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Influence of a storage protocol on sarcosine levels in the human urinary specimens

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Urinary metabolomic profiles have recently drawn a lot of attention owing to a debate regarding their possible role as potential clinical markers for prostate cancer. As was shown, amino acid metabolism in cancer patients differs from that in healthy people, and it can be thus utilized in early diagnostics. In this study, we monitored the behavior of potential non-invasive biomarker for prostate carcinoma, sarcosine, involved in the folate metabolism and DNA methylation processes, linked to the progression of prostate carcinoma. To obtain the maximum amount of information, the biochemical parameters (total protein, creatinine, ions, conductivity) were determined using spectrophotometry and electrochemistry. All results were subjected to statistical processing for revealing different correlations between the studied parameters. These metabolites were observed in the urine obtained from healthy subjects and influence of storage conditions (freezing and thawing) on the concentration of addition of sarcosine was monitored.

Keywords: Biomarker; Ion-exchange chromatography; Electrochemistry; Prostate cancer; Stability

1. Introduction

Urine is a very popular biofluid for metabolomic investigations due to its non-invasive collection, the complex metabolic nature and the ability to collect multiple specimens over a period of time [1]. Quantification of free amino acids, present in the biological fluids is an important tool in biomedical research and the diagnosis of various diseases state. As urinary amino acid content circadian varies during 24 h, precise methodic approach must be used for quantitative analysis of amino acids [2-4]. It is essential to avoid fecal and bacterial contamination, which may increased or decrease the concentration many amino acids in urine [5]. Even the smallest changes in the sample treatment, storage or analysis can confound the desired effects and lead to noise in corresponding data that adds to the underlying biological variations and complicates data interpretation. Many different methods of detection have been used for metabolomic studies or urinary specimens, such as high performance liquid chromatography (HPLC) [6], ion exchange chromatography (IEC) [7, 8], high-resolution nuclear magnetic resonance (HR-NMR) [9], mass spectrometry [10] or fluorimetry [11]. Practically all studies on biological systems involve freezing and storage steps, and freezing conditions may vary even within a study usually

because of local conditions, like the accessibility or inaccessibility of a freezer in close proximity to the site where the samples are generated and the corresponding use of dry ice for freezing or intermediate storage at a step [12]. In epidemiological studies in patients with diabetes, urine samples are often stored frozen prior to assessment of urinary albumin concentration (UAC). However, prolonged frozen storage may result in a falsely low urinary albumin (UA) [12, 13]. Detection of albumin by immunonephelometry appears to be significantly less influenced by freezing than detection by HPLC. Storage at -80 ºC appears to prevent loss when using immunonephelometry, whereas HPLC still shows considerable loss even when urine is frozen at -80 ºC. It was demonstrated that for reliable measurement of urine albumin, fresh samples should be used [14]. Urinary excretion of the pyridinium crosslinks pyridinoline (Pyr) and deoxypyridinoline (Dpyr) can be used as biochemical marker of bone resorption and predict stability of urine stored for 10-20 years at -20 ºC in the dark. Also, freezing and thawing as many as 10 times had no effect on the concentrations of the crosslinks [15]. The study by Garde et al. implies that samples for analysis of creatinine should be kept at a temperature of -20 ºC or lower and frozen and thawed only once [16]. Hence, it is obvious that various urinary metabolites require different storage and processing to provide the ideal unbiased data.

In our study, we focused on potential non-invasive biomarker of prostate cancer - sarcosine. Even though the linkage of sarcosine with PCa development and its potential in a diagnosis of early stages of tumors was described [17, 18], its usage as a marker is still under discussion [7, 19, 20]. These contradictory findings may be due to the cumulative effects of samples selection, urine storage, the normalization way or analytical methods. We decided to use IEC with post-column ninhydrin derivatization and Vis detection, as it minimizes the sample pre-cleaning requirement [6, 8]. The goal of this study was to determine, whether the storage conditions by means of freezing and thawing interfere with detemination sarcosine and other biochemical parameters (K^+, Na^+, Cl^-, creatinine and total proteins).

2. Material and methods

Chemicals and pH measurement

Buffers and standard solutions were prepared daily by dilution of the stock solutions. Standards and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) meeting the specification of American Chemical Society (ACS), unless noted otherwise. Methylcellosolve and tin chloride were purchased from Ingos (Prague, Czech Republic).

Volunteers, sample collection, freezing and storage

Morning urinary specimens were collected from four healthy volunteers (male) and were aliquoted into falcon vials and immediately analyzed. Aliquots were also stored at +25; +4, -20 and -80 ºC under sterile conditions in dark and analyzed after 24; 48 and 72 hours.

Measurements of pH and conductivity

For conductivity and pH measurements, the 913 pH meter (Metrohm, Herissau, Switzerland) was employed.

Determination of Na^+, K^+, Cl^- ions

Na^+, K^+, Cl^- ions were determined electrochemically, using ion-selective electrodes , the AgCl electrode was employed as a reference one (Metrohm).

