AUTHOR'S REVIEW OF HER RESEARCH

(*Annex 2b*)

PhD Grażyna Chwatko

Department of Environmental Chemistry

Faculty of Chemistry

University of Lodz

2014

1. Personal details

Name: Grażyna Chwatko

Diplomas and degrees:

- MSc in 1994, University of Lodz, Poland. Thesis: "Analysis of captopril in biological samples by high performance liquid chromatography with solid phase extraction".
- PhD in 2002, University of Lodz, Poland. Thesis: "Estimation of thiol redox status in human plasma by high performance liquid chromatography".

Employment:

- 1994 – 1996, Teacher in Secondary School in Monki.
- 1996 – 2002, Assistant in the Department of Chemical Technology and Environmental Protection, Faculty of Chemistry, University of Lodz.
- 2002 – ongoing, Adjunct in the Department of Environmental Chemistry, Faculty of Chemistry, University of Lodz.

Research achievements

- Number of publications before obtaining a doctoral degree – 9
- Number of publications after obtaining a doctoral degree – 33, including:
  - number of original articles from Journal Citation Reports (JCR) – 26
  - number of original articles not included in JCR – 4
  - number of review articles from JCR – 2
  - number of review articles not included in JCR – 1
- Number of publications which are scientific achievents submitted for the habilitation procedure – 7
- Summarized impact factor of all articles:
  - according to the data from 2012 – 107.048
  - according to year of publication of the articles – 93.296
- Summarized impact factor of publications which are scientific achievements submitted for the habilitation procedure
  - according to the data from 2012 – 19.737
  - according to year of publication of the articles – 19.646
- Number of citations according to Web of Science:
- total citations – 667 (Hirsch index – 14)
- citations without self-citations- 636 (Hirsch index – 14)

2. Scientific achievement submitted for the habilitation procedure

As an achievement resulting from Article 16, section 2 of the Act on Academic Degrees and Titles and about Degrees and Titles in the Field of Fine Arts of March 14, 2003 (Dz.U. No 65/2003, item 595, with subsequent amendments) seven of scientific publications designed.

2.1. Title of scientific achievement

Analysis of biological samples for the content of metabolically related sulfur compounds

2.2. List of publication

The articles are presented in the order that they will be discussed in the text. The impact factor (IF) has been specified according to year of publication of the articles but for the articles published in 2013 and 2014 according to the data from 2012. The number of citations of works are presented according to the Web of Science database. An asterisk next to the name indicates that the author of achievement is a corresponding author.

H1 G. Chwatko*, V.M. Darras, E. Bald, A method for the determination of total and reduced methimazole in various biological samples. Food Addit. Contam. A, DOI: 10.1080/19440049.2014.905878.

IF(2012) 2.22, number of citations 0

My contribution is related to: planning and carrying out all the experiments, describing and interpreting the results, discussing with co-authors on the research results, preparation of the manuscript, corresponding with the editor of the journal. I declare my contribution to be equal to 65%.

H2 G. Chwatko, E. Bald, Determination of thiosulfate in human urine by high performance liquid chromatography, Talanta, 79 (2009) 229-234.

IF 3.498, number of citations 7

My contribution is related to: planning and carrying out all the experiments, describing and interpreting the results, discussing with co-author on the research results, preparation of the manuscript. I declare my contribution to be equal to 80%.

H3 K. Kuśmerek, G. Chwatko, E. Bald, Redox status of main urinary sulfur amino acids evaluation by liquid chromatography, Chromatographia, 68 (2008) S91-S95.
IF 1.312, number of citations 6
My contribution is related to: planning an experiment for the determination of aminothiols in urine, describing and interpreting the results, participation in the manuscript preparation. I declare my contribution to be equal to 50%.

H4 G. Chwatko*, Spectrophotometric method for the determination of total thiols in human urine. Ann. Clin. Lab. Sci. 43 (2013) 424-428.
IF(2012) 0.879, number of citations 0
My contribution is related to: planning and carrying out all of the experiments, describing and interpreting the results, preparation of the manuscript, corresponding with the editor of the journal. I declare my contribution to be equal to 100%.

H5 G. Chwatko*, P. Kubalczyk, E. Bald, Determination of lipoic acid in the form of 2-S-pyridinium derivative by high-performance liquid chromatography with ultraviolet detection. Curr. Anal. Chem. DOI: 10.2174/157341101099913121910134O.
IF(2012) 1.558, number of citations 0
My contribution is related to: planning all the experiments and carrying out most of them, describing and interpreting the results, discussing with co-authors on the research results, preparation of the manuscript, corresponding with the editor of the journal. I declare my contribution to be equal to 75%.

H6 G. Chwatko, H. Jakubowski, The determination of homocysteine-thiolactone in human plasma, Anal. Biochem. 337 (2005) 271-277.
IF 2.996, number of citations 66
My contribution is related to: carrying out all the experiments, calculating all results, discussing with co-author on the research results. I declare my contribution to be equal to 33%.

H7 G. Chwatko, H. Jakubowski, Urinary excretion of homocysteine-thiolactone in humans, Clin. Chem. 51 (2005) 408-415.
IF 7.149, number of citations 46
My contribution is related to: carrying out all the experiments, calculating all results, discussing with co-author on the research results. I declare my contribution to be equal to 33%.

2.3. Description of the scientific aim and the results achieved

Abbreviations

BCPB – 1-benzyl-2-chloropyridinium bromide

CMQT – 2-chloro-1-methylquinolinium tetrafluoroborate

CGSH – cysteinylglycine

CSH – cysteine

DHLA –dihydrolipoic acid

Hcy – homocysteine

HTL – homocysteine thiolactone

LA – lipoic acid

MMI – methimazole

OPA – *o*-phthaldialdehyde

TCEP – tris(2-carboxyethyl)phosphine

Introduction

Sulfur-containing compounds have been of continuing interest to many biomedical research centers because of their importance in several biological and pharmacological processes [1, 2].

Biological samples are one of the most complex matrices. The biological matrices consists of a large number of components which have different concentrations. The analysis of the biological samples presents a variety of problems: large number of individual compounds in the sample, leading to difficulty in resolving the analytes of interest, low concentrations of exogenous or endogenous compounds of interest, leading to detection difficulties, and conjugation of analytes to protein and/or low-molecular-mass components of the analyzed mixture. In order to obtain reliable results of the chemical analysis of complex biological samples, the analyst should pay attention to all steps of the analytical procedure. These steps include: collection and storage of the sample, sample preparation, method validation, sample analysis and data handling. In the analytical procedure, the measurement is the shortest step, which takes ~6% of the total time of analysis, while the data handling consumes up to 27% of the time [3]. The correct collection and sample preparation are one of the most difficult and time-consuming steps and take ~67% of the total analysis time. Furthermore, these steps are the largest source of

the total error of the analytical procedure, and it is estimated for 60%. The remaining 40% of the total error results from the measurement and method validation (30%), and data handling (10%).

The major objective of my research was to provide research tools which will precisely and accurately analyze the relationship between the presence of high levels of sulfur-containing compounds and the aging process, the presence of cardiovascular diseases and neurodegenerative disorders. Furthermore these methods will allow one to follow the conversion of some drugs or dietary supplements in living organisms, that include the maternal transfer and pharmacokinetics.

