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Spectrometric and Electrochemical Analysis of Sarcosine as a Potential Prostate Carcinoma Marker

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Sarcosine, also known as N-methylglycine, is a natural ubiquitous non/protein amino acid which occurs as intermediate and side product in glycine synthesis and degradation. Recently, sarcosine has been investigated as a new putative marker in relation to prostate cancer. Sarcosine was identified as a differential metabolite that was greatly increased during prostate cancer progression to metastasis and could be detected in urine. It was also shown, that sarcosine addition to benign prostate cell cultures caused increase of their invasivity and motility. The aim of our study was to propose a low-cost, robust and simple method suitable for sarcosine determination in biological samples such as urine or blood plasma. For this purpose an ion exchange liquid chromatography as robust separation method was tested however this method suffers from insufficient limit of detection at the level of 70 μM of sarcosine. For more sensitive detection we optimized the off-line approach to ninhydrin derivatization of collected fractions. The fractions were collected and after addition of ninhydrin incubation of the mixture under the optimised temperature and time were done. Analysis of mixture was carried out by the simple UV-VIS spectrometer. The obtained limit of detection for this optimised procedure was promising 1.7 μM of sarcosine but this value is similar to physiologically occurring concentration of sarcosine in prostate cancer patients. Therefore we optimized the electrochemical method based on analysis of collected fractions by coulometric detection integrated as FIA-ED. Here we obtained the limits of detection of 110 nM of sarcosine, which is more than satisfactory for its determination in various matrixes such as urine or plasma of cancer patients.

Keywords: Metabolomics; Cancer marker; Ninhydrin; UV-VIS detection; Ionic separation; Electrochemical detection; Carbon porous electrode

1. INTRODUCTION

1.1 Role of sarcosine in cancer

Prostate cancer (CaP) continues to be one of the leading causes of cancer-related deaths among men. CaP more often affects elderly men and is thus a bigger health concern in developed countries. In 2004, there were 1 534 700 incident cases of cancer of all forms (except non-melanoma skin cancer) diagnosed in men in European Union. There were 298 600 new cases of lung cancer (19.4%), and CaP was the second most frequent incident form of cancer with 237 800 new cases estimated (15.5%). Mortality reflects the cancer prognosis, and in men there were 962 600 cancer deaths recorded, from which 85 200 deaths were estimated by CaP (8.9%) [1]. Histologically, more than 90% of tumours are adenocarcinomas, and the remaining 10% are small cell, squamous and transitional cell carcinomas [2]. Adenocarcinoma has a small metastasizing potential and if it does, it affects lymphatic system and bones. Prostate cancer tumours are usually asymptomatic and slowly growing; thus tumours are often detected by autopsy only [3]. However, a minor part of patients suffers from aggressive form of CaP, which forms metastases readily and affects men in younger age and leads to death from CaP. The early and accurate diagnosis is essential especially for those patients. The current diagnostic procedure of CaP is based on prostatic specific antigen (PSA) screening in blood, and also on digital rectal examination and biopsy [4,5]. PSA is a kallikrein-like serine protease produced almost exclusively by the epithelial cells of the prostate. Therefore PSA is organ specific, but not cancer-specific, and serum levels may be elevated in the presence of benign prostatic hypertrophy, prostatitis and other non-malignant conditions [6]. Thus searching for new potential CaP markers is highly desirable.

New molecules, which can be used as CaP markers, were recently reviewed [7,8]. Prostate cancer antigen 3 (PCA3), early prostate cancer antigen (EPCA) and gene fusions of TRMPSS2 and ETS gene family belong to the most perspective serum CaP markers. These markers are, however, blood ones. Urine is more easily available and contains many molecules connected to numerous malignancies. Hence urine proteomics and metabolomics are widely investigated. Approximately 583 metabolites in urine were analysed for prostate cancer [9]. Technological tools for proteome-wide research include mass spectrometry, two-dimensional electrophoresis, high performance liquid chromatography (HPLC), capillary electrophoresis, protein array technologies and bioinformatics [10]. Urinary biomarkers include non-protein nitrogen metabolites, carbohydrates, proteins and amino acids and those biomarkers are searched for various diseases as nephropathies, transplanted kidney failures, diabetic nephropathy, renal and prostate carcinoma [11]. The major advantages of urine-based assays are their non-invasive character and ability to monitor CaP coming from heterogeneous foci. Approximately 30 urine-detectable prostate-specific markers at DNA, RNA, protein and metabolite level have recently been reviewed. Alpha-methyl coenzyme A racemase (AMACR), prostate cancer antigen 1 (PCA1), PCA3, 8-hydroxydeoxyguanosine (8-OhdG), matrix metalloproteinase 9 (MMP9) and sarcosine belong to the most promising ones [12]. Recently, sarcosine also known as N-methylglycine, a natural ubiquitous non-protein amino acid has been investigated as a new putative CaP marker. Sarcosine, occurs as intermediate and by product in glycine synthesis and degradation and acts as an antagonist of glycine transporter I [13]. Published by Sreekumar et al., sarcosine was

identified as a differential metabolite that was greatly increased during prostate cancer progression to metastasis and could be detected in urine [9]. Sarcosine was warmly welcomed by professionals in the scientific field [14], however consequent studies bring contrary results. Measurements of sarcosine in blood serum by Struys et al. did not confirm Sreekumar's findings and conclusions and show that there are no major alterations in the extracellular concentrations of sarcosine, implying that the assessment of sarcosine in easily obtainable body fluids like urine and serum has limited potential in the diagnostic of prostate cancer [15]. Moreover, in another reported study authors measured sarcosine levels in urine samples from prostate cancer patients and concluded that sarcosine level in urine fails as a marker of prostate cancer detection and identification of aggressive tumours [16]. On the other hand, recently, Jiang et al. used liquid chromatography with tandem mass spectrometry for determination of sarcosine levels and other four putative CaP markers in urine samples and the results obtained show significant difference in sarcosine urine levels between CaP patients and healthy subjects [17]. Some authors attempted to detect sarcosine also in blood, but the results published are in well agreement that this way of determination of this marker cannot be used for prostate cancer diagnostics [15].Moreover, the influence of sarcosine on prostatic cancer cell cultures was also investigated. A significantly elevated level of sarcosine in prostatic cancer cell lines was found compared to benign cells. It has also been shown that addition of sarcosine to non-invasive benign prostate epithelial cells caused increasing of their invasivity and motility. This indicates that sarcosine has a direct role in changing of the cells phenotype [9].