Sample preparation for determination of sarcosine

Urinary specimens with spiked sarcosine (250 µg/mL) were pipetted into a 96-well evaporation plate (Deepwell plate 96, Eppendorf AG, Hamburg, Germany) and evaporated by the nitrogen blow-down evaporator Ultravap 96 with spiral needles (Porvair Sciences Ltd., Leatherhead, UK). After that, the sample was diluted with 300 µL of dilution buffer and was subsequently used for analysis by IEC.

Ion-exchange liquid chromatography

For determination of sarcosine, an ion-exchange chromatography (IEC) (Model AAA-400; Ingos,Czech Republic) with post column

derivatization by ninhydrin and an absorbance detector in visible light range (Vis) was used. A glass column with an inner diameter of 3.7 mm and length of 350 mm was filled manually with strong cation exchanger (Ostion LG ANB; Ingos,Czech Republic) in sodium cycle with ~12 µm particles and 8% porosity. The column was thermostated at 60 ˚C. Double channel Vis detector with an inner cell of 5 µL was set to two wavelengths: 440 and 570 nm. Prepared solution of ninhydrin was stored under nitrogen atmosphere in the dark at 4 ˚C. Elution of sarcosine was carried out by buffer containing 10.0 g of citric acid, 5.6 g of sodium citrate, and 8.4 g of natrium chloride per liter of solution (pH 3.0). The flow rate was 0.25 mL/min. The reactor temperature was set to 120 ˚C.

Determination of total proteins and creatinine

Total proteins and creatinine were quantified using pyrogallol red protein assay (Skalab, Svitavy, Czech Republic) and creatinine assay kit (Sigma-Aldrich), respectively, according to manufacturers intstructions. Analyses were performed on automated spectrophotometer BS-400 (Mindray, Shenzhen, China).

Square wave voltammetry

Square wave voltammetry (SWV) was used for determination of urinary antioxidant activity. Electrode system was designed and fabricated as a disposable planar three-electrode sensor in LabSensNano laboratories (University of Technology, Brno, Czech Republic). Working electrode was designed to be as large as possible (in this case geometrically comparable with the diameter of the 3 mm^2 glassy carbon electrode with working area of 7.1 mm^2), reference electrode 1.3 mm^2 and auxiliary electrode 6.2 mm^2. The assay of low molecular weight antioxidants using square wave voltammetry was done in a slight modification of previously optimized protocol [21]. Changes in the electrochemical signals were recorded with a PGSTAT 101 potentiostat (Metrohm, Herisau, Switzerland) and the results were evaluated by the NOVA 1.8 software (Metrohm). The voltage was applied within the range from 0 to 1.1 V with potential step as well as voltage amplitude 5 mV. Frequency of the waves was 10 Hz. In a total, 20 µL of the urine samples was spread over the electrodes and voltammetry was run immediately.

Cyclic voltametry

For determination of urinary antioxidant activity by cyclic voltammetry (CV), the same electrochemical apparatus as for SWV was utilized. The parameters of the measurement were as follows: start potential of 0 V, upper vertex potential 1 V, lower vertex potential -1 V, stop potential 0 V, step potential 5 mV and scan rate 0.2 V/s were used.

Descriptive Statistics

Mathematical analysis of the data and their graphical interpretation were realized by Microsoft Excel®, Microsoft Word® and Microsoft PowerPoint®. Results are expressed as mean ± standard deviation (S. D.) unless noted otherwise. The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [22], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. Results and discussion

3.1 IEC with Vis detection

Determination of sarcosine in the urinary specimens is not an easy task, regarding the demands on sensitivity and accuracy of measurements and the influences of the matrix interferents. Sarcosine (N-methylglycine) is not present in proteins and therefore the samples do not require acidic hydrolysis prior its analyses. Due to this sample preparation procedure has been simplified on simple evaporation of 250 µL of urine with subsequent resuspension in the same volume of dilution buffer. The advantage of the procedure is that is not time-consuming, eliminates potential errors occuring during sample preparation and cost-effectivity. By the analysis of sarcosine it is crucial to distinguish it from mass identical alanine (89.0932 Da) [23]. Widely used methods - GC/MS [24] LC/MS [25] attempt to charge high sensitivity, but there are cost complicated pre-treatments of samples with a need to involve an experienced

and well-trained staff. Overlay of chromatograms and calibration curve of sarcosine are shown in (Fig. 1A, B).

Overlay of chromatograms representing the urine amino acid profiles with addition of sarcosine (250 µg/mL) into urine samples of four volunteers, freezing at -80 ˚C during 24; 48