Sample preparation

The techniques used for the analysis of the biological samples do not allow to determine of the trace components in the sample without its appropriate pretreatment. There is no single, universal method of the sample preparation that would be versatile for all analyzed samples. The method of the sample preparation depends on physical state of the matter. The ease with which biological samples can be analyzed increase with the degree of fluidity. Liquid samples, such as urine and plasma, are analyzed easily, meanwhile solid tissues such as brain, liver and thyroid gland make more difficulties. Solid tissues must be shredded and homogenized before analysis by liquid phase separation techniques. I noticed that gradual dilution of the sample during homogenization decreases the total error of the method (Fig. 1). An advantage is that, the chemical reactions occurs better in non-viscous solutions and measurement of the diluted sample gives a smaller error. On the other hand, with increasing dilution of the sample inferior limits of detection and quantitation are obtained. In my research the parameters of homogenization of hens' eggs (white and yolk), chicken embryo, hens' brain, liver and thyroid tissue have been optimized [**H1**].

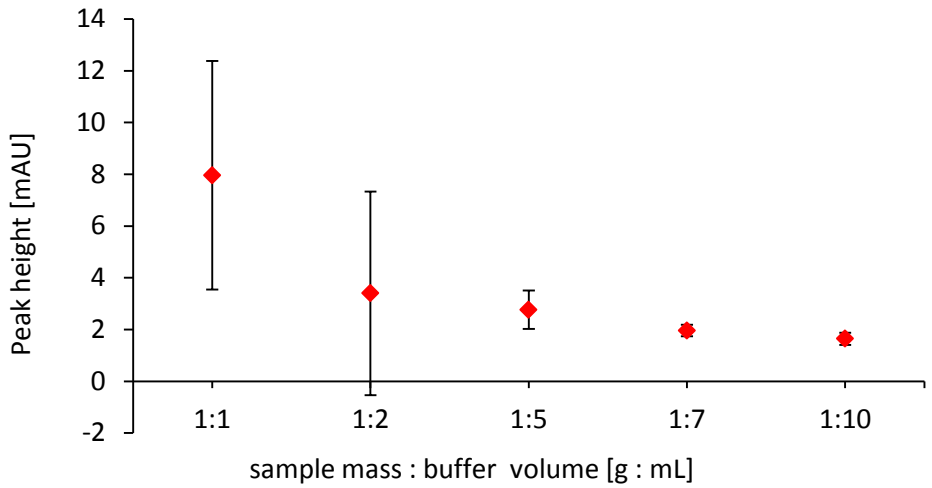


Fig. 1. The influence of the sample dilution on the accuracy of the assay. (G. Chwatko, unpublished data)

Biologically important sulfur compounds, such as aminothiols and thiosulfate, lack the structural properties necessary for the production of signals compatible with the spectrophotometric and fluorescence detectors. Homocysteine thiolactone (HTL) has a well-defined absorption maximum at 240 nm with the low molar absorption coefficient of 3,500 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ [4]. The analytical methods based on measurement of absorbance at 240 nm characterize a high limit of detection and are of little use for the determination of HTL in plasma samples [4]. Therefore, I have used the derivatization reaction for the determination of the sulfur compounds, during sample preparation step. For this purpose, I have used the following derivatization reagents: 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) [**H1-H3**], 1-benzyl-2-chloropyridinium bromide (BCPB) [**H4, H5**] and *o*-phthaldialdehyde (OPA) [**H6, H7**]. CMQT, widely used for derivatization of compounds contains –SH group [5, 6], react rapidly and quantitatively with hydrophilic thiols to form stable *S*-quinolinium derivatives, which have the molar absorption coefficient ~20,000 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ [7]. I conducted research using CMQT as derivatizing reagent and I have demonstrated that CMQT reacts with the sulfane sulfur of thiosulfate. Therefore, I have applied CMQT to the determination of thiosulfate in urine [**H2**]. Thiosulfate reacts with CMQT, under water conditions to form 1-methyl-2-thioquinolone (Fig. 2), in the stoichiometric ratio of 1:1 as was proven by continuous variation method. The reaction was completed after 2 min at pH 7 with 10-fold reagent excess at room temperature [**H2**].

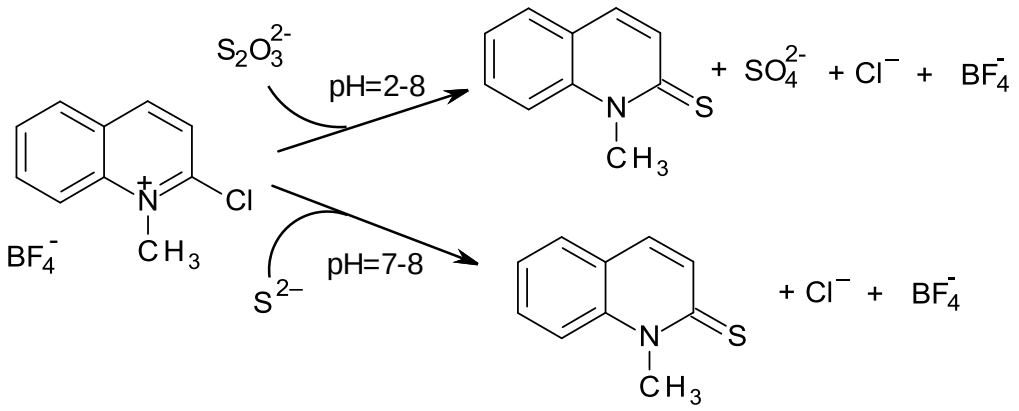
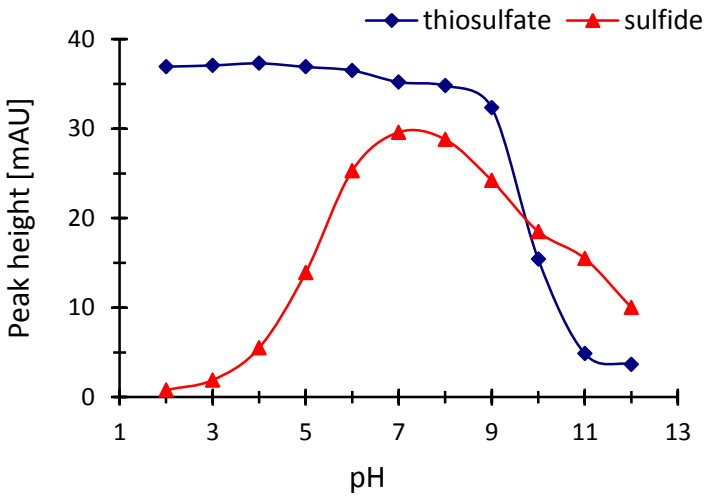


Fig. 2. Scheme of derivatization reaction of thiosulfate and sulfide with CMQT.

As can be seen on Fig. 2, both sulfide and thiosulfate react with CMQT to give the same derivative, 1-methyl-2-thioquinolone. If sulfide and thiosulfate are present in the testing sample, the reaction conditions allowing the differentiation of these compounds should be found. I proved that in the acidic environment (pH 2) thiosulfate forms easily 1-methyl-2-thioquinolone derivative whereas sulfide practically does not reacts with CMQT (Fig. 3). When derivatization takes place at pH 7.4, the detector response corresponds to the sum of thiosulfate and sulfide. The subtraction between the results of the analysis provides way to calculate the amount of sulfide.



