glycine subserves both inhibitory and excitatory functions within the CNS[8].

Alanine is a non-essential amino acid, which is biosynthetized from pyruvate, branched chain amino acids like valine, leucine or isoleucine or by the breakdown of DNA and the dipeptides carnosine and anserine[9].

However the most common synthesis pathway is the reductive amination of pyruvate via alanine transaminase. This reaction is reversible and involves interconversion of alpha-ketoglutarate (2-oxoglutarate) and glutamate. Because transamination reactions are readily reversible and pyruvate is widespread, alanine can be easily formed in most tissues.

Another way of producing alanine is via the enzyme alanine-glyoxylate transaminase and is coupled to the interconversion of glyoxylate and glycine[10] (Fig.3).

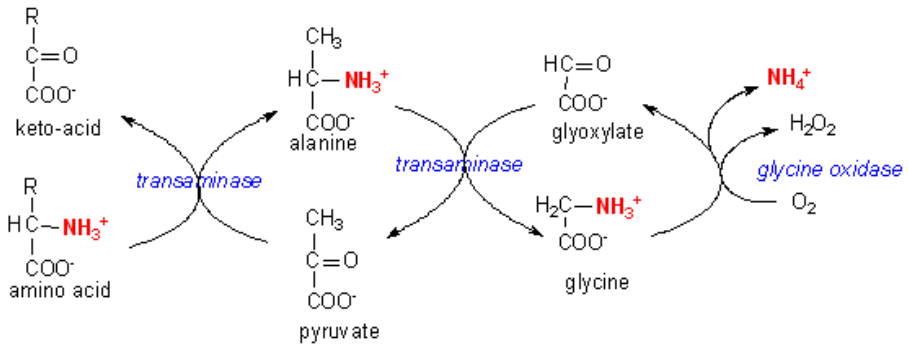


Fig. 3: Alanine formation upon deamination of glyoxylate

Alanine can be coupled to alanyl tRNA via alanyl-tRNA synthetase and used by the body in protein synthesis. It constitutes about 8% of human proteins[10]. Under fasting conditions, alanine, derived from protein breakdown, can be converted to pyruvate and used to synthesize glucose via gluconeogenesis in the liver. Alternately, alanine, after conversion to pyruvate, can be fully oxidized via the TCA cycle in other tissues.

Aside from its role in protein synthesis, alanine is functioning in the transfer of nitrogen from peripheral tissue to the liver. Its main transfer to the circulation is by muscle, where branched-chained amino acids are degraded for fuel. Thereby released excess nitrogen is collected in the form of glutamate by transamination. The glutamate amino group is further transferred to pyruvate at a rate proportional to intracellular pyruvate levels, catalyzed by alanine aminotransferase (ALT), to form alanine. Alanine then enters the blood stream and is transported to the liver, where a reverse of the alanine aminotransferase reaction takes place. Pyruvate is being regenerated and used for gluconeogenesis. The newly formed glucose can then enter the blood for delivery back to the muscle. The amino group transported from the muscle to the liver in the form of alanine enters the urea cycle and is finally excreted.

The key feature of this cycle is that via alanine, peripheral tissue exports pyruvate and ammonia (which are potentially rate-limiting for metabolism) to the liver, where the carbon skeleton is recycled and most nitrogen eliminated (Fig.4).

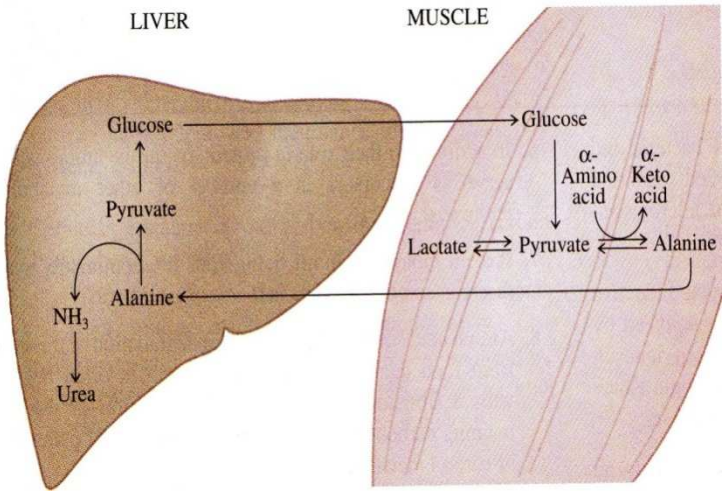


Fig. 4: Alanine-Cycle

2.1.2. Amino acids and disease

Levels of amino acids and their metabolites may be considered in various physiological matrices like plasma, serum, saliva, urine, and cerebral spinal fluid (CSF). Normal physiological concentration ranges for healthy adult individuals in body fluids are given in Tab.1.