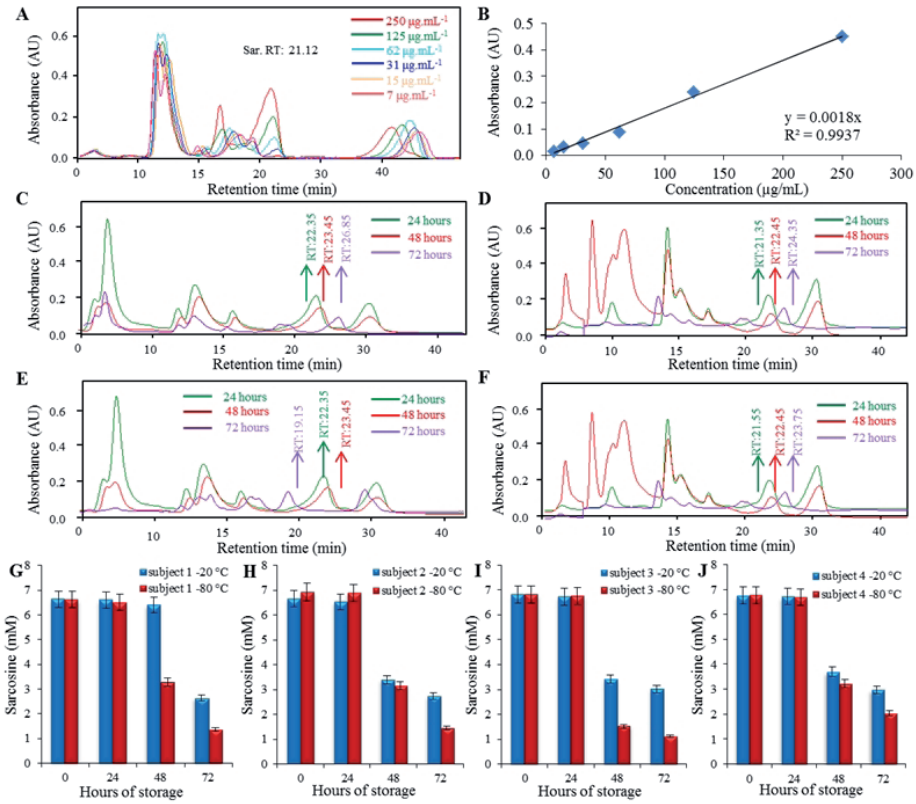

Figure 1: (A) Chromatograms of amino acids obtained from the measurements of urine samples of volunteers with addition of sarcosine (7 - 250 µg/mL). The amino acids are eluted with a gradient with increasing pH 0-5 min (pH 2.7), 10-28 min (pH 3). (B) Calibration curve and overlay of chromatograms of sarcosine within the range from – 7 µg/mL to 250 µg/mL). (C,D,E,F) Chromatograms of amino acids obtained from the measurements of urinary specimens of 4 volunteers with addition of sarcosine (250 µg/mL) and freezing at -80 ˚C. (G-H,I,J) Influence of temperature (-20 ˚C = blue and -80 ˚C = red) and time of storage (0; 24; 48 and 72 hours) on sarcosine concentration in 4 volunteers. Comparison of different storage conditions -20 ˚C and -80 ˚C and their influence on the concentration of amino acid sarcosine in the samples of urine of tested volunteers.

Interfering compounds commonly occurring in urine had no significant effect to sarcosine concentration and retention time (Fig. 1A), the calibration curve was linear within the range from – 7 µg/mL to 250 µg/mL (Fig. 1B) determined as dependence of the peak area on the sarcosine concentration and exhibited an excellent linearity (R^2 = 0.9937). For analytical parameters see Table 1.

and 72 hours and thawing 3 times are shown in (Fig. 1C-F). Levels of sarcosine changed when compared to the control sample (spiked fresh urine). Results from sarcosine analysis exhibit decrease in concentration of added sarcosine in volunteer´s urine after 72 hours and changes in retention time. The significant increase in RT was observed mainly in the measurents after 48 and 72 hours (up to 2 minute). After compari-

son of different storage conditions -20 ˚C and -80 ˚C, changes in the concentration of sarcosine were found. Determined concentration of sarcosine in all samples decreased with prolonged time of storage. After 72 hours of storage at -20 ˚C the determined sarcosine concentration was less than 50% of original concentration. Storage temperature of -80 ˚C had more deleterious effect on sarcosine determination; after 48 hours of storage at -80 ˚C the determined sarcosine concentration was less than 45% with decrease up to 37% of original concentration after 72 hours of storage (Fig. 1G-J).

Compound	Retention time	Linear regression	Linear dynamic range	$R^{1,2}$	LOD	LOQ	LOD (nmol)[3]	LOQ (nmol)[4]	RSD[5]
	(min)	Equation	(µg/mL)	R^2	(µg/mL)	(µg/mL)	per injection	per injection	(%)
Sarcosine	21.12	y = 0.0018x	7 - 250	0.9855	7	22	0.03	0.1	3.2

Table 1: Analytical parameters of IEC detection of sarcosine in urinary samples where [1] stands for regression coefficient, [2] for limit of detection (3 *S/N*), [3] for limit of quantification (10 S/N), [4] for injection of 5 µL volume, [5] for relative standard deviation.