Rys. 3. Derivatization reaction yield for thiosulfate and sulfide as a function of the pH. Conditions: ten-fold CMQT, time 5min, concentration of each analyte 10 nmol/mL. Figure 4 in the article **H2**.

BCPB was used for determination of the sum of total thiols [**H4**] and also lipoic acid (LA) [**H5**] in urine samples. I have developed procedures for conversion of LA to its thiol counterpart, dihydrolipoic acid (DHLA), by reductive cleavage with

tris(2-carboxyethyl)phosphine (TCEP) prior to precolumn derivatization with BCPB. DHLA reacts rapidly with BCPB in the stoichiometric ratio of 1:2, as it was proven by continuous variation method, according to the nucleophilic displacement scheme of chlorine atom by sulfur from DHLA sulfhydryl group leading to the formation of the stable thioether linkage, 2-S-pyridinium derivative as depicted in Fig. 4 [**H5**].

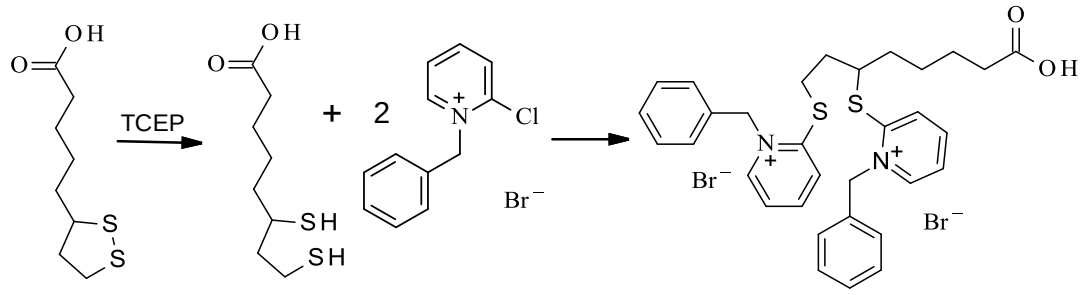


Fig. 4. Scheme of reduction of LA and conversion of DHLA to 2-*S*-pyridinium derivative.

The optimal reaction pH for derivatization of DHLA with BCPB was 9. In the case of fivefold BCPB excess at room temperature the derivatization reaction came to the end within 5 min. I have also proved that, BCPB reacts with other compounds containing –SH group in alkaline water solution in the pH range 8-12 (Fig. 5) [**H4**].

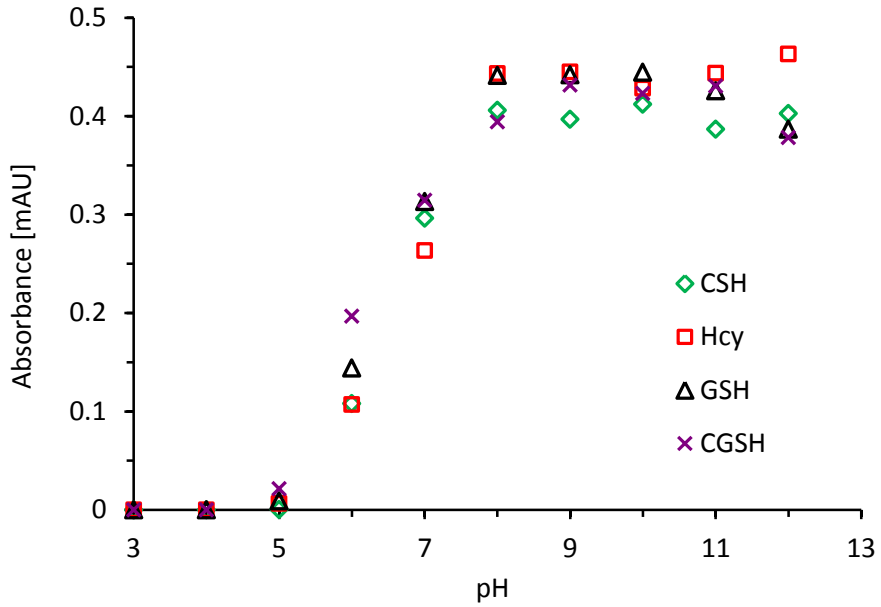


Fig. 5. Derivatization reaction yield for thiols as a function of the pH. Conditions: ten-fold BCPB, derivatization reagent excess, time 5 min, concentration of each analyte 50 µmol/L. CSH – cysteine, Hcy – homocysteine, GSH – glutathione, CGSH – cysteinylglycine. Figure 4 in the article **H4**.

The reaction of BCPB with thiols is accompanied by an analytically advantageous bathochromic shift of about 45 nm from reagent maximum to that of derivative. Because of this phenomenon, I used BCPB to determine the sum of total thiols in human urine by UV spectrophotometry. Importantly, I simplified the sample preparation procedure to use reduction and derivatization reactions only [**H4**].

OPA has been earlier used for post-column derivatization of homocysteine (Hcy) and homocysteine thiolactone (HTL) during determination of Hcy and HTL in the media of Hep G2 cells [8]. This method, due to the limit of detection equal to 10 nmol/L, is useless for the direct determination of HTL in plasma, because its average concentration is smaller. HTL concentrations in men and women plasma is 1.9 ± 3.9 and 3.3 ± 7.3 nmol/L, respectively [**H6**]. Therefore, during the development of methods for determination of HTL in plasma and urine samples the, liquid-liquid [**H6**] and liquid-solid phase [**H7**] extraction was used in sample preparation steps, respectively. Selective HTL extraction from biological sample was possible due to chemical properties of HTL. In contrast to most amino acids whose α-amino groups have a pKa ~9.5, the α-amino group of HTL has a pKa = 7.1 [9]. Thus, HTL is chiefly neutral at pH near 8 and mostly positively charged at slightly acid environment (Fig. 6). Ionic properties of other amino acids, except histidine, are essentially not affected by changes in the pH range from 6 to 8.

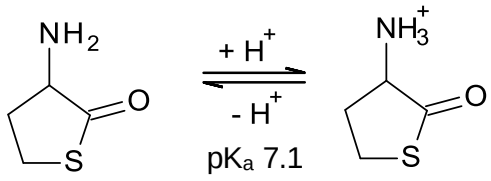


Fig. 6. Effect of pH on the change in the net charge of the amino group of HTL.

Human urine and plasma samples were deproteinized by ultrafiltration through 10-kDa cutoff membranes, adjusted to pH 8.0 and HTL was adsorbed on activated charcoal or extracted with chloroform/methanol mixture, respectively. HTL was re-extracted from the organic phase or charcoal with hydrochloric acid. The efficiency of HTL extraction using the chloroform/methanol procedure, monitored with plasma samples supplemented with range 1-20 nmol/L HTL or [^{35}S]HTL and immediately analyzed, was 61.8 ± 7.7% (n = 30) [**H6**]. Chloroform/methanol mixture is more selective than charcoal for extracting HTL from plasma samples. The use of extraction procedure allows to lower the detection limit of the method.