The distribution in physiological fluids may slightly vary according to age, sex and diet, however,

more aberrant levels of one or more amino acids usually are indicative of some disease state.

	Plasma [μM]	Urine [mmol/mol creatinine]	CSF [μM]	Saliva [μM]
Sarcosine	3.7	69	Undetectable	0.4-11.0
Glycine	122-322	330	10	24.5-425.9
Alanine	200-483	9-67	1-107	7.6-39.6

Tab.1: Common concentrations of sarcosine, glycine and alanine[11]

Sarcosine has been proposed to be clinically useful in the treatment of schizophrenia, causing significant alleviation of neurocognitive and general psychopathological symptoms. This was explained by the action of sarcosine as a competitive inhibitor of the type I glycine transporter (GlyT1)[12] which increases the glycine concentration[13] in the brain and has regulatory functions at NMDAR-containing excitatory synapses [14, 15], which may result in a reduction of symptoms. Consequently, the sarcosine treatment in patients with depressions has been taken into consideration[16].

Sarcosine was recently proposed as a potential biomarker for the detection of the progressive prostate cancer (PCa). Its levels seem to strongly correlate with cancer malignancy, enabling to monitor cancer progression to metastasis. This feature would render sarcosine particularly precious for diagnostics, since the currently majorly used biomarker PSA is increased also in other prostate diseases and does not enable to distinguish invasive cancer from benign[17].

However, skepticism about the usability of sarcosine for the PCa diagnostics has still been present. A German research team found that urinary sarcosine levels failed as a marker in prostate cancer detection and identification of aggressive tumors. Referring to a study on 106 prostate cancer patients and 33 patients with no evidence of malignancy they reported that "sarcosine in urine (…) is not suitable for improving the PCa diagnostic performance in comparison with routine markers such as %fPSA"[18]. It was concluded that serum sarcosine is not a marker suitabla for the PCa diagnosis [19,20].

Alanine has effects on both insulin and glucagon circulation, being closely linked to the events such as glycolysis, gluconeogenesis and the citric acid cycl. It enables glucose generation from proteins. It was shown to raise plasma glucose concentrations in diabetics, and to produce sustained glucose recovery from hypoglycemia[21]. Further, alanine has been proven to decrease proteolysis[22] and stimulate protein biosynthesis, rendering it a popular supplement for athletes and bodybuilders. This may be secondary to the provision of an energy source, though the mechanism of this action has not yet been determined[23].

Alanine was also proposed as a prospective therapeutic marker for schizophrenia severity and improvement. Plasma alanine levels were observed to be lower during the acute stage and to increase upon easing of symptoms[24]. Alanine was considered as potential biomarkers for prostate adenocarcinoma. Its concentration was shown to be significantly higher in benign compared to malignant prostate tissue, which has been partly explained by the role of alanine as an end-product of glutamate oxidation, a major source of respiratory energy in cancer[25]. Not only alanine itself, but also levels of ALT (alanine aminotransferase) are measured routinely in screening for liver abnormalities or muscle damage[26].

2.2. Analytical chemistry of Sarcosine, Glycine and related small amino acids

Numerous methods have been developed and applied to determine small amino acids in biological fluids. Routine strategies involve capillary electrophoresis[27–30], high-performance liquid chromatography and ion-exchange chromatography with UV or fluorometric detection[31–34]. Recently also GC-MS and LC-MS based methods for the quantitative determination of sarcosine and related compounds in urinary samples by LC and mass-spectrometric detection have been reported[35–41]. Although direct analysis by amino acid analysers based on the ion-exchange LC principal predominates in clinical laboratories there are still more and more applications employing reversed-phase and normal phase separation principles.