In the next step we studied the effect of above--zero temperatures on sarcosine determination in urine matrix. Overlay of chromatograms representing the urine amino acid profiles with addition of sarcosine (250 µg/mL) into urinary samples of healthy volunteers, stored at +25 ˚C during 24; 48 and 72 h are shown in (Fig. 2A-D). The minimal increase in RT was observed mainly in the measurents after 48 and 72 h (up to 1 minute). Determined concentration of sarcosine in all samples during first 48 h of storage both at +25 and +4 ˚C varied in range of measurement error (5%). After 72 hours up to 40% increase of determined sarcosine concentration was observed (Fig. 2E–H). This indicates that urinary specimens provided by healthy and motivated volunteers was possible to use for sarcosine determination even after 2-day storage in above-zero temperatures in dark and under sterile conditions. However, in urine, as well as in other biological fluids, decaying cellular elements can affect subsequent processing, and thus result in the false positive or negative results [5]. During storage, it is necessary to keep the material well enclosed and to prevent the microbial contamination, the influence of light and diffusion of gases and of course metabolism urine elements. On the other hand, freezing that is frequently used for slowering of samples decay may affect the determination of some analytes much more than appropriate storage without state change.

3.2 Biochemical parameters of the urine samples.

Using various spectrophotometric methods, the concentrations of K^+ , Na^+ ,Cl^- ions , total proteins, conductivity, creatinine and pH were measured in the urine with the addition of sarcosine after its storage at -20 ˚C (**Table 2**), -80 ˚C (**Table 3**), +4 ˚C (**Table 4**) and +25 ˚C (**Table 5**). Higher differences after urine storage and 3 times freezing and thawing of urine samples from healthy subjects were observed for levels of conductivity, creatinine and total levels of proteins. Minimal differences were observed also in the pH of the urine (mean 6.49), and concentration of ions. A little acidic pH corresponds with higher levels of proteins; nevertheless, this value is still within the physiological range [26].

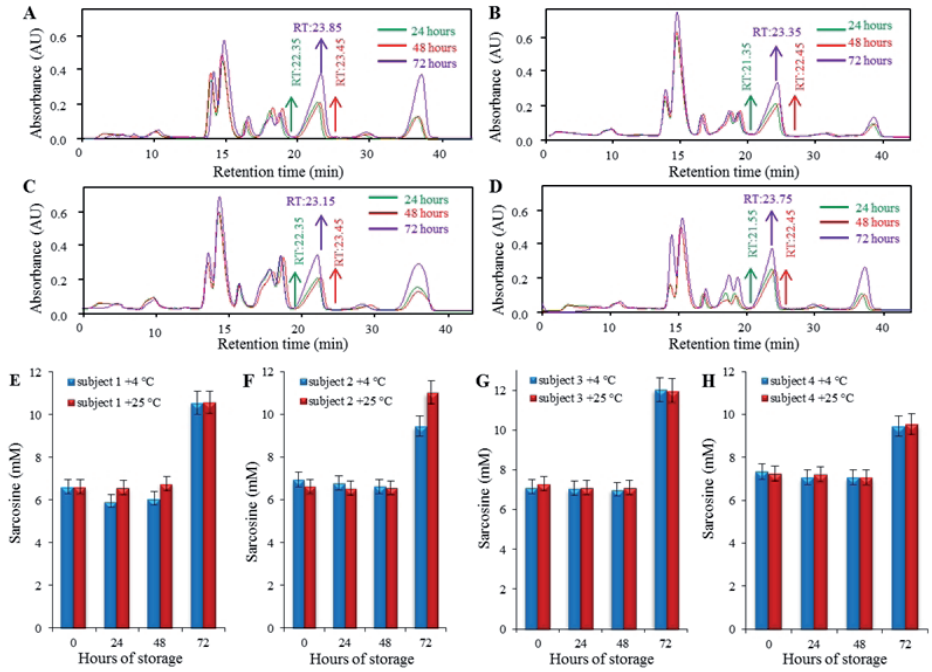

Figure 2: (A-D) Chromatograms of amino acids obtained from the measurements of urinary samples of volunteers with addition of sarcosine (250 µg/mL) which were stored at +25 ˚C. The amino acids are eluted with a gradient with increasing pH 0-5 min (pH 2.7), 10 - 28 min (pH 3). (E - H) Influence of temperature (+4 ˚C = blue and +25 ˚C = red) and time of storage (0; 24; 48 and 72 hours) on sarcosine concentration in four volunteers. Comparison of different storage conditions +4 ˚C and +25 ˚C and their influence on the concentration of amino acid sarcosine in the samples of urine of volunteers.

	-20 ˚C (48; 72 h) Conductivity	K^+ (mmol/mmol crea)	Na^+ (mmol/mmol crea)	p*H*	Cl^- (mmol/mmol crea)	Creatinine (mmol/L)	Pyrogal (g/L)
Subject1	29±0.0	25.4±0.05	45.6±0.05	5.95	407.7±0.05	8±0.05	11.9±0.65
Subject1	29.9±0.05	25.45±0.05	45.62±0.05	5.8	407.8±0.05	7.9±0.1	11±1.19
Subject2	28.81±0.0	96.7±0.05	112.5±0.05	6.3	393.3±0.05	6.6±0.0	9.5±2.9
Subject2	28.83±0.0	96.75±0.05	112.3±0.05	6.1	393.4±0.05	6.4±0.05	6±0.41
Subject3	15.1±0.0	62.8±0.05	108±0.05	6.3	339.4±0.05	12±0.0	34.72±0.11
Subject3	15.3±0.05	62.9±0.05	108.5±0.05	6.1	339.5±0.05	11.9±0.05	27±1.72
Subject4	8.5±0.0	30.5±0.05	102±0.05	6.95	506.9±0.05	3.6±0.0	10.9±0.55
Subject4	8.7±0.05	30.6±0.05	102.1±0.05	6.92	506.10±0.05	2.9±0.05	10±0.5