The developed methods include the sample preparation step that involves various processes, such as homogenization, derivatization and reduction reaction, extraction and deproteinization. Depending on the method, the sample preparation step takes from 62 to 88% of the total analysis time (Fig. 7).

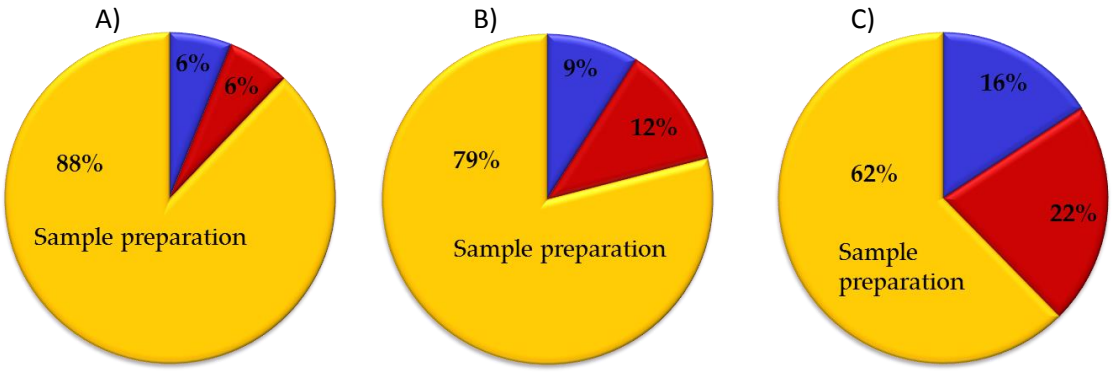


Fig. 7. Sample preparation (yellow), measurement and calibration (blue) and data processing (red) contribution to a total time of analysis. A) The method for the determination of HTL in urine and plasma [**H6**, **H7**], B) the method for the determination of MMI in tissue homogenates [**H1**], C) the method for the determination of thiosulfate in urine [**H2**].

Chromatographic conditions

During the research I have developed the new methods for the determination of thiosulfate and thiols [**H2**, **H3**] homocysteine thiolactone [**H6**, **H7**], lipoic acid [**H5**] and methimazole [**H1**] in biological samples with the use of high performance liquid chromatography (HPLC).

As I mentioned above, thiosulfate reacts with CMQT to form non-polar derivative, 1-methyl-2-thioquinolone, which was determined by reversed phase HPLC technique [**H2**]. 1-methyl-2-thioquinolone is retained effectively at the non-polar column (C-18) and separation of the urine components, under isocratic elution takes 3.5 minutes (Fig. 8A). For the simultaneous determination of thiols and thiosulfate in urine the gradient elution was applied (Fig. 8B). 1-methyl-2-thioquinolone elutes as a symmetric peak after 2.9 min (isocratic elution) and 8.7 min (gradient elution) without any disturbances from the urine matrix. The 2-*S*-quinolinium derivatives of thiols have a net positive charge in contrast to the 1-methyl-2-tioquinolone, therefore the addition of pairing agent (trichloroacetic acid) to the mobile phase is needed. Derivatives, separated on a column, exhibited significantly different hydrophobicity, so the gradient profile allowing elution of lipophilic analyte from the column was necessary. The total chromatographic analysis time of urine samples containing thiosulfate, cysteine (CSH) and cysteinylglycine (CGSH) was

11 minutes [**H2**]. The absorption maximun of 1-methyl-2-thioquinolon and 2-*S*-quinolinium derivatives of thiols differ significantly. Therefore, chromatogram profiles should be obtained at two different wavelengths 375 and 355 nm for thiosulfate and thiols, respectively.

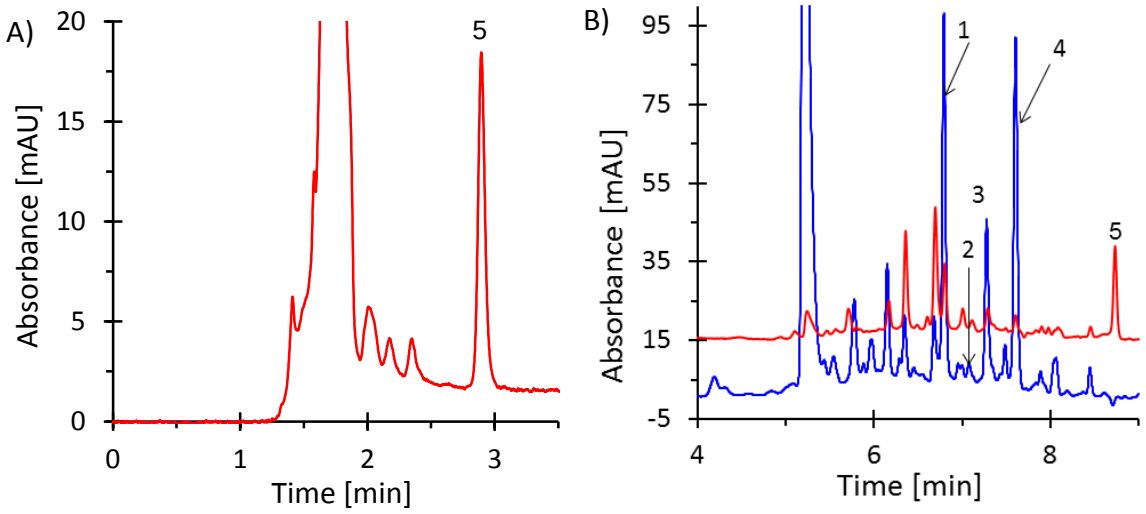


Fig. 8. The representative chromatograms of urine sample: (A) isocratic elution, (B) gradient elution. The peaks: 1 – CSH-CMQT (20.26 µmol/L), 2 – CGSH-CMQT (1.39 µmol/L), 3 – internal standard, 4 - CMQT, 5 – 1-methyl-2-thioquinolone (18.7 µmol/L). Chromatographic conditions: Zorbax C-18 column, temp. 25 °C, flow-rate 1.2 mL/min, analytical wavelength 355 nm for thiol (blue line) and 375 nm for thiosulfate (red line). A – isocratic elution 60/40 (v/v) acetonitrile/water; B – gradient elution, mobile phase TCA/acetonitrile. Figure 5B and 5C in the article **H2**.

The method developed for determination of methimazole (MMI) [**H1**] and LA [**H5**] in biological samples was based on the same scheme as the method elaborated for thiols. The samples containing MMI or LA were derivatized with the use of CMQT or BCPB before chromatographic separation. MMI concentration in homogenates of chicken tissue and plasma was determined after derivatization with CMQT at the analytical wavelength of 345 nm (Fig. 9). The content of LA in urine and pharmaceutical products was determined using 321 nm as the analytical wavelength (Fig. 10).