1.2 Methods for sarcosine determination

To obtain more data about sarcosine significance for CaP diagnostics, robust analytical methods are needed. 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 [17]. Except above-mentioned gas chromatography-mass spectrometry [9,16] and liquid chromatography with tandem mass spectrometry [17], electrospray ionization and tandem differential mobility analysis-mass spectrometry [18] were used for the same purpose. Sarcosine was also semiquantitatively evaluated in urine and serum by thin layer chromatography (TLC) [19], which was the first mention about level of this aminoacid in urine and blood serum of healthy persons. Previously mentioned instruments are accurate but expensive and analysis can be laborious, but to routinely determine sarcosine in large sets of clinical samples simple and inexpensive methods are needed. Therefore, techniques based on chromatographic and/or electrophoretic separation coupled with non-mass spectrometric detector are tested. Reversed-phase HPLC with UV detector and TLC was used to separate and detect dansyl derivatives of aminoacids and amine containing phosphonic acids produced by microbial conversion of N-phosphonomethylglycine. For HPLC-UV, the detection limit was estimated as 19.3 ng and isomers L-alanine and sarcosine were well separated [20]. A glass electrophoresis microchip integrated a flow-type chemluminescence (CL) detection cell was developed and evaluated and tested for dansyl-sarcosine determination [21]. Laser-induced fluorescence (LIF) was applied to the detection of 9-

fluorenylmethyl chloroformate (FMOC-Cl) derivatized amino acids separated by capillary electrophoresis. A limit of detection of 0.5 nM was obtained for FMOC-alanine. Separation of FMOC-derivatized proline, hydroxyproline and sarcosine was achieved [22]. In addition, there were also suggested methods for detection of aminoacids, which can be also used for sarcosine. The chiral separation of enantiomeric forms of derivatized amino acids were achieved based on a metal-chelate chiral capillary electrophoretic method and a cyclodextrin mediated host-guest interaction approach in micellar electrokinetic chromatography (MEKC) mode with laser-induced fluorescence detection. Amino acids were analysed by complete hydrolysis and the hydrolysate was derivatized with either dansyl chloride for UV absorbance detection or fluorescein isothiocyanate for laser based fluorescence detection. This approach has been applied for the determination of enantiomeric forms of amino acids derived from novel peptide antitumour antibiotics, BMY-45012 and its analogues [23]. Construction of miniaturized sensors and biosensors has a great potential for simple determination of a wide spectrum of analytes in complex biological matrices. Electrochemical biosensors were also fabricated and tested for sarcosine determination [24-26].

Electrochemistry has numerous advantages including low operation cost, possibility to be miniaturized and sufficient sensitivity. There is numerous solid electrodes, which are useful for various types of analytes as glassy carbon [27,28]or various easily modified carbon paste electrodes [29,30]. It is not surprising that many approaches based on changing of structure of working electrode surface and material are being developed to enhance sensitivity [31-35]. Good robustness with sufficient sensitivity is provided by porous graphite working electrode, which is suitable to be used in electrochemical detectors coupled with HPLC [36-38]. The aim of this study was to suggest and optimize a simple and robust method for sarcosine determination in biological samples with minimal needs for sample pretreatment. For this purpose ion exchange liquid chromatography with post column derivatization by ninhydrin and VIS detection and flow injection analysis coupled with electrochemical detection were tested.

2. EXPERIMENTAL PART

2.1 Chemicals

Working solutions as buffers or standard solution of sarcosine were prepared daily by diluting the stock solutions. Standard of sarcosine and others were in ACS purity purchased from Sigma Aldrich (USA) unless noted otherwise. The chemicals for Aminoacid analyser were prepared according the manufacturer's instructions and were purchased from INGOS (Prague, Czech Republic). All solutions were prepared in deionised water obtained using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.2 Ion-exchange liquid chromatography

For determination of sarcosine an ion-exchange liquid chromatography (Model AAA-400) with post column derivatization by ninhydrin and absorbance detector in visible light range (Vis) was used (Fig. 1). Glass column with inner diameter of 3.7 mm and 350 mm length was filed manually with strong cation exchanger in sodium cycle LG ANB with approximately 12 μm particles and 8% porosity. Column was tempered within the range 35 - 95°C. Double channel Vis detector with inner cell of 5 μl volume was set to two wavelengths: 440 and 570 nm. Solution of ninhydrin (Ingos, Czech Republic) was prepared in 75% *v*/*v* methylcelosolve (Ingos, Czech Republic) and in 2 % *v*/*v* 4 M acetic buffer (pH 5.5). Tin chloride ($SnCl_2$) was used as a reducing agent. Prepared solution of ninhydrin was stored under inert atmosphere (N_2) in dark at 4 °C. Elution of sarcosine was done by buffer containing 10.0 g of citric acid, 5.6 g of sodium citrate, and 8.36 g of NaCl per litre of solution and pH was 3.0. Flow rate was 0.25 ml/min. Reactor temperature was 120 °C. Fractions were collected by automated fraction collector (Biorad, USA).

2.3 Spectrophotometric analysis

Spectrophotometric analysis was carried out using UV-Vis spectrophotometer Specord-210 (Jena Analytic, Germany). The instrument is equipped by the carrousel for eight cuvettes. Plastic disposable cuvettes with the optical pathlength of 1 cm and volume of 1.5 ml were used Microcuvette (Kartell, Italy). The carrousel was tempered to the required temperature (50-100 °C) using flow-through thermostat JULABO F12/ED (Labortechnik GmbH, Germany) with distilled water as a medium. The wavelength range was set to 450-800 nm.