2.2.1. Reversed-Phase High Pressure Liquid Chromatography (RP-HPLC)

RP-HPLC employs a non-polar support particle, either a resin or carbon particle, or a silica particle bonded with a non-polar substituent such as alkyl chains or phenyl rings. Common RP-HPLC eluents are polar solvents such as water, methanol, or acetonitrile. Analytes are retained through a combination of adsorption/desorption and a partitioning type mechanism. The use of RP-HPLC commonly involves some derivatization step to ease and improve detection of the analytes. However , several RP-HPLC methods for the analysis of the underivatized amino acids have been described, employing direct detection by UV absorbance[42–46], conductivity detection[47–49] electrospray MS methods, in simple and tandem MS mode[43, 5–52], fluorometric detection [53], and other techniques such as evaporative light scattering (ELS) or NMR detection[43] as well as a combination of different methods[54–56].

2.2.2.Hydrophobic interaction liquid ion chromatography (HILIC)

In the HILIC separation process, the mobile phase forms an aqueous layer on the surface of the polar stationary phase and the HILIC retention mechanism involves partitioning of the injected analyte solute molecules between the mobile phase eluent and the water-enriched layer (**Fig.4**). However, the separation mode has been suggested to occur in a more multimodal fashion. Under the

proper pH conditions, an ion exchange mechanism may contribute to the analyte retention, as a positively charged (basic) analyte undergoes the cation exchange with the negatively charged silanol groups[57]. Another, athough a rather weak mechanism is gentle reversed-phase retention by the analyte interaction with the siloxane bridges of the stationary phase surface[58].
Elution is done by increasing the hydrophilicity of the mobile phase by increasing the water content.

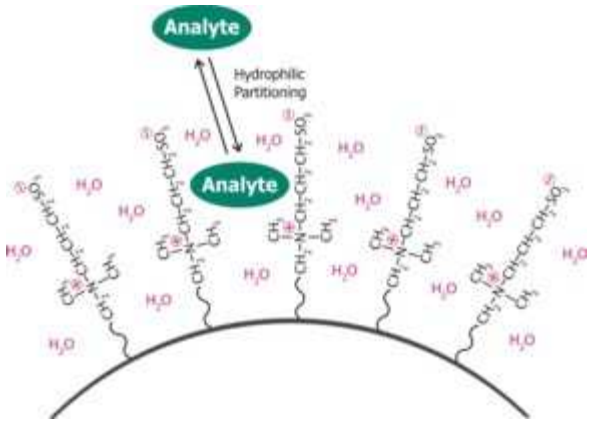


Fig.4: Interaction of analyte with the stationary phase in HILIC

The described HILIC mechanism may also involve weak electrostatic and hydrogen donor interactions, which is specifically utilized in the concept of electrostatic repulsion–hydrophilic interaction chromatography (ERLIC)[59]. Highly charged compounds are significantly better retained than weakly or non-ionic ones, and retention is overwhelmingly driven by the analyte charge status. This makes the gradient elution indispensable in order to elute both types of ionic compounds (positively, negatively charged) in the same time frame. ERLIC is performed by means of the stationary phases carrying an almost equal charge as the analytes, to be separated. Consequently, selectively antagonized electrostatic repulsion will shift more retained ions back into the main elution timeframe, allowing isocratic resolution[60]. Separation by differently polar functional groups is facilitated, as the dominating influence of the ionic group is minimized[61]

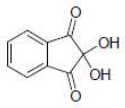
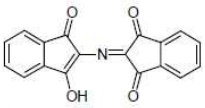
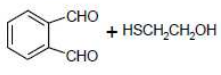
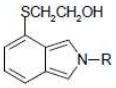
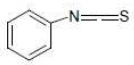
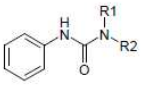
2.2.3. Derivatization

Chemical modification of the analyte by derivatization is a common analytical strategy increasing its analytical sensitivity or selectivity. Most amino acids lack a suitable chromophore, fluorophore or electroactive group which makes their detection difficult. Photometric, fluorometric or amperometric detection have been most commonly used in conjunction with the derivatization methods. Similarly, derivatization is also useful in combination with LC-MS technology minimizing thus matrix effects of ion supression and adduct formation[62]. Furthermore, the derivatization approach may influence the analyte polarity, increasing retention in the RP-LC mechanism. For example, benzylation provides UV absorbing and much less polar analytes. Common reagents for this procedure are benzoyl chloride, m-toluol chloride or p-nitrobenzoyl chloride.