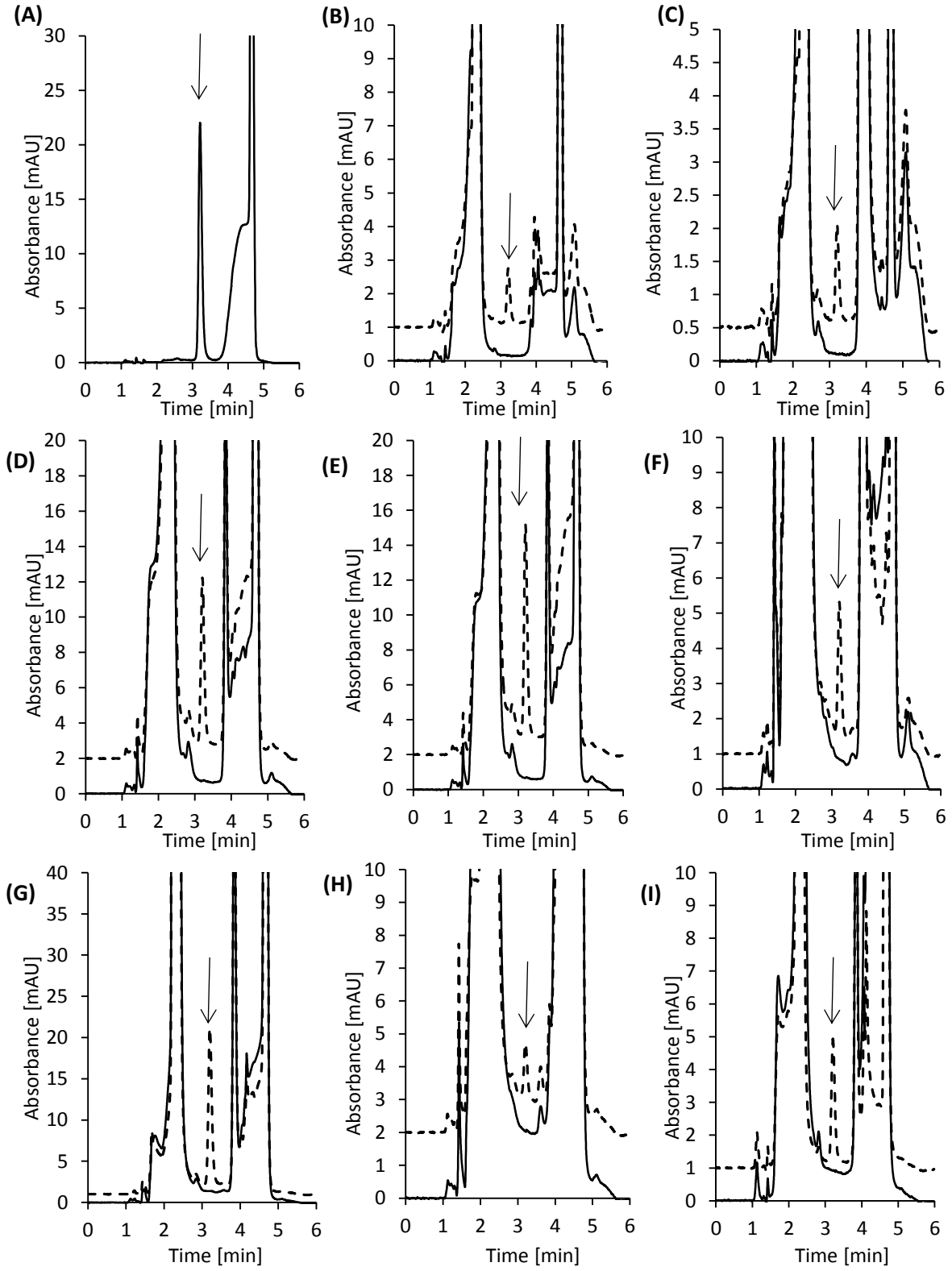


Fig. 9. The representative chromatograms of tissue homogenate. Solid line – blank samples, dotted line – samples spiked with MMI. The peak of MMI-CMQT derivative is indicated by the arrow on the chromatograms. A) standard water solution of MMI, concentration 1 μg/mL (20 ng in peak), B) white homogenate, MMI concentration 2 μg/g (3.6 ng in peak), C) yolk homogenate, MMI concentration 2 μg/g (3.6 ng in peak), D) homogenate of chicken embryo head, MMI concentration 13.1 μg/g (23.3 ng in peak), E) homogenate of chicken embryo trunk, MMI concentration 15.6 μg/g (27.8 ng in peak), F) homogenate of hen liver, MMI concentration 5.1 μg/g (9.0 ng in peak), G) homogenate of hen thyroid, MMI concentration 25.2 μg/g (44.9 ng in peak), H) homogenate of hen brain, MMI concentration 1.9 μg/g (3.4 ng in peak), I) hen plasma, MMI concentration 4.9 μg/mL (8.7 ng in peak) Figure 2 in the article **H1.**

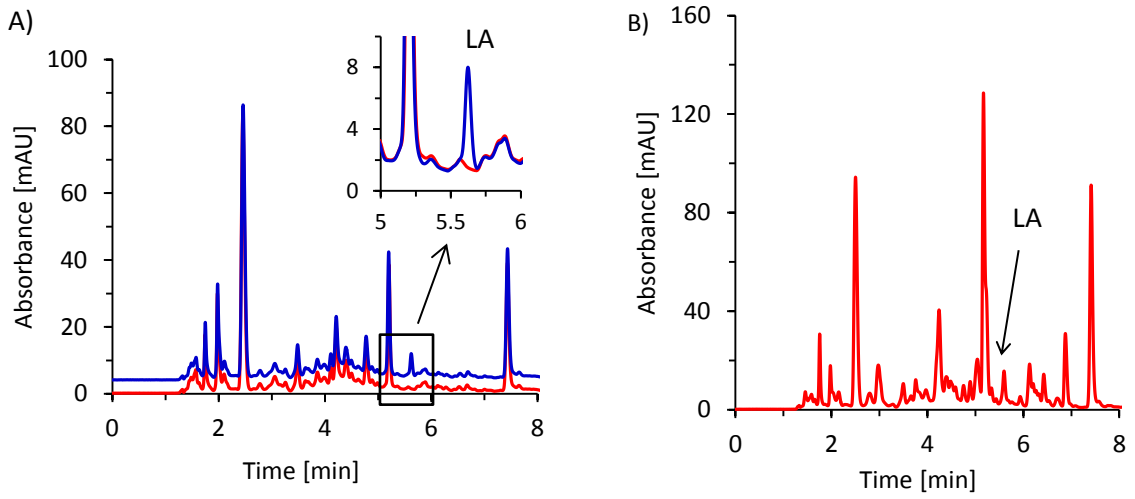


Fig. 10. The representative chromatograms of urine sample: (A) morning urine sample of a volunteer not taking LA (red line) and spiked with LA (blue line), concentration 1 μmol/L, (B) urine obtained from a volunteer at 1 hour after oral administration of 600 mg of LA. Chromatographic conditions: Zorbax C-18 column; gradient elution: 0–6 min, 10–48% B; 6–10 min, 48–10% B; 10–12 min, 10% B; Solvents: (A) 2% acetic acid solution, pH 2.54 and (B) acetonitrile; temp. 25 °C, flow rate 1 mL/min, analytical wavelength 321 nm. Figure 4a and 4b in the article **H5**.