2.4 Flow injection analysis with electrochemical detection

Flow injection analysis with electrochemical detection (FIA-ED) system consists of two chromatographic pumps Model 582 ESA (ESA Inc., Chelmsford, MA) (working range 0.001-9.999 ml/min) and CoulArray electrochemical detector (Model 5600A, ESA, USA). Detector consists of flow analytical chamber (Model 6210, ESA, USA). Chamber contains four analytical cells. One analytical cell contains two referent (hydrogen-palladium), and two counter electrodes and one porous graphite working electrode. Electrochemical detector is situated in control module, which is thermostated. Sample (5 μl) was injected by manual valve (Rheodyne, USA). Flow rate of mobile phase was 1 ml/min.

2.5 Mathematical data treatment and calculation of detection limits

Mathematical analysis of the data and their graphical interpretation was realized by software Matlab (version 7.11.). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). The detection limits (3 signal/noise, S/N) were calculated according to Long

and Winefordner [39], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

Simple analytes such as amino acids may play a very important role in cancer diagnostics and/or in monitoring of disease progression. Based on the previously published papers identifying urinary sarcosine as CaP marker, we tested whether methionine metabolites in urine and serum could serve as pre-surgical markers for aggressive disease [9,40]. Even though the applicability of sarcosine as a tumour marker can be considered [16,17,41-43], detailed investigation of its role in cancer development is required.

3.1 Sarcosine separation

It is well known that due to the amphiphilic properties of amino acids they can be separated by ion-exchange liquid chromatography (IELC) [44]. Under ideal condition, the isoelectric point is the main factor defining the theoretical retention of the amino acid. The scheme of the IELC instrument using cation exchanger in the sodium cycle as a stationary phase is shown in Fig. 1. Determined amino acids are injected into the system under buffer flow rate of 0.5 ml/min (pH of the used buffer was 2.7). Subsequently the elution takes place by the increasing gradient of pH and ionic strength (Fig. 2). Due to the fact that sarcosine does not contain an amino but the imino group in the structure, slightly acidic properties can be expected. Its theoretical pI is 6.2.

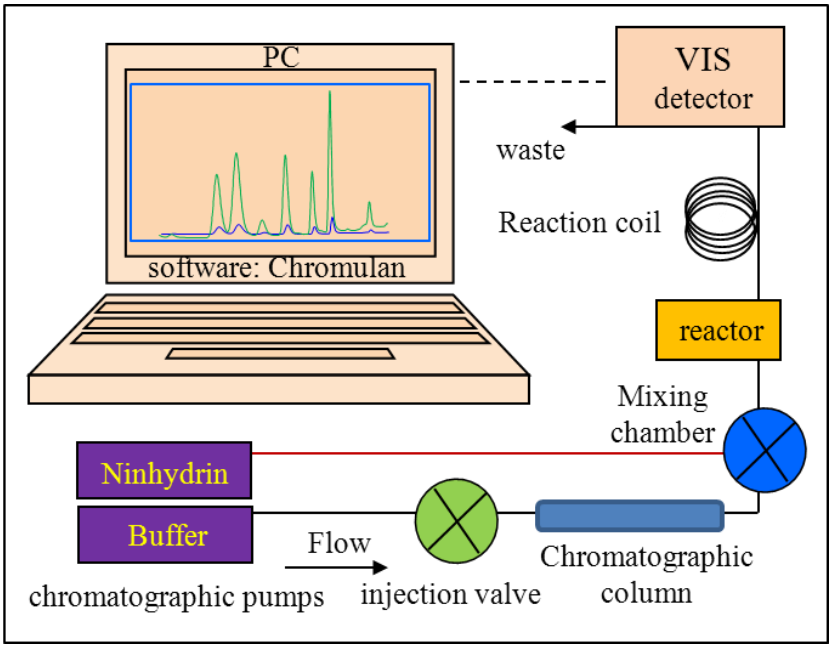

Figure 1. Scheme of IELC-Vis with post column derivatization with ninhydrin.

During the separation of mixture of 17 amino acids with addition of sarcosine its retention time was 32.93 minutes (Fig. 2). It clearly follows from the chromatogram that sarcosine elutes between serine (pI 5.76) and glutamic acid (pI 3.22). The sarcosine peak is symmetrical and baseline separated.