Derivatization can be done in either of two modes: **Pre-column** modification reactions are mostly performed manually in vials before injection, which allows for more flexibility regarding reaction time, solvent reaction or elimination of excess reagent. An essential feature of the resulting derivatives is stability. Pre-column methods have been considered to be generally more sensitive than most post-column approaches[62, 63] [36, 37]. A frequently used reagent is phenylisothiocyanate (PITC), which yields UV-detectable, stabile derivatives and is integral part of the IDQ analytical kit of Biocrates Co. (Innsbruck, Austria [cit].

Automatization is easier with the **post-column** derivatizations. This is done in between separation and detection in a reaction cell with a mixer. The derivatization reaction has to be fast and compatible with the mobile phase. Classical reagents are ninhydrin, which enables photometric detection as it will react with amino acids to give a blue or purple color (known as Ruhemann's purple) and o-Phtalaldehyde (Fig.), which yields highly fluorescent products and was shown to allow even more sensitive detection than ninhydrin[64].

Tab.2: Structures of common derivatization reagents

Structure of the reagent	Structure of derivate or detected compound
 Ninhydrin	 Ruhemann's Purple
 OPA (o-phthalaldehyde)	
 PITC=Phenyliso-thiocyanate; Waters: Pico•Tag	

The pre-column derivatization methods commonly involve acylation/esterification reactions. For such reactions, the successful use of various anhydride/alcohol combinations has been reported[65]. As an alternative, alkyl chloroformates and some alcohol may be employed to gain esters from carboxylic groups and carbamates from amino groups (Fig. 5).

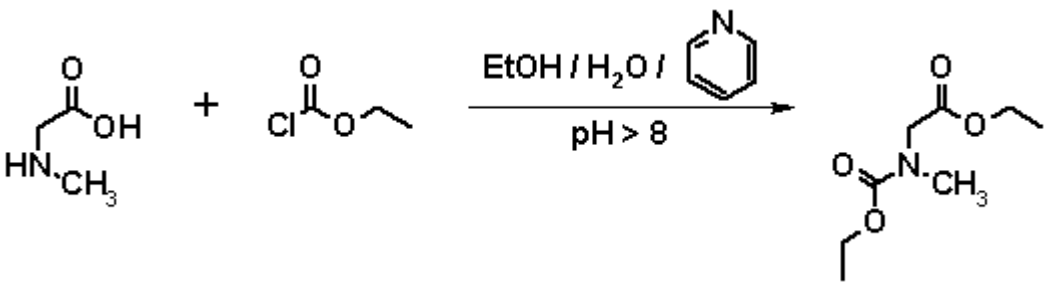


Fig.5: Derivatization employing ethyl chloroformate

The amino acid metabolites are typically derivatized directly in slightly alkaline (pH 8) aqueous solution, followed by the extraction of the arising, much less polar product. The derivatization reaction can be catalysed by pyridine, which is removed from the organic phase by adding HCl into the reaction medium.

In addition to the now common GC/MS applications, RCF represent perspective derivatization reagents in the field of LC-MS analysis. RCF have been recently applied to the determination of sarcosine and related amino acids by GC-MS[66–67]. In this work, we examined simultaneous derivatization with ethyl chloroformate (ECF) and with liquid liquid extraction of the arising amino acid derivatives into chloroform for the analysis of sarcosine and other small amino acids by the LC-MS.

3. EXPERIMENTAL

3.1. Chemicals

All chemicals were purchase from Sigma-Aldrich (Prague, Czech Republic) and are summarized in Table 3.

Tab.3:Reagents and chemicals

Chemical	Quality, Producer
3d-Sarcosine (ISTD)	<99%, Isotec
Ethylchloroformate	97%, Sigma Aldrich
Pyridine	Molecular sieve dried, Sigma-Aldrich
Chloroform/Ethanol	99.9%, Sigma Aldrich
NaOH	<99%, Riedel-deHaën
HCl	37%, Fluca
Artificial Urine	1% urea, 8000 μM creatinine, 1000 μM hippuric acid, 1000 μM glycine, 500 μM histidine, 100 μmol/L lysine, 50 μM tyrosine, 50 μM tryptophan, 250 μM citric acid, μM cystine

3.2. Calibration

10 ml of a stock solution of sarcosine (10 nmol/μl) was prepared and diluted to give seven standard solutions of sarcosine in synthetic urine with the respective concentrations 0.5, 2, 5, 20, 50, 200 and 500 nmol/μl. After the derivatization and extraction step, the extracts containing the standards were measured by LC-MS.