Table 2. Spectrophotometric and ISE determination of the concentrations of K^+, Na^+, Cl^- ions, total proteins, conductivity, creatinine and pH in urine samples from healthy subjects 48 and 72 h after sample storage at -20 ˚C.

-80 °C (48; 72 h)	Conductivity	K^+ (mmol/mmol crea)	Na^+ (mmol/mmol crea)	p*H*	Cl^- (mmol/mmol crea)	Creatinine (mmol/L)	Pyrogal (g/L)
Subject1	29±0.0	25.4±0.05	45.6±0.05	5.92	407.3±0.05	5.8±0.10	4.16±1.18
Subject1	29.9±0.05	25.45±0.05	45.63±0.05	5.86	407.8±0.05	4.6±0.057	2.22±0.85
Subject2	29.1±0.0	96.75±0.06	112.6±0.05	6.32	393.5±0.05	8.3±0.17	27.4±0.15
Subject2	29.32±0.05	96.8±0.05	112.8±0.05	6.12	393.7±0.05	7.3±0.17	24.03±2.19
Subject3	15.1±0.0	62.8±0.06	108±0.05	6.3	339.4±0.05	8.3±0.15	65.6±1.40
Subject3	15.3±0.05	62.9±0.05	108±0.05	6.1	339.5±0.05	7.3±0.15	54.46±1.40
Subject4	8.5±0.0	30.5±0.06	102±0.05	6.95	506.9±0.05	7.5±0.05	11.5±05
Subject4	8.7±0.05	30.6±0.05	102.5±0.05	6.92	506.10±0.05	6.5±0.05	9.8±0.63

Table 3: Spectrophotometric and ISE determination of the concentrations of K^+, Na^+, Cl^- ions, total proteins, conductivity, creatinine and pH in urine samples from healthy subjects 48 and 72 h after sample storage at -80 °C.

+4 °C (48; 72 h)	Conductivity	K^+ (mmol/mmol crea)	Na^+ (mmol/mmol crea)	p*H*	Cl^- (mmol/mmol crea)	Creatinine (mmol/L)	Pyrogal (g/L)
Subject1	28.8±0.0	25.4±0.05	28.6±0.05	5.6	155.7±0.05	3.58±0.05	0.3±0.65
Subject1	28.9±0.05	25.45±0.05	18.62±0.05	5.4	174.8±0.05	3.58±0.1	0.01±1.19
Subject2	28.9±0.0	211.5±0.05	125.5±0.05	5.4	308.3±0.05	4.67±0.0	9.84±2.9
Subject2	28.0±0.0	203.6±0.05	121.3±0.05	5.1	329.4±0.05	4.64±0.05	10.2±0.41
Subject3	20.1±0.0	107.4±0.05	59±0.05	5.3	295.4±0.05	7.51±0.0	20.94±0.11
Subject3	21.3±0.05	96.5±0.05	56.5±0.05	5.2	293.5±0.05	7.44±0.05	19.72±1.72
Subject4	12.8±0.0	121.5±0.05	98±0.05	6.3	203.9±0.05	13.44±0.0	2.62±0.55
Subject4	12.9±0.05	100.6±0.05	101.1±0.05	6.5	206.10±0.05	4.39±0.05	3.73±0.5

Table 4: Spectrophotometric and ISE determination of the concentrations of K^+, Na^+, Cl^- ions, total proteins, conductivity, creatinine and pH in urine samples from healthy subjects 48 and 72 h after sample storage at +4 °C.

+4 °C (48; 72 h)	Conductivity	K^+ (mmol/mmol crea)	Na^+ (mmol/mmol crea)	p*H*	Cl^- (mmol/mmol crea)	Creatinine (mmol/L)	Pyrogal (g/L)
Subject1	28.8±0.0	25.4±0.05	28.6±0.05	5.6	155.7±0.05	3.58±0.05	0.3±0.65
Subject1	28.9±0.05	25.45±0.05	18.62±0.05	5.4	174.8±0.05	3.58±0.1	0.01±1.19
Subject2	28.9±0.0	211.5±0.05	125.5±0.05	5.4	308.3±0.05	4.67±0.0	9.84±2.9
Subject2	28.0±0.0	203.6±0.05	121.3±0.05	5.1	329.4±0.05	4.64±0.05	10.2±0.41
Subject3	20.1±0.0	107.4±0.05	59±0.05	5.3	295.4±0.05	7.51±0.0	20.94±0.11
Subject3	21.3±0.05	96.5±0.05	56.5±0.05	5.2	293.5±0.05	7.44±0.05	19.72±1.72
Subject4	12.8±0.0	121.5±0.05	98±0.05	6.3	203.9±0.05	13.44±0.0	2.62±0.55
Subject4	12.9±0.05	100.6±0.05	101.1±0.05	6.5	206.10±0.05	4.39±0.05	3.73±0.5

Table 4: Spectrophotometric and ISE determination of the concentrations of K^+ , Na^+ ,Cl^- ions, total proteins, conductivity, creatinine and pH in urine samples from healthy subjects 48 and 72 h after sample storage at +4 ˚C.