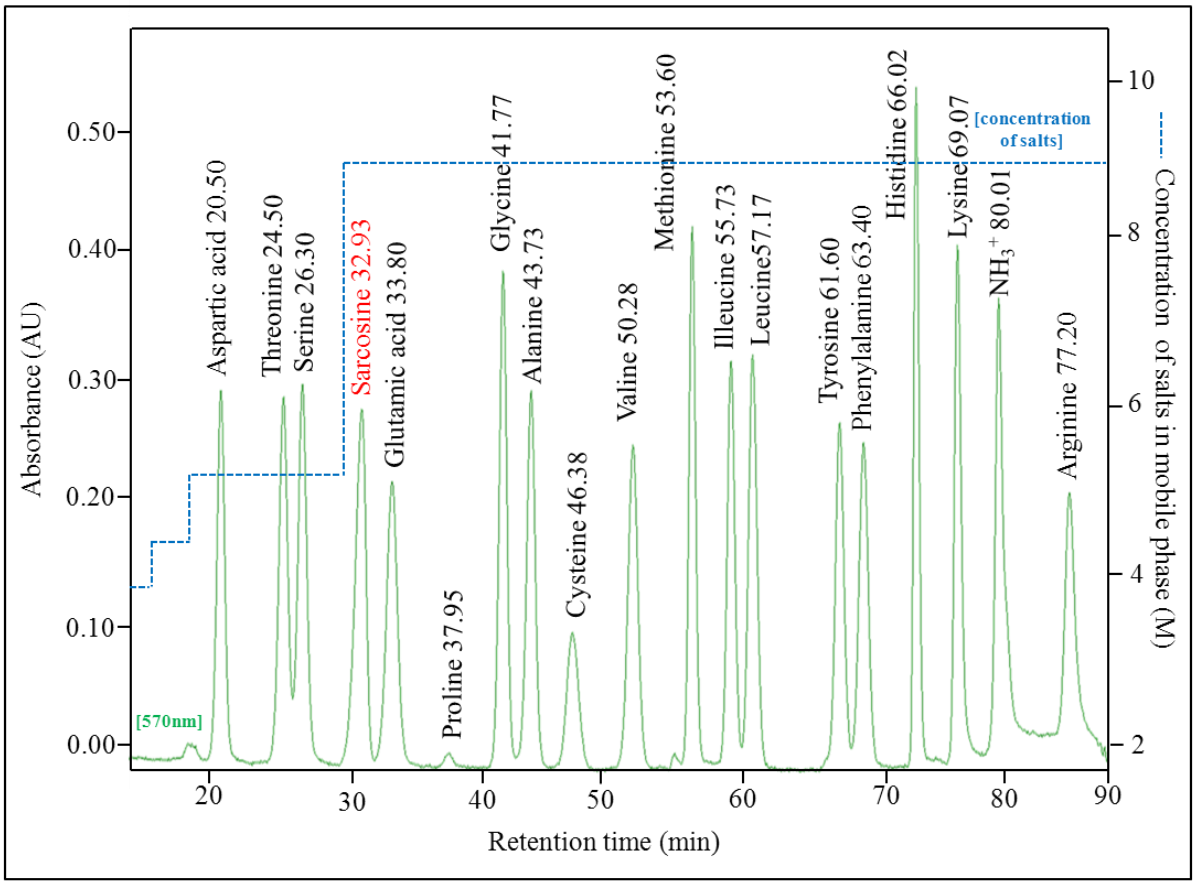

Figure 2. Chromatogram of standard mixture of 17 aminoacids (50 µM) with addition of sarcosine 100 µM. Aminoacids were eluted by the increasing gradient of buffers with increasing pH and concentration of salts: 0-7 min (pH 2.7, 4.06 M), 7-20 min (pH 3, 4.24 M), 20-31 min (pH 4.25, 5.30 M), 31-90 min (pH 9.7, 8.96 M), which is highlighted as blue dashed line. For other separation parameters see in chapter 2.3.

The analysis of real biological samples is usually complicated by the sample composition with high content of both organic and inorganic components. Therefore the influence of NaCl, uric acid, proline (Pro) and glycine (Gly) was studied. Sarcosine sample (500 µM) was adjusted to contain required concentrations of studied compounds (0.1 – 0.9 M) and analysed immediately. The influence of the NaCl and uric acid at concentrations present in real urine samples was investigated. Additionally the influence of Pro (pI 6.3) and Gly (pI 5.97) was tested due to their high similarity in chemical properties (pI). It was found that Gly and Pro elute from the column more than 5 minutes later (Gly RT: 41.77 min, Pro RT: 37.95 min) than sarcosine (RT: 32.93 min). It follows from the results that the increased NaCl concentration had significant impact on the retention time of sarcosine. The linear increase of the ionic strength led to the linear decrease of the retention time (Fig. 3A). Within the

concentration range between 0 and 800 mM NaCl the retention time of sarcosine decreased for 2 minutes. The addition of uric acid into the sarcosine sample led, on the other hand, to the increase in retention time. The highest retention time change (0.51 min) was observed for concentration of 100 mM of uric acid (Fig. 3B). Higher concentrations of uric acid caused less significant RT shift. The addition of proline also led to the increase of RT, however, the trend is nonlinear (Fig. 3C). The highest RT shift (2.24 min) was reached for 900 mM proline solution. The increase of sarcosine RT can be caused by the high concentration of nonpolar proline side chain. This caused the increase of the affinity of more polar molecules such as sarcosine to the stationary phase similarly as in the normal phase separation. The addition of glycine to the sarcosine sample led to the significant increase of the RT mainly in the concentrations 100 – 400 mM (up to 1 minute). Concentrations above 400 mM caused less significant RT increase (Fig. 3D). It was found that the influence of physiological concentrations of NaCl (120 – 260 mM) and uric acid (0.4 – 4.5 mM) is app. 15 %. Moreover it was found that addition of these compounds has no significant impact on the peak height (relative standard deviation (RSD) 1 %, n = 10).