3.3. Derivatization-Extraction Step

For each measurement, 100 µl of urine sample were added 8 µl of internal standard, 75 µl of a 4:1 mixture of ethanol and pyridine and the same amount of chloroform/ECF (9:1). The mixture was emulsified and added another 75 µl of ECF/chloroform, as well as 75 µl of 1.5 M NaOH. After mixing and centrifuging, 50 µl of the organic layer were evaporated using N_2 and the residue was dissolved in 100 µl of MeOH (30%).

3.4. Instrumentation

The metabolites derivatized and extracted from the ECF – ethanol - pyridine- chloroform aqueous medium were subjected to LC/MS analysis on an LTQ linear ion trap mass spectrometer (Thermo-Fisher Scientific, San Jose, USA) equipped with a CTC-PAL autosampler (CTC Analytics, Basel, Switzerland) and an Accela gradient LC pump (Thermo-Fisher). The experimental conditions examined in this work are summarized in Table 4.

Tab.4: Instrumental Settings for LC-MS

LC	Pump: Rheos Allegro, Autosampler: CTC/PAL
Column	Gemini-C18, 150x2 mm, particle diameter: 3µm
Injection volume	5 µl
Temperature	35°C
Mobile Phase	A: $HCOONH_4$ in MeOH, B: $HCOONH_4/H_20$
Gradient	30%A→100%A (0–12min) 100%A (2min) 30%A (4min)
Flow rate	200 µl/min
MS	LTQ (Thermo Scientific)
Source type	HESI
Capillary temperature	150°C
APCI vaporizer temperature	150°C
Sheat gas flow	40.00
Aux gas flow	30.00
Source voltage	3.00 kV
SIM masses monitored	116, 119, 189, 192

4.RESULTS AND DISCUSSION

Prior to analysis, sarcosine and the internal standard 3d-sarcosine were converted into the respective N-ethoxycarbonyl-O-ethyl esters as depicted in Fig. 5. The most abundand fragments of sarcosine (m/z = 116, 189) and 3d-sarcosine (m/z = 119, 192) were monitored.