+25 °C (48; 72 h)	Conductivity	K^+ (mmol/mmol crea)	Na^+ (mmol/mmol crea)	p*H*	Cl^- (mmol/mmol crea)	Creatinine (mmol/L)	Pyrogal (g/L)
Subject1	28.6±0.05	38.4±0.05	25.6±0.05	5	178.7±0.05	4.56±0.05	13.19±0.65
Subject1	28.9±0.05	28.45±0.05	26.62±0.05	5.4	159.8±0.05	4.42±0.1	23.2±1.19
Subject2	29.2±0.0	242.7±0.05	116.5±0.05	5.5	311.3±0.05	6.74±0.0	24.81±2.9
Subject2	28.3±0.0	211.75±0.05	115.3±0.05	5	333.4±0.05	4.63±0.05	25.7±0.41
Subject3	22.1±0.0	108.8±0.05	65±0.05	5.2	301.4±0.05	4.46±0.0	5.25±0.11
Subject3	21.3±0.05	89.9±0.05	60.5±0.05	5	284.5±0.05	6.67±0.05	33.2±1.72
Subject4	13.2±0.0	115.5±0.05	99±0.05	6.7	210.9±0.05	4.61±0.0	14.21±0.55
Subject4	12.8±0.05	101.6±0.05	99.1±0.05	6.5	202.10±0.05	4.52±0.05	18.54±0.5

Table 5: Spectrophotometric and ISE determination of the concentrations of K+ , Na+ ,Cl- ions, total proteins, conductivity, creatinine and pH in urine samples from healthy subjects 48 and 72 h after sample storage at +25 ˚C.

3.3 Electrochemical determination of urine antioxidant activity

Reactive oxygen species influence the organism, potentially causing oxidative cell damage. They can be produced by exogenous sources, or be a product of a variety of not only physiological metabolic processes, such as immune response, but also pathological processes. The analysis of antioxidant activity in the urine is therefore becoming increasingly important for the diagnosis [27].

Oxidative stress biomarkers such as superoxide dismutase (CuZnSOD) [28], catalase (CAT) [29] and malondialdehyde (MDA) [30] play an important role in the pathogenesis or progression of numerous diseases. Photometric assays (ABTS, FRAP, DMPD and FR) of low molecular weight antioxidants level are usualy used [31-34]. Antioxidant activity can be directly determined by voltammetry [35]. In this way can be quickly and cheaply monitored degradation processes of biomolecules in time. In our study, we focused on electrochemical monitoring of urine, which were frozen at +25, +4, -20 and -80 ˚C. Square wave voltammetry and cylic voltammetry of urinary specimens of volunteers on graphite screen printed electrodes provided two peaks marked as CVi1 and CVi2. Peaks CVi1 and CVi2 were positioned at about 0.5 V and 0.8 V (Fig. 3A and B), which is consistent with a previous publication [36]. The peak CVi1 was detected in all tested samples, but the peak CVi2 was detected only in some samples. For this reason urine was evaluated based on the CVi1 peak height (SWV) or area (CV).

The height and position of CVi1 peak in dependence on concentration of oxidative agent hydrogen peroxide (0 – 430 mM) and antioxidant ascorbic acid (0 – 6 mM) was monitored in the urine. The height of CVi1 peak decreased in dependence on hydrogen peroxide concentration (Fig. 3C) and increased with increasing concentration of ascorbic acid (Fig. 3D). The position of the CVi1 peak slightly shifted towards positive potential in the presence of H_2O_2 and towards negative potential in the presence of ascorbic acid. This indicates, that decrease of electrochemical signals (CVi1) is usable for monitoring of urine antioxidant activity.

After freezing the time-dependent decrease of the CVi1 peak was observed both in case of +4, +25, -20 ˚C and -80 ˚C. This indicates, that the samples are oxidized during storage. Even after 24 hours freezing of sample from subject 1 the 75 % decrease of the peak was observed after freezing at -20 ˚C and 25 % decrease after storage in -80 ˚C (Fig. 3E).With prolonged storage the peaks height continually decreased, but the differences between storage at -20 ˚C and -80 ˚C were not so markable. The noticable decrease of CVi1 peak height was observed also in other subjects (Fig. 3F–H). Individual rate of peak CVi1 decrease deflects individual content of pro-oxidative and antioxidative compounds and implicate the necessity of immediate samples processing.