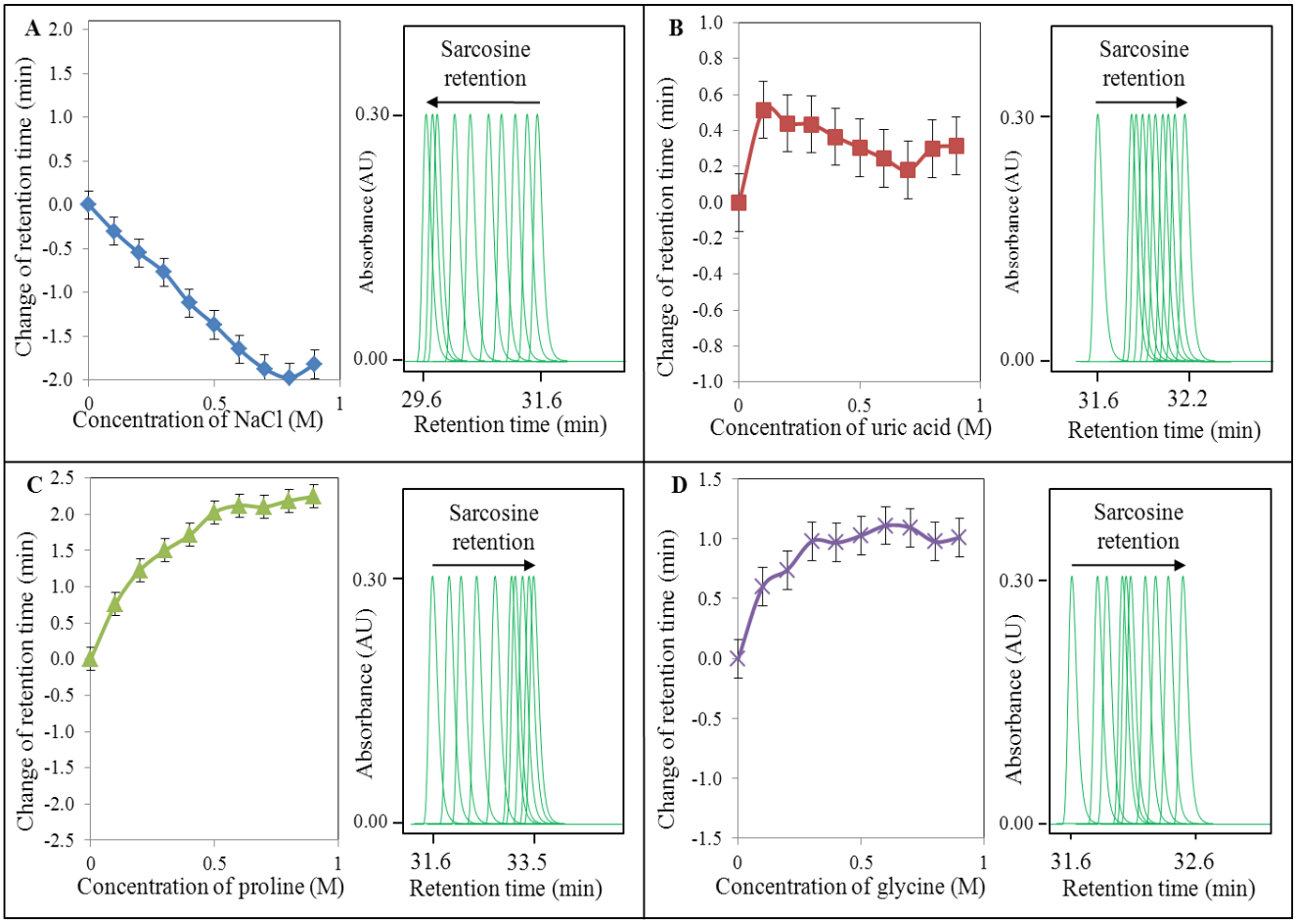

Figure 3. Change of retention time of peak of sarcosine in dependence on addition of various concentrations of (**A**) NaCl, (**B**) Uric acid, (**C**) Proline and (**D**) Glycine.

3.2 Detection of sarcosine

Amino acid detection eluting from the chromatographic column is carried out using post-column derivatization by ninhydrin and detected by photometric detector at 570 nm. The device is mixing the mobile phase coming out from the chromatographic column with the ninhydrin solution in the ratio 1:1 and this mixture is subsequently heated up to 120 °C in the reaction loop for the time required for the reaction (Fig. 1). Derivatization product is then transported to the photometric detector (Fig. 1). Using this instrument, the analysis of sarcosine within the concentration range from 100 to 5600 µM was performed reaching RSD of 3.4 % (n = 5). The overlay of the chromatographic signals is shown in Fig. 4A. The calibration curve determined as a dependence of the peak height on the concentration exhibited an excellent linearity (R^2 = 0.999, n = 5, RSD = 1.4%, Fig. 4B). Limit of detection for sarcosine of 70 µM was calculated (3 S/N). Obtained figures of merit are summarized in Tab. 1.

It clearly follows from the results obtained that used ion-exchange liquid chromatography is robust and reliable method, however, the sensitivity expressed as limit of detection obtained using post-column derivatization with photometric detection is insufficient for detection of sarcosine in real samples. In biological samples, 1-20 µM (correlated to creatinine) and 1.59±1.08 µM of sarcosine was determined in urine and in blood plasma, respectively [19]. For these reason, the possibility of photometric detection optimization was tested.