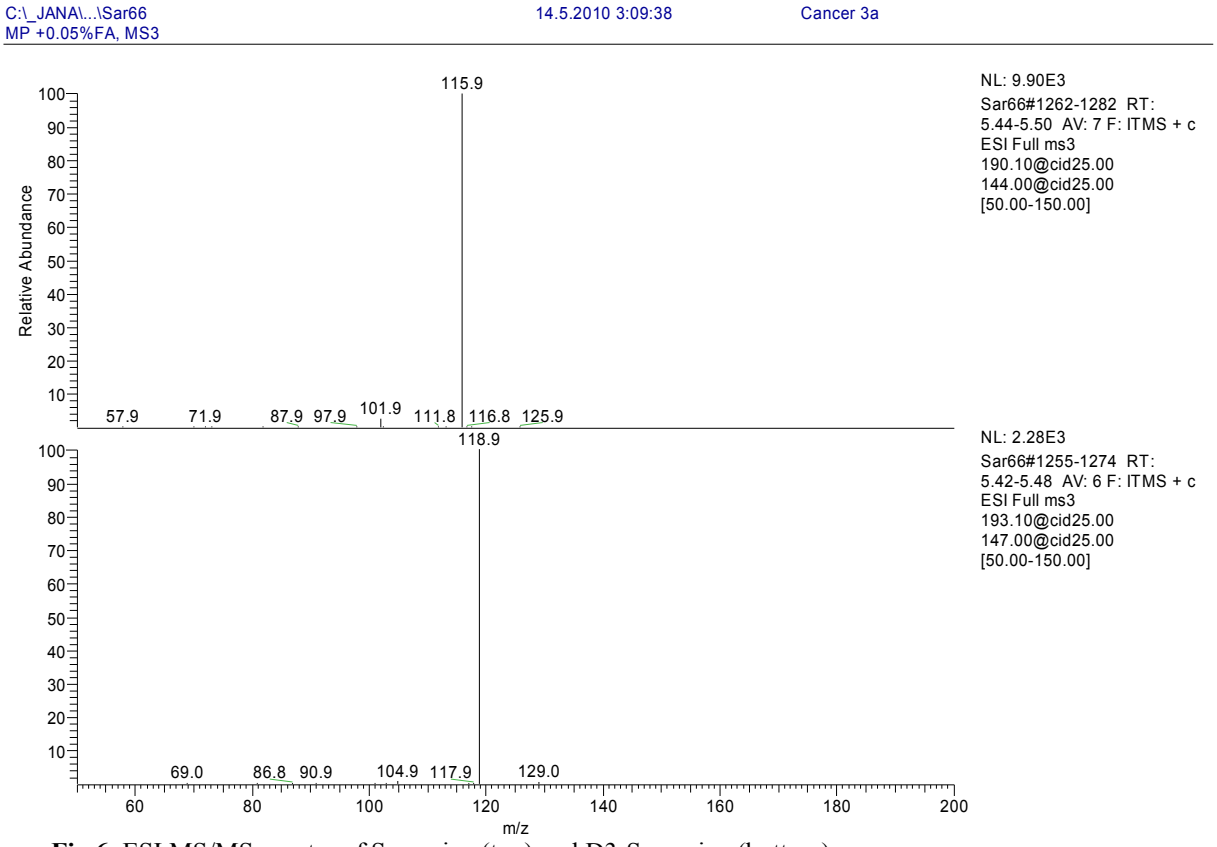


Fig.6: ESI MS/MS spectra of Sarcosine (top) and D3-Sarcosine (bottom)

Calibration

The matrix of the calibration solutions was simulated by a "synthetic" urine. Derivatization was done using ECF in chloroform; 3d-sarcosine served as an internal standard. The calibration curve was set up using the program Xcalibur 2.07, thereby the measurement of the 500 nmol/µl standard was excluded, as it obviously was above the linear range. The curve was based on the relative peak area (sarcosine peak area/ISTD peak area) versus the concentration of sarcosine. It was linear with $R^2 = 0.9980$. (Fig.7). The limit of detection (signal/noise = 3) was estimated to be at a concentration of ~0.05 nmol/µl.

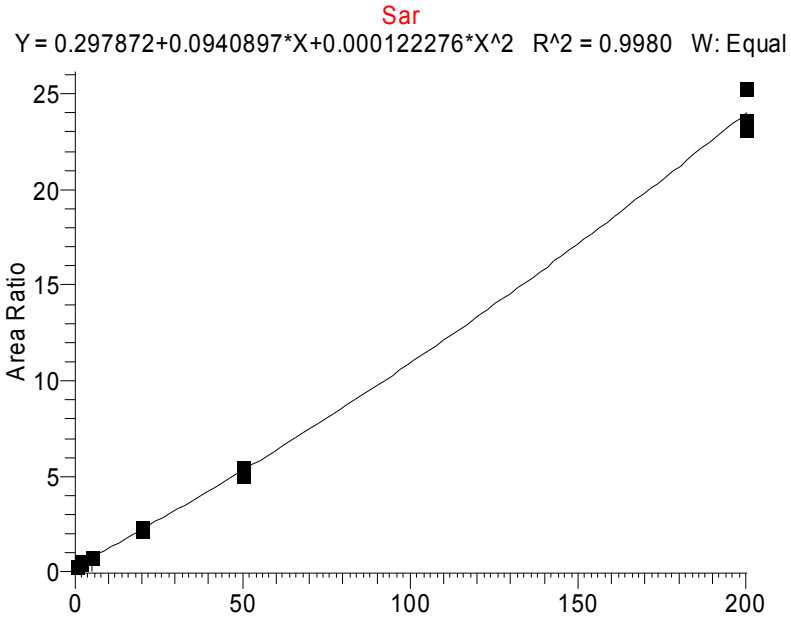


Fig.7: Calibration curve for LC-MS

Urine Samples

Sarcosine and alanine were measured in the urine of 5 patients with prostate cancer diagnosis and 5 healthy volunteers. Each sample was measured 3 times.

For calculation of the concentrations linear regression was performed using the prepared calibration curve. To obtain the actual levels of sarcosine in the urine samples, the measured concentrations were multiplied by the factor 4.66 to correct for the dilution during the derivatization/extraction procedure. The retention times of sarcosine were in average 5.46 min. (Fig.8)

The same urine samples were further analyzed by GC-MS which will be a subject of another diploma study. Thereby, an equal calibration and derivatization/extraction procedure were used (data not shown here).