For the determination of HTL in plasma and urine I have developed a new method using ion-exchanging chromatography [**H6**, **H7**]. Because of its positive charge at pH below 7.1 (Fig. 6), HTL was expected to be retained on a cation exchange column. Good chromatographic separation of HTL from possible interfering substances (glutathione, histidine and Hcy) was obtained using isocratic elution with mobile phase containing phosphate buffer, pH 6.6 and sodium chloride (Fig. 11).

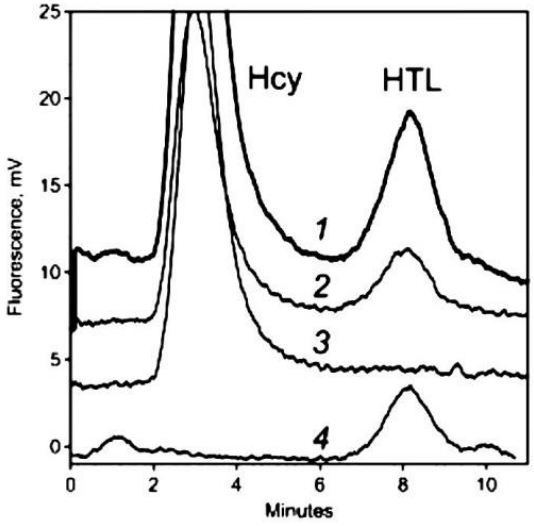


Fig. 11. The representative chromatograms of plasma and standard solution of HTL. 1–plasma sample, HTL concentration 6.7 nmol/L, 2– plasma sample, HTL concentration 2.8 nmol/L, 3– plasma sample treated with NaOH before HPLC analysis, 4–HTL standard solution (0.1pmol in peak). Chromatographic conditions: column: PolySULFOETHYL A, mobile phase: 10 mmol/L phosphate buffer, pH 6.6 containing 5 mmol/L NaCl, flow rate 0.15 mL/min. Detection was by fluorescence emission at 480 nm (excitation at 370 nm) after postcolumn derivatization with OPA. Figure 1 in the article **H6**.

The combination of the chromatographic separation with extraction technique allowed accurate determination of HTL in the plasma and urine. Elaborated methodology can help to clarify the role of HTL in certain diseases.

Method validation

The analytical methods have been validated according to the procedures recommended in the literature [10].The methods were approved by evaluating the linearity, precision, recovery, limit of quantification (LOQ) and detection (LOD), and stability. Detailed data are inserted in Table1. The developed methods have good accuracy (recovery) and precision (relative standard deviation, RSD). The obtained LOQ allow to follow the concentration of the examined analytes in biological samples.

Table 1. The results of the validation process.

Analyte	Matrix	Linearity	RSD [%]		Recovery [%]		LOD [a]	LOQ [a]	Ref.
			min	max	min	max			
MMI	Plasma, tissue	0.5 – 20 (mg/L, mg/kg)	0.5	12.1	88.5	109.7	0.1	0.5	**H1**
$S_2O_3^{2-}$	Urine	0.5 – 50 (µmol/L)	1.4	7.9	91.2	108.2	0.3	0.5	**H2**
Thiols	Urine	50 – 500 (µmol/L)	2.1	8.4	95.7	102.9	14.5	48.4	**H4**
LA	Urine	0.2 – 50 (µmol/L)	0.9	7.0	96.5	111.5	0.1	0.2	**H5**
HTL	Urine, plasma	1 – 40 (nmol/L)	6.7	10.5	93.4	113.5	0.36	1	**H6** **H7**

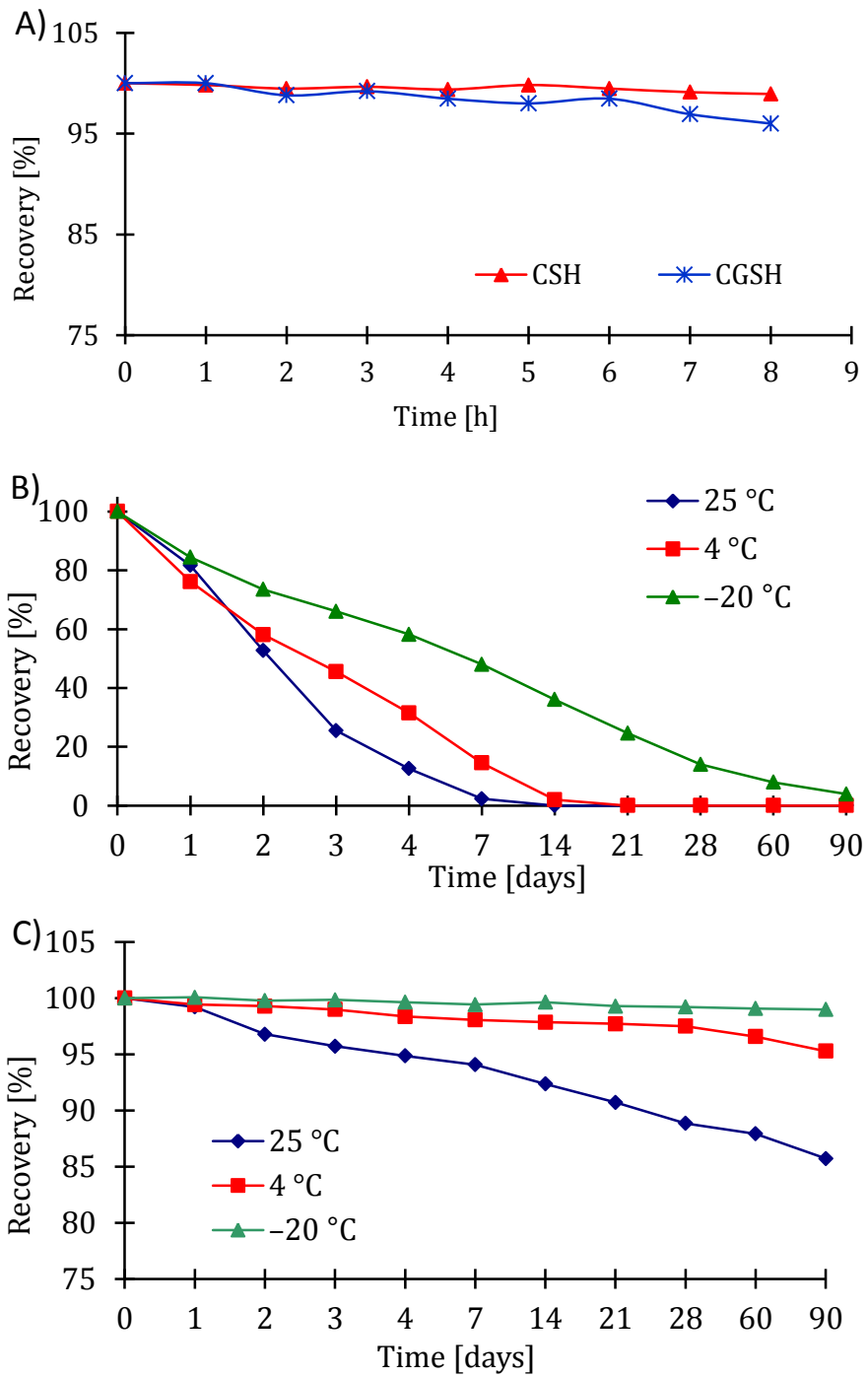
[a] LOD and LOQ values are given in the same units as the linearity