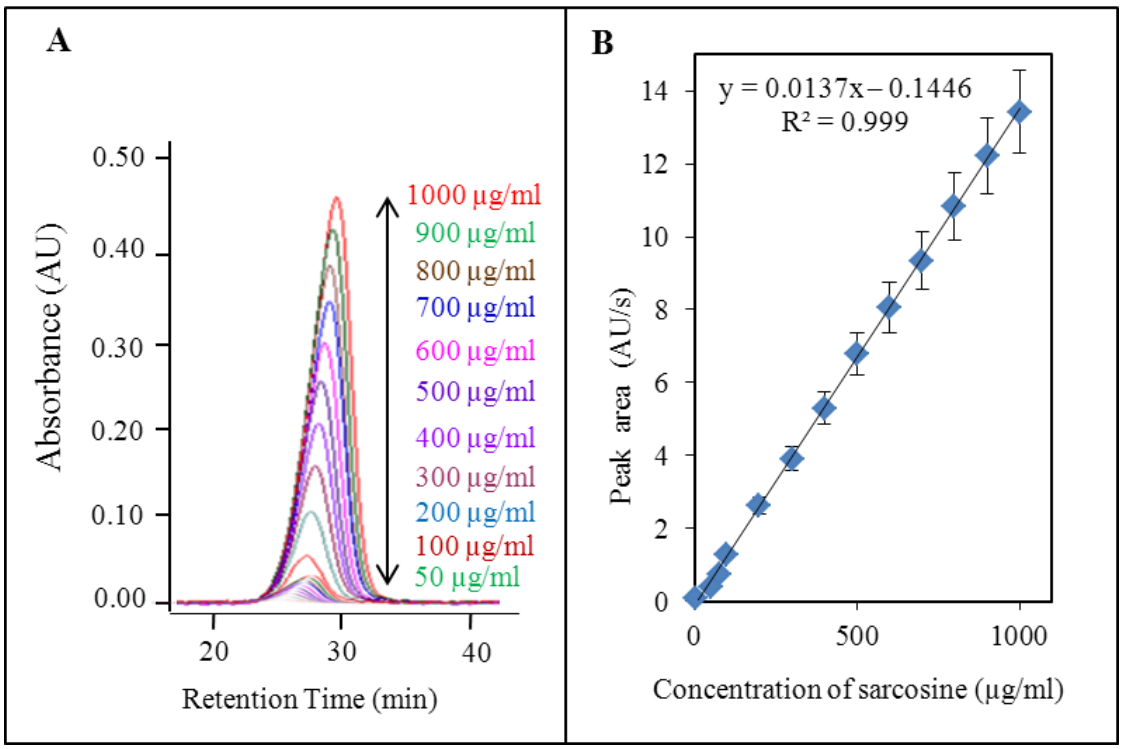

Figure 4. (**A**) Overlaying chromatograms from calibration dependence of sarcosine determined by AAA 400 and (**B**) calibration curve measured within the range from 5 to 1000 µg/ml under the experimental conditions mentioned in section 2.3.

3.3 Optimization of spectrophotometric detection

To optimize the sarcosine detection, several parameters were tested. It is known that the highest yield of the derivatization reaction of ninhydrin is reached for amino-group containing compounds. Therefore, it was primarily required to confirm that the absorption maximum of the derivatization product of sarcosine and ninhydrin is corresponding. In Fig. 5A, the reaction between ninhydrin and sarcosine is shown. Based the comparison of absorption spectra of the glycine-ninhydrin and sarcosine-ninhydrin reaction products within the range from 400 to 800 nm it can be concluded that the there is no shift in the absorption maximum. However, it was found that there is a 50% decrease of the intensity for sarcosine-ninhydrin product in comparison to glycine-ninhydrin product of the same concentration (100 µM of glycine/sarcosine +101 mM ninhydrin) (Fig. 5B). Due to the fact that the molar ratio of nitrogen is same for both, glycine as well as sarcosine, the decreased intensity can be explained only by the structural difference.

Table 1. Analytical parameters of AAA-400.

Substance	Regression equation	Linear dynamic range	Linear dynamic range	R2[1]	LOD[2]	LOD	LOD	LOQ[3]	LOQ	LOQ	RSD[4]
		(µM)	(µg/ml)		(µM)	(ug/ml)	(nmol per injection)	(µM)	(µg/ml)	(nmol per injection)	(%)
Sarcosine	y = 0.0098x + 1.7625	89 - 5611	8 - 500	0.999	70	6	7	232	21	23	3.4

1 … regression coefficients
2 … limits of detection of detector (3 S/N)
3 … limits of quantification of detector (10 S/N)
4 … relative standard deviations

The influence of the sarcosine/ninhydrin ratio was also studied. Ninhydrin solutions of various concentrations (5.6, 11.2, 16.9, 22.5, 28.1, 33.7, 39.3, 44.9, 50.5, 56.1, 61.7, 67.4, 73.0, 78.9, 84.2, 89.8 and 101 mM) were added to the sarcosine sample (100 µM) and the absorption spectra were measured within the range from 450 to 800 nm. The absorbance signal increased significantly (200%) with increased ninhydrin concentration (Fig. 5C). As noted previously, the absorption maximum is at 570 nm and therefore this wavelength was used to obtain the dependency of the absorbance signal on temperature and incubation time. Automated spectrophotometer was programmed to acquire the absorbance in 1 minute intervals and temperatures 50 °C, 60 °C, 70 °C, 80 °C, 90 °C, 100 °C were kept constant during the measurement using an external thermostatic unit. The highest absorbance signal was obtained at 90 °C after 10 minute incubation (Fig. 5D). Using these optimised conditions, a calibration curve was determined within the concentration range from 1 to 50 µM. The calibration curve exhibited a good linearity with correlation coefficient R^2 = 0.994, R.S.D. = 2.1% (n = 5) (Fig. 5E). Limit of detection was improved by the factor of 35 (figures of merit are summarized in Tab 2), however, this is still insufficient for the analysis of biological samples.