The associated results are summarized in Tab.6.

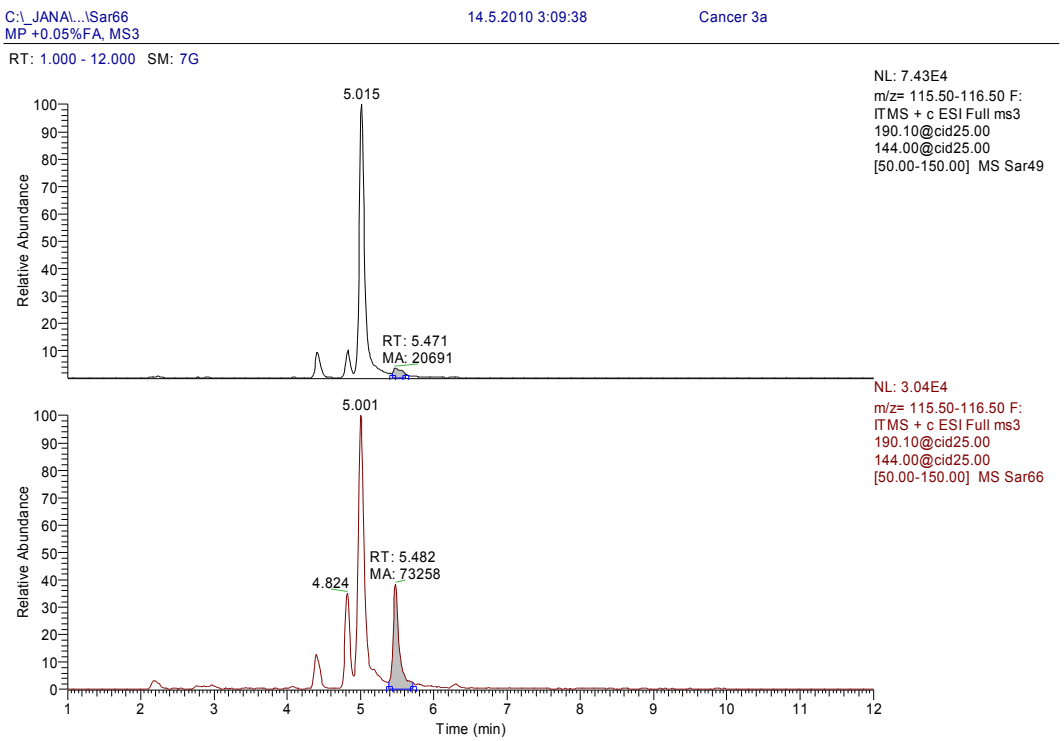


Fig.8: Chromatograms of Sarcosine (top: healthy volunteer, bottom: patient with prostate cancer)