I also checked the stability of the analytes or their derivatives in the studied samples. Estimation of stability of the analyte is important because it determines the conditions and time of storage of biological samples. As a thioester, HTL is chemically reactive and therefore inherently unstable. In human plasma 98.0 ± 1.8% and 86.5 ± 3.8% of the originally present HTL were detected, when plasma sample was kept at 4 and 23 °C, respectively, for 1 hour. In plasma kept for 5 h at 4 and 23 °C remained 87.8 ± 3.8% and 43.9 ± 2.0% of HTL, respectively [**H6**]. For reliable assays, blood should be collected in EDTA-containing tubes and processed at 4 °C immediately after collection or obtained plasma should be frozen. HTL was essentially stable in deep-frozen (-80 °C) plasma

samples stored for up to 18 months. Because most of urine samples were slightly acidic (pH < 7.0) and almost protein-free (compared with plasma), HTL was expected to be much more stable in urine than in plasma. The hydrolysis of HTL to Hcy depends on sample pH and rises with increasing sample pH. I observed no significant loss of HTL in urine samples (pH 5.2 – 6.3) kept for up to 24 h at 4 °C or 9 h at 23 °C, whereas ~20% was lost after 24 h at 23 °C. In the urine samples with a pH of 7.3 I observed a faster loss of HTL concentration than in slightly acidic samples. In a urine sample with a pH of 7.3, significant diminution of HTL after 9 h of storage at 4 °C was not observed, whereas ~30% was lost after 24 h at 4 °C. However, when the pH 7.3 urine sample was kept at 23 °C for 9 and 24 h, 40% and 70% of the original HTL was lost, respectively [**H7**]. Urinary HTL is much more stable and can be reliably assayed, even in samples kept at room temperature for a few hours.

The amino acids possessing a free –SH group exhibit similar reactivity as the HTL. In urine samples stored at room temperature CSH and CGSH are fairly stable during 4-5 hours after collection (Fig. 12A) [**H3**]. Total loss of reduced CSH in urine was observed after 14 days at room temperature and after 21 days at 4 °C (Fig. 12B). Immediate freezing of urine samples at -20 °C slows the oxidation of –SH groups, but does not inhibit. A decrease of 96% was observed within 3 months in urine stored at -20 °C. Then I checked the stability of 2-*S*-quinolinium derivative of CSH in the final analytical solution obtained after urine pretreatment. As shown in Fig. 12C, 2-*S*-quinolinium derivative of CSH was found to be stable at room temperature for 1 day, unfrozen at low temperature (4 °C) for at least 3 days and in the frozen (-20 °C) state for 90 days. After 3 months the recovery for CSH derivative at 25, 4 and -20 °C was 85, 96 and over 99%, respectively [**H3**].

Moreover, I tested the stability of endogenous thiosulfate in urine ex vivo at different temperatures. Thiosulfate in urine was found to be stable at room temperature for 8 h and at 4 °C for at least 24 h [**H2**]. After 24 h the recovery for thiosulfate at 25 and 4 °C was 67.9 and 95.8%, respectively. As shown above the stability of endogenous thiols was less. Therefore, the urine samples were processed as soon as possible, but not later than 4 h after collection, when reduced thiols were estimated together with thiosulfate. The final analytical solution obtained after urine processing can be stored for up to 3 months in -20 °C [**H3**]. This fact allows to collect the urine samples from a large group of patients.



Rys. 12. Stability study. A) Stability of the endogenous reduced CSH and CGSH in urine at room temperature in the early hours after collection. B) Loss of endogenous reduced CSH in urine ex vivo, within 90 days. C) Stability of the 2-*S*-quinolinium derivative of CSH in final analytical solution within 90 days. Figure 1 in the article **H3**.

LA exhibit the highest stability in the urine than other tested analytes. LA in urine was found to be stable at room temperature and 4 °C for at least 24 h [**H5**]. 2-*S*-quinolinium derivative of MMI is stable in plasma and tissue homogenates matrix for 24 h at room temperature and for 10 days at 4 °C [**H1**].

Results

Endogenous thiols and thiosulfate in urine

The biological thiols are critical cellular components that play numerous important roles in the metabolism and homeostasis [11, 12]. Therefore, a simple, sensitive and accurate method for the determination of thiols in biological samples is required. The developed spectrophotometric method for determining the sum of the total thiols in urine [**H4**] fulfils these criteria and allowed me to examine the relationship between concentrations of plasma thiols and age. Urine was received from 38 volunteers (19 women and 19 men), 14 – 82 years old. To facilitate comparison for different individuals, the analytical results for the urinary total thiols were normalized against creatinine. Concentrations of total thiols in human urine varied from 17.2 to 83.6 mmol/mol creatinine and the obtained results are in good agreement with data reported by other authors [6, 13, 14]. There was no correlation between urinary excretion of total thiols in groups of women and men. However, there was a significant statistical correlation between urine total thiols and age in the studied group ($r = 0.661$, $p < 0.05$) [**H4**]. Although, the previous data has shown that there were no significant differences between age and concentration of CSH and CGSH excreted in urine [**H3**]. The difference may be due to the fact, that the proposed method allows for the determination of the sum of all compounds possessing the –SH function excreted in urine, not only sulfur amino acids.

Moreover, I evaluated CSH and CGSH redox state in urine of 45 volunteers [**H3**]. Contents of reduced and total CSH and CGSH were measured directly by HPLC, but oxidized forms of thiols for each urine specimen were calculated as a difference between total and reduced amounts. The research group of volunteers was divided into 6 subgroups due to age. The results are shown in Table 2 and 3.

Table 2. Total urinary contents of CSH and CGSH in different age groups [**H3**].

Age [years]	N	CSH [a]		CGSH [a]	
		Mean ± SD	Median	Mean ± SD	Median
< 10	11	30.37 ± 6.96	28.57	1.72 ± 0.46	1.70
20-29	7	30.39 ±11.54	22.87	1.58 ±0.35	1.42
30-39	6	29.99 ± 7.80	31.03	1.70 ± 0.40	1.73
40-49	3	29.63 ± 1.32	29.22	1.67 ± 0.10	1.70
50-59	8	30.49 ± 9.53	27.88	1.70 ± 0.47	1.67
> 60	10	30.01 ± 5.25	30.81	1.76 ± 0.73	1.72

[a] Values are expressed in mmol/mol creatinine

Total CSH and CGSH contents for all age subgroups are very similar, and are not significantly different ($p > 0.05$). A mean value, calculated for all volunteers ($N = 45$), is 30.15 and 1.69 mmol/mol creatinine for CSH and CGSH, respectively. These values are consistent with results reported by other authors [6, 14 - 16]. A total pool of CSH in urine consisted of 6.1% in reduced and 93.9% in oxidized form. There was no significant differences in reduced and oxidized form of CSH in children (6.0 and 94%) and adults (6.3 and 93.7%). In the case of CGSH, in children about 9.6% of the total CGSH existed in reduced and 90.4% as oxidized, whereas in adults 13.9% representing reduced and 86.1% the oxidized forms.

Table 3. Redox status of CSH and CGSH in human urine [**H3**].