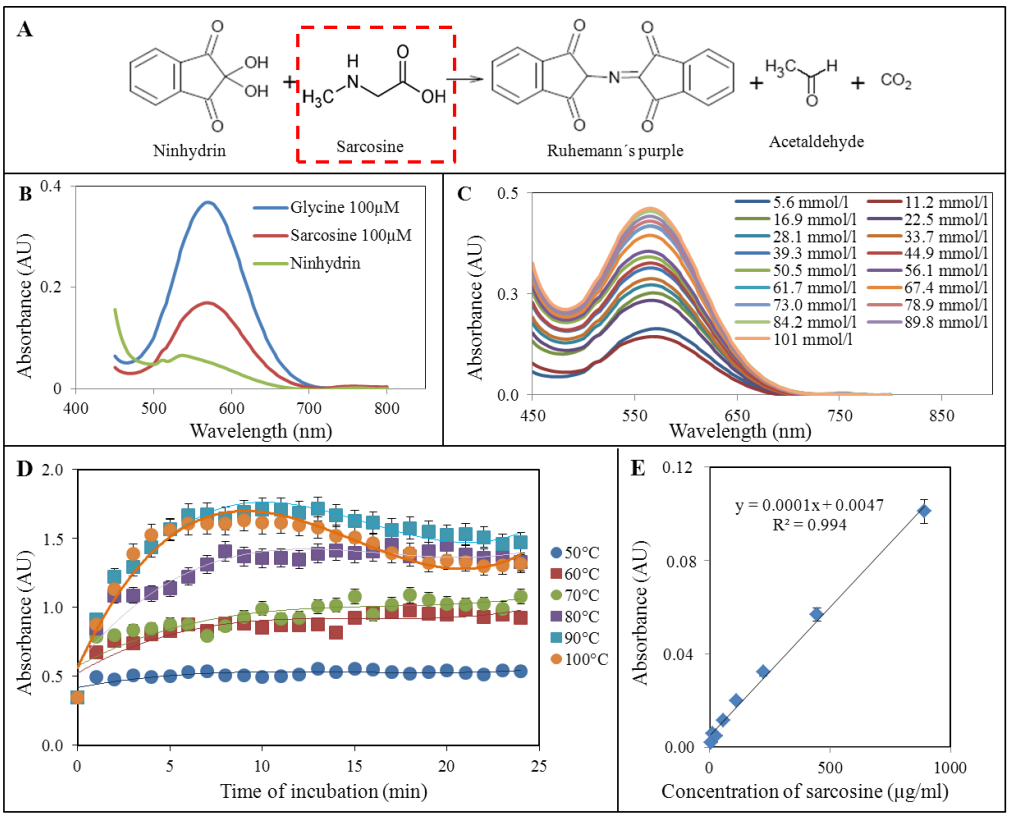

Figure 5. (**A**) Chemical reaction of ninhydrin with sarcosine, after heating 110 °C there is colour product. (**B**) Absorption spectrum (450-800 nm) of 100 µM glycine and 100 µM sarcosine with ninhydrin after heating 90 °C 10 min under 400 rpm. (**C**) Influence of concentration of ninhydrin (5.6-101 µM) on sarcosine 100 µM. (**D**) Change of absorption of complex of sarcosine (100 µM) with ninhydrin (56.1 mM) under various temperatures 50, 60, 70, 80, 90 and 100 °C in time manner. (**E**) Calibration curve under the optimized conditions (570 nm, 10 min incubation and temperature of heating 90 °C) within the range from 5to 891 µg/ml).

Table 2. Analytical parameters of optimized UV-Vis detection.

Substance	Regression equation	Linear dynamic range (µM)	Linear dynamic range (µg/ml)	R2[1]	LOD[2] (µM)	LOD (ng/ml)	LOD (fmol per volume)	LOQ[3] (µM)	LOQ (ng/ml)	LOQ (fmol per volume)	RSD[4] (%)
Sarcosine	y = 0.2471x + 1.9937	0.56 - 10	0.05 - 891	0.986	1.7	150	168	6	500	561	6.2

1 … regression coefficients
2 … limits of detection of detector (3 S/N)
3 … limits of quantification of detector (10 S/N)
4 … relative standard deviations

Due to the fact that the instrument of ion exchange chromatography is a compact automated appliance unsuitable for changing of the derivatization parameters, the separated analyte fractions were collected immediately after exiting the separation column (Fig. 6) and spectrophotometricaly analysed off-line. By abovementioned approach we were able to determine of sarcosine with maximal 10% error of determination.

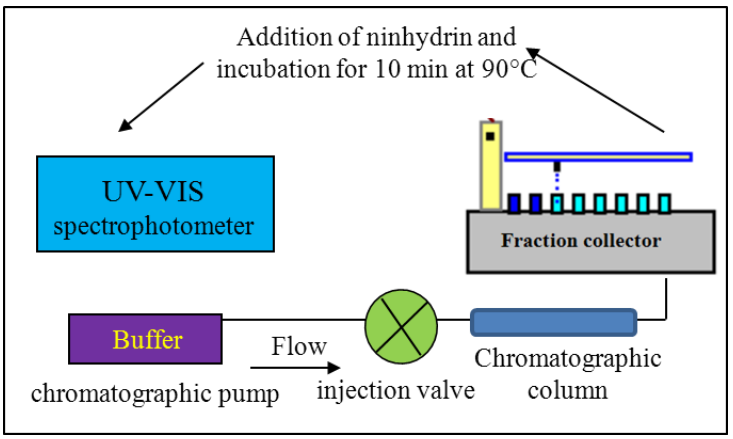

Figure 6. Scheme of ion exchange chromatography connected to fraction collector. Fractions were incubated according the optimized conditions mentioned in caption 3.2. and then analysed using UV-VIS spectrometer.

3.4 Optimization of electrochemical detection

Previously described procedure enabled to decrease the LOD of sarcosine from 70 µM to 1.7 µM, however even this method would be applicable to the real samples only after employing a powerful sample pre-treatment method. Such procedure would however increase the analysis time as well as costs. Therefore the electrochemical detection was tested as an alternative detection technique. First electrochemical experiments were presented in the papers by Cukrowski et al. [45,46].

Flow injection analysis setup with electrochemical detector CoulArray, containing analytical cells with applied potentials in the range from -1000 to +1000 mV was used. First, two hydrodynamic voltammograms (HDV) [47] of sarcosine sample (100 µM) were obtained for the whole potential range (-1000 – +1000 mV, 100 mV steps) using the Britton-Robinson buffer (buffering range 1.8-9) with concentration 100 mM and pH 2 and 8. The highest detector response was obtained for potential range +700 - +1000 mV at pH 8. Based on the obtained data it can be concluded that for the coulometric detection of sarcosine basic pH and high oxidation potential are preferable (Fig. 7). To investigate the influence conditions in details, three buffers as Britton-Robinson buffer (pH 7-9), borate buffer (pH 7-9) and phosphate buffer (pH 7-9) were tested. Using all buffers at pH values 7, 7.5, 8, 8.5, and 9, HDV were measured within the potential range from +400 to +1000 mV. Measured HDVs are shown in Fig. 8. The highest response using Britton-Robinson buffer was observed at pH 8.5 (Fig. 8A) where the peak height of 3 µA was reached. Using borate and phosphate buffer pH 9 the maximal reached peak heights were 1.8 and 6 µA, respectively (Fig. 8B and 8C, respectively). The maximum current values were obtained at +1000 mV for all three buffers. Finally, the flow rate was optimized as shown in Fig. 8D.

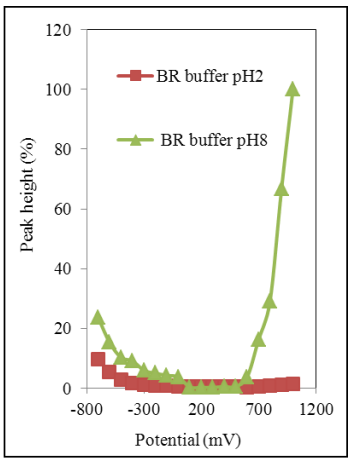

Figure 7. Influence of pH 2 and 8 of Britton Robinson buffer on HDV of sarcosine (100 µg/ml), flow rate 1 ml/min, working electrode potential from -700 to +1100 mV.