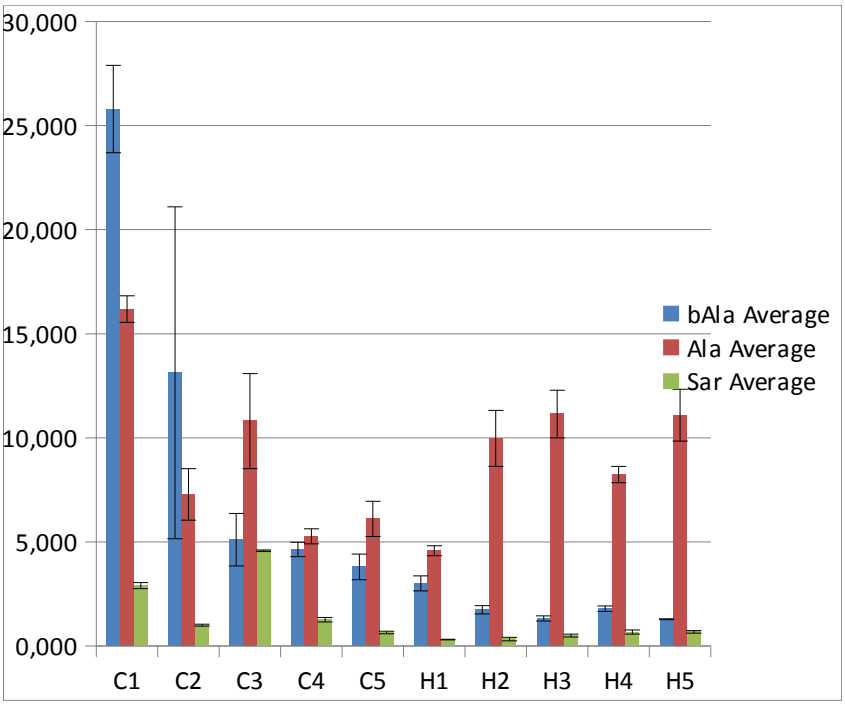


Fig. 9: Measurement results from LC-MS

Comparison with GC-MS

Remarkably, the quality of chromatographic separation was much better in GC than in LC. LC spectra showed severely tailing peaks, deviating strongly from the symmetrical shape of a Gaussian

peak, whereas resolution and peak shape were of better quality in GC/MS. Also the relative standard deviations were lower in these measurements. (Tab.6). Both LC-MS and GC-MS revealed elevated levels of sarcosine in the urine of PCa patients (Fig.10).

Tab.6: Measured average concentrations of sarcosine and relative standard deviations. Samples C1–5 were from PCa patients, H1–5 from healthy persons

		GC-MS		LC-MS	
Sample	**PSA [ng/ml]**	**Sarcosine [nmol/µl]**	**RSD [%]**	**Sarcosine [nmol/µl]**	**RSD [%]**
C1	12,7	16,95348	7,6	13,4822	5,2
C2	98	5,234802	4,1	4,62077	5,2
C3	151	25,23249	7,7	21,312	0,9
C4	51	7,413401	3,4	5,83315	8,5
C5		3,233428	8,1	2,97142	9,6
H1		1,652964	3,7	1,38894	5,4
H2		1,714351	7,9	1,52889	25,8
H3		3,14736	7,8	2,30291	14,7
H4		3,27381	3,7	3,0615	14,8
H5		3,895719	6,2	3,13048	9,3

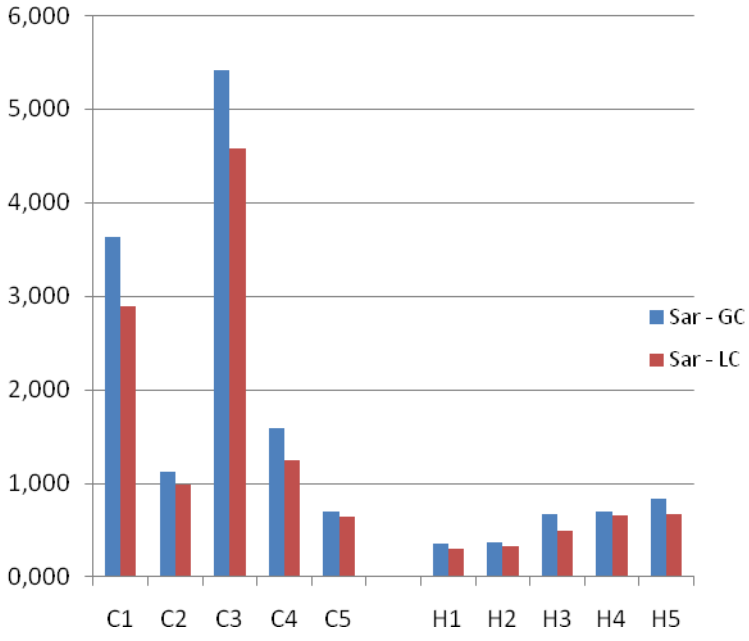


Fig.10: Sarcosine levels measured by LC-MS and GC-MS

5.CONCLUSION

A novel LC/MS method was examined in this study and proved that measured of sarcosine in urine can be accomplished well by using the described pre-column derivatization mode. Sarcosine could be separated perfectly from the isomeric analytes alanine and ß-alanine However, the separation process on a particular LC column hast to be optimized carefully in order to develop a robust and reliable method for amino acid analysis. The developed LC-MS method was compared with a parallel GC-MS developed in a complementory dimploma study and the data matched well the levels in a series of five clinical and five control samples. Sarcosine was clearly elevated in patients compared to the small control cohort. Anyway, the number of measured samples was too small to allow judging the biomarker quality of sarcosine.

In summary, an easy and fast sample preparation protocol was examined in this study and proved that ECF/EtOH may be a convenient and promising reagent for the pre-column derivatization of sarcosine and other related amino acid metabolites in human urine.

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