In this work, we have systematically screened different storage temperatures of urine samples and investigated their effects on the sarcosine determination analyzed by IEC. Electrochemical detection of urine antioxidant activity using square wave voltammetry is a suitable tool for monitoring of the antioxidant capacity in biological samples. Due to the continual increasing interest on sarcosine can approach his noninvasive determination in urine serve as screening, a low cost method, useful for prebioptic testing of patients with suspected presence prostate cancer. Reliability of the method was demonstrated and it well correlated to the standard test. It has been demonstrated from the experimental data urine samples intended for sarcosine determination has to be processed during 48 h after sampling in the case of samples frozen at -20 ˚C and during 72 h for samples stored at 4 ˚C. On the other hand, storage at above-zero conditions leads to decreasing of antioxidant activity of urine samples, that indicates the intactness of the sample and possibility of using for other analyses.

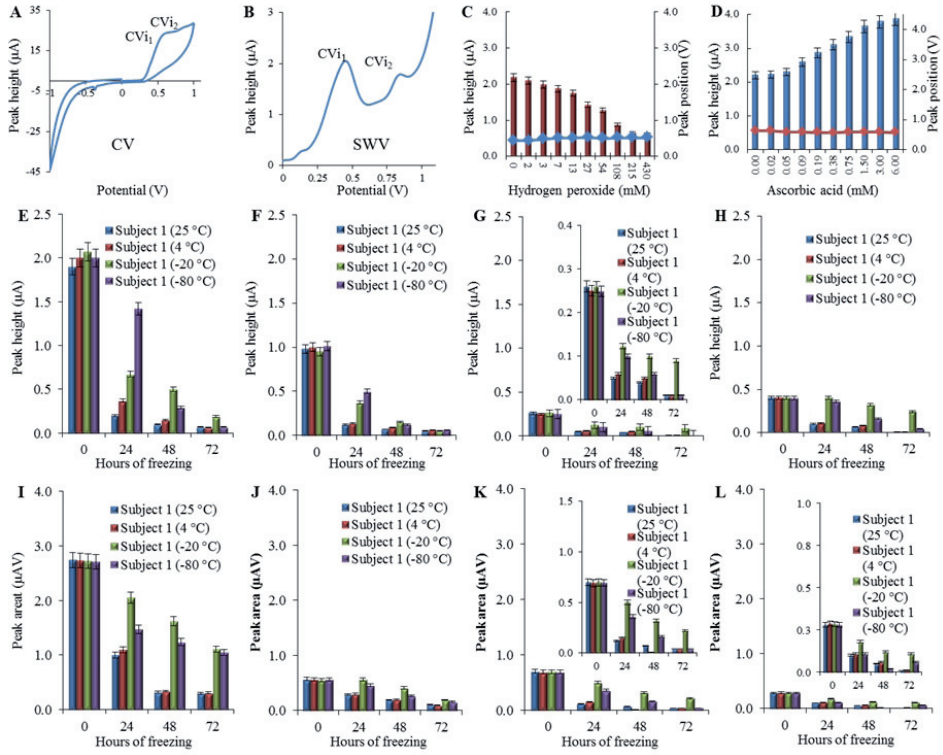

Figure 3: (A) Cyclic voltammogram and (B) square wave voltammogram of urine. (C) Influence of different concentration hydrogen peroxide (0 - 430 mM) and (D) ascorbic acid (0 – 6 mM) on the electrochemical signal CVi1 in urine (columns = height CVi1 and rhombus = CVi1 position). Influence of temperature (25 ˚C = blue, 4 ˚C = red, -20 ˚C = green and - 80 ˚C = purple) and time (0; 24; 48 and 72 h) on CVi1 signal height in the urine of volunteers recorded by square wave voltammetry (E-H) and by cyclic voltammetry (I-L). Total 40 µL of the samples (20 uL of urine with 20 uL of 0.2 M acetate buffer pH 5) was spread over the electrodes and voltammetry was run immediately. Setting of potentiostat is shown in the section materials and methods.

4. Conclusion

In metabolomics studies, often subtle changes need to be detected in order to find physiologically relevant differences between study groups. Every step along the way, from sample collection to data analysis, needs to be carefully controlled, and there are many efforts to standardize protocols and reporting them for sample collection and storage. For practical reasons, the actual freezing procedure of collected samples may involve conditions (temperature) that are different from long-term storage, there are only a few examples in the literature where freezing conditions are given.

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Conflicts of Interest

The authors declare no potential conflicts of interests.