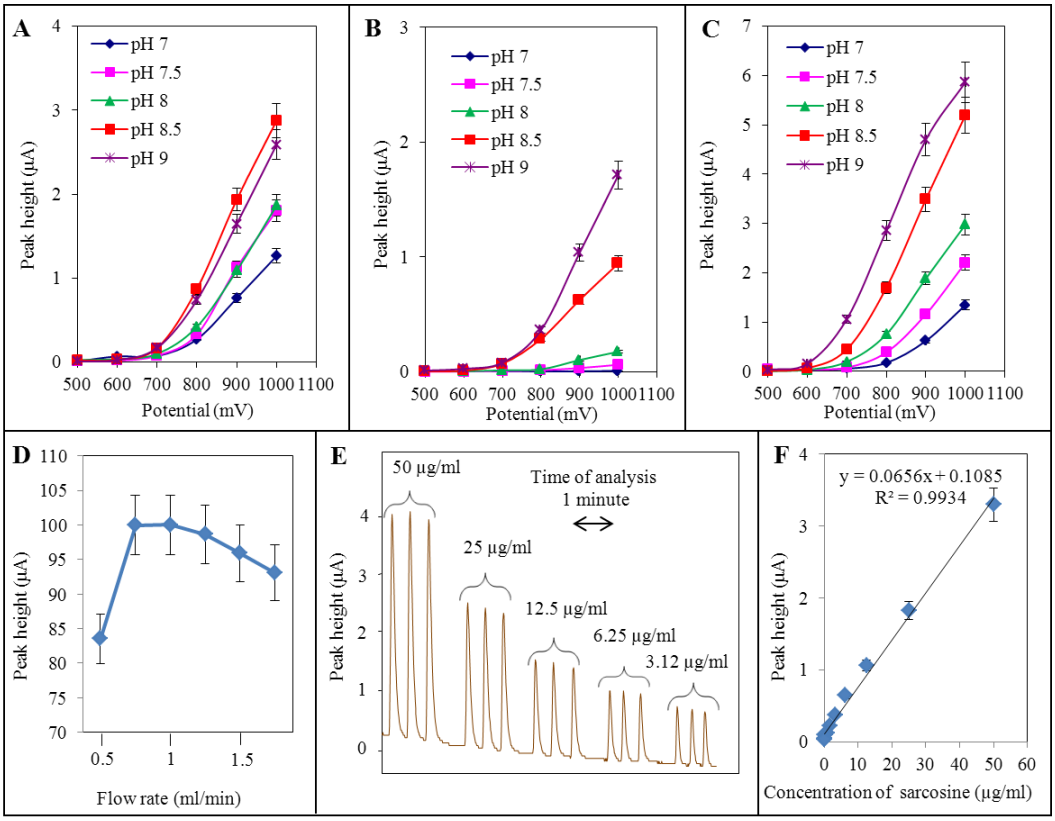

Figure 8. Influence of (**A**) Britton-Robinson buffer, (**B**) borate buffer and (**C**) phosphate buffer on HDV of sarcosine (100 µg/ml). (**D**) Influence of flow rate on peak height. (**E**) The typical FIA-ED record of calibration dependence of sarcosine as triplicate of each concentration under the best detection options (phosphate buffer pH 9 and flow rate 1 ml/min). (F) Calibration of sarcosine measured within the range from 0.024 to 50 µg/ml.

The highest signal of sarcosine was obtained using the flow rate of 0.75 ml/min. In addition for optimal detection we had to test the influence of concentration of phosphate buffer on electrochemical response. We found 100 mM concentration of the phosphate buffer as the best for sarcosine detection.

Under these optimal conditions, the calibration curve was measured. The peaks of sarcosine at different concentrations are shown in Fig. 8E and the calibration curve in the concentration range between 0.1 and 560 µM is shown in Fig. 8F. The correlation coefficient of 0.9934 was obtained. Calculated limit of detection for sarcosine using the electrochemical method was 110 nM (3 S/N). Figures of merit including limits of quantification and standard deviations are summarized in Tab. 3. Limits of detection obtained by flow injection analysis with electrochemical detection are sufficient for analysis of sarcosine in real biological samples. The main advantage of this method is that no derivatization of sarcosine is required. Moreover its application as an off-line detection techniques following ion-exchange liquid chromatographic separation minimizes the sample pre-treatment requirements.

Table 3. Analytical parameters of optimized FIA-ED.

Substance	Regression equation	Linear dynamic range	Linear dynamic range	R2[1]	LOD[2]	LOD	LOD	LOQ[3]	LOQ	LOQ	RSD[4]
		(µM)	(µg/ml)		(nM)	(ng/ml)	(fmol per injection)	(nM)	(µg/ml)	(fmol per injection)	(%)
Sarcosine	y = 0.2956x + 1.7625	0.07 - 561	0.024 - 50	0.997	110	10	1	380	34	4	2.5

1 … regression coefficients
2 … limits of detection of detector (3 S/N)
3 … limits of quantification of detector (10 S/N)
4 … relative standard deviations

4. CONCLUSIONS

Early on in the search for tumour markers, the hope was that someday all cancers could be detected early with a blood test. A simple blood test that could find cancers in their earliest stages could prevent the deaths of millions of people. But very few tumour markers are useful for finding cancer at a very early stage and there are several reasons for this including methodology one, because we need simple and accurate tests for screening of population. There have been shown various applications of metabolomics to cancer and highlight clinical associations and potential challenges [48-52]. It was proved that flow-injection analysis with electrochemical detection is a suitable method for analyses of sarcosine in low concentrations and can be used as a detection method for analysis of real samples as urine.

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