Age [years]	N	CSH redox status		CGSH redox status	
		Mean ± SD	Median	Mean ± SD	Median
< 10	11	0.0577 ± 0.0371	0.0478	0.0805 ± 0.0208[a]	0.0808
20-29	7	0.0668 ± 0.0282	0.0719	0.1408 ± 0.0374	0.1307
30-39	6	0.0698 ± 0.0365	0.0800	0.1549 ± 0.0638	0.1519
40-49	3	0.0704 ± 0.0054	0.0705	0.1493 ± 0.0189	0.1455
50-59	8	0.0709 ± 0.0467	0.0639	0.1553 ± 0.0336	0.1663
> 60	10	0.0703 ± 0.0316	0.0762	0.1545 ± 0.0805	0.1413

[a] Mean is significantly different between children and other age group at $p < 0.01$

Urinary CSH/CSSC ratio (redox status) increases in all age groups from 0.0577 to 0.0709, however mean values are not significantly different ($p > 0.05$). I observed a significantly positive correlation between CGSH redox state and age ($p = 0.0013$, $r = 0.4976$). Moreover, CGSH redox status in children was significantly lower than in adults ($p < 0.01$), whereas there was no significant difference between all adult subgroups ($p > 0.05$). The reduced to oxidized CGSH ratio in children is more oxidized than that in adults [**H3**].

The HPLC method [**H2**] was applied to the determination of thiosulfate urinary excretion in apparently healthy volunteers. The amount of urinary thiosulfate varied from 0.7 to 4.5 mmol/mol creatinine and the mean value for 98 man was 1.9 mmol/mol creatinine [**H2**, 17]. Value for thiosulfate was consistent with results reported by Belardinelli et al [18].

Relationship between homocysteine thiolactone and homocysteine in urine and plasma

Homocysteine thiolactone (HTL) is a highly reactive metabolite of homocysteine (Hcy) that has been implicated in cardiovascular disease [19, 20]. Metabolism of HTL in the human body has remained unexplored. Using the HPLC method [**H6**], the levels of HTL in plasma from normal healthy human subjects were determined (Table 4). Concentrations of HTL in human plasma from 60 subjects varied from 0 to 34.8 nmol/L with an average 2.82 ± 6.13 nmol/L and represented from 0 to 0.28% of plasma total Hcy [**H6**]. I didn't find correlation between plasma HTL and plasma tHcy in groups of women ($r = 0.21$, $p > 0.05$) and men ($r = 0.19$, $p > 0.05$). There was a weak correlation between plasma HTL and age in men, but it did not reach statistical significance ($r = 0.295$, $p > 0.05$). There was no correlation between HTL and age in the group of women ($r = 0.02$).

Table 4. Concentrations of HTL and Hcy in human plasma [**H6**].

Analyte	Gender [a] (N)	Range	Median	Average ± SD
HTL (nmol/L)	M (35)	0 – 34.8[b]	0.5	3.3 ± 7.3
	K (25)	0 – 16.0[b]	0.0	1.9 ± 3.9
Hcy (μmol/L)	M (35)	8.0 – 35.4	13.5	14.9 ± 5.2
	K (25)	5.25 – 24.4	12.5	13.1 ± 4.7
HTL/Hcy (%)	M (35)	0 – 0.281	0.000	0.020 ± 0.051
	K (25)	0 – 0.1444	0.013	0.035 ± 0.047

[a] Differences by gender are no significant ($p > 0.05$)
[b] In plasma samples from 16 men and 13 women HTL was undetectable

Using the HPLC method [**H7**] I have discovered a previously unknown pool of HTL in human urine. HTL was found in all analyzed human urine samples in the range from 11.0 to 473.7 nmol/L (Table 5). Concentration of Hcy was also determined and ranged from 0.4 to 5.3 μmol/L. In tested urine samples, HTL represented 2.5 – 28.3% of total urinary Hcy.

Table 5. HTL and Hcy concentrations in human urine and plasma [**H7**].

Analyte	Urine (n = 19)			Plasma (n = 20)		
	Mean (SD)	Median	Range	Mean (SD)	Median	Range
HTL (nmol/L)	168 (127)	144.8	11.0-473.7	4.1 (6.7)	0.56	<0.1-22.6
Hcy (μmol/L)	2.5 (1.6)	2.3	0.4-5.3	10.9 (4.6)	12.2	3.5-18.6
HTL/Hcy (%)	7.7 (7.0)	6.0	1.7-28.3	0.03 (0.04)	0.01	<0.001-0.29

Next, the relationship between HTL and Hcy in plasma and urine were tested. There was no correlation between HTL and Hcy concentrations in urine (Fig. 13A), whereas there was a weak but significant correlation (Fig. 13B) between HTL and Hcy in plasma ($r = 0.51$, $p = 0.02$). These results suggest that Hcy, a metabolic precursor of HTL, is not a major determinant of HTL concentrations in humans. Apparently, other determinants of HTL concentrations, such as folic acid, methionine, and the Hcy-thiolactonase activity, may also be important.

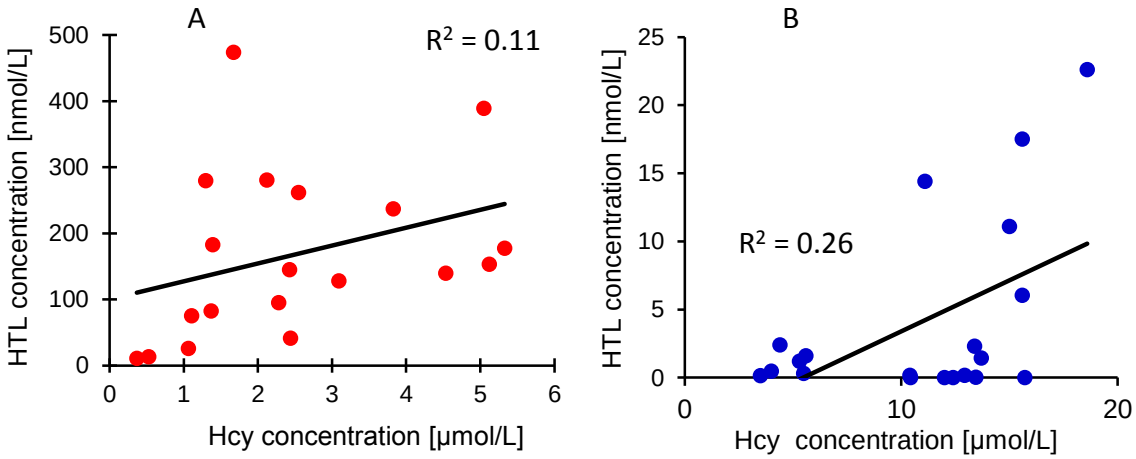


Fig. 13. Relationships between HTL and Hcy in human urine (A) and plasma (B). Figure 2 in the article **H7**.

I also examined the relationship between urinary HTL and Hcy concentrations and pH. There was a significant negative correlation ($r = -0.63$, $p < 0.01$) between urinary HTL and pH (Fig. 14A), suggesting a pH-dependent mechanism of excretion. In contrast, there was no correlation between Hcy and pH in urine (Fig. 14B), suggesting a different, pH-independent mechanism of excretion.