References

1. Saude E, Sykes B (2007) Metabolomics 3:19-27
2. Deyl Z, Hyanek J, Horakova M (1986) Journal of Chromatography 379:177-250
3. Deyl Z, Sweeley CC (1986) Journal of

Chromatography 379:1-2
4. Cernei N, Heger Z, Kopel P, Skladanka J, Zitka O, Adam V, Kizek R (2014) Chromatographia 77:1451-1459
5. Walker V, Mills GA (1995) Ann. Clin. Biochem. 32:28-57
6. Cernei N, Zitka O, Ryvolova M, Adam V, Masarik M, Hubalek J, Kizek R (2012) Int. J. Electrochem. Sci. 7:4286-4301
7. Heger Z, Cernei N, Gumulec J, Masarik M, Eckschlager T, Hrabec R, Zitka O, Adam V, Kizek R (2014) Oncol. Rep. 31:1846-1854
8. Zitka O, Cernei N, Heger Z, Matousek M, Kopel P, Kynicky J, Masarik M, Kizek R, Adam V (2013) Electrophoresis 34:2639-2647
9. Barton RH, Nicholson JK, Elliott P, Holmes E (2008) International journal of epidemiology 37:i31-i40
10. Chadha V, Garg U, Alon US (2001) Pediatr Nephrol 16:374-382
11. Heger Z, Cernei N, Krizkova S, Masarik M, Kopel P, Hodek P, Zitka O, Adam V, Kizek R (2015) Scientific reports 5
12. Rist MJ, Muhle-Goll C, Gorling B, Bub A, Heissler S, Watzl B, Luy B (2013) Metabolites 3:243-258
13. Lambers Heerspink HJ, Nauta FL, van der Zee CP, Brinkman JW, Gansevoort RT, de Zeeuw D, Bakker SJ (2009) Diabet Med 26:556-559
14. Brinkman JW, de Zeeuw D, Lambers Heerspink HJ, Gansevoort RT, Kema IP, de Jong PE, Bakker SJ (2007) Clin Chem 53:1520-1526
15. Gerrits MI, Thijssen JH, van Rijn HJ (1995) Clin Chem 41:571-574
16. Garde AH, Hansen AM, Kristiansen J (2003) Scand. J. Clin. Lab. Invest. 63:521-524
17. Armstrong AJ, Eisenberger MA, Halabi S, Oudard S, Nanus DM, Petrylak DP, Sartor AO, Scher HI (2012) Eur. Urol. 61:549-559
18. Lucarelli G, Fanelli M, Larocca AMV, Germinario CA, Rutigliano M, Vavallo A, Selvaggi FP, Bettocchi C, Battaglia M, Ditonno P (2012) Prostate 72:1611-1621
19. Cernei N, Heger Z, Gumulec J, Zitka O, Masarik M, Babula P, Eckschlager T, Stiborova M, Kizek R, Adam V (2013) International Journal of Molecular Sciences 14:13893-13908
20. Zitka O, Heger Z, Kominkova M, Skalickova S, Krizkova S, Adam V, Kizek R (2014) J. Sep. Sci. 37:465-475
21. Pohanka M, Hynek D, Kracmarova A, Kruseova J, Ruttkay-Nedecky B, Sochor J, Adam V, Hubalek J, Masarik M, Eckschlager T (2012) Int. J. Electrochem. Sci. 7:11978-11992
22. Long GL, Winefordner JD (1983) Anal. Chem. 55:A712-A724
23. Chen J, Zhang J, Zhang W, Chen Z (2014) Journal of Separation Science 37:14-19
24. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, Laxman B, Mehra R, Lonigro RJ, Li Y, Nyati MK, Ahsan A, Kalyana-Sundaram S, Han B, Cao X, Byun J, Omenn GS, Ghosh D, Pennathur S, Alexander DC, Berger A, Shuster JR, Wei JT, Varambally S, Beecher C, Chinnaiyan AM (2009) Nature 457:910-914
25. Jiang Y, Cheng X, Wang C, Ma Y (2010) Analytical Chemistry 82:9022-9027
26. Alaya A, Nouri A, Belgith M, Saad H, Jouini R, Najjar MF (2012) Annals of laboratory medicine 32:177-183
27. Benkhai H, Kohler F, Lademann J, Lemanski S, Bornewasser M, Below E, Below H, Kramer A (2011) GMS Krankenhaushygiene interdisziplinar 6:Doc02-Doc02
28. Elchuri S, Oberley TD, Qi WB, Eisenstein RS, Roberts LJ, Van Remmen H, Jepstein CJ, Huang TT (2005) Oncogene 24:367-380
29. Zamocky M, Koller F (1999) Prog. Biophys. Mol. Biol. 72:19-66
30. Jentzsch AM, Bachmann H, Furst P, Biesalski HK (1996) Free Radic. Biol. Med. 20:251-256
31. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Free Radic. Biol. Med. 26:1231-1237
32. Benzie IFF, Strain JJ (1996) Analytical Biochemistry 239:70-76
33. Gulcin I (2008) J. Enzym. Inhib. Med. Chem. 23:871-876
34. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J (2007) Int. J. Biochem. Cell Biol. 39:44-84
35. Chudobova D, Dobes J, Nejdl L, Maskova D, Rodrigo MAM, Nedecky BR, Krystofova O, Kynicky J, Konecna M, Pohanka M, Hubalek J, Zehnalek J, Klejdus B, Kizek R, Adam V (2013) Int. J. Electrochem. Sci. 8:4422-4440
36. Pohanka M, Hynek D, Kracmarova A, Kruseova J, Ruttkay-Nedecky B, Sochor J, Adam V, Hubalek J, Masarik M, Eckschlager T, Kizek R (2012) Int. J. Electrochem. Sci. 7:11978-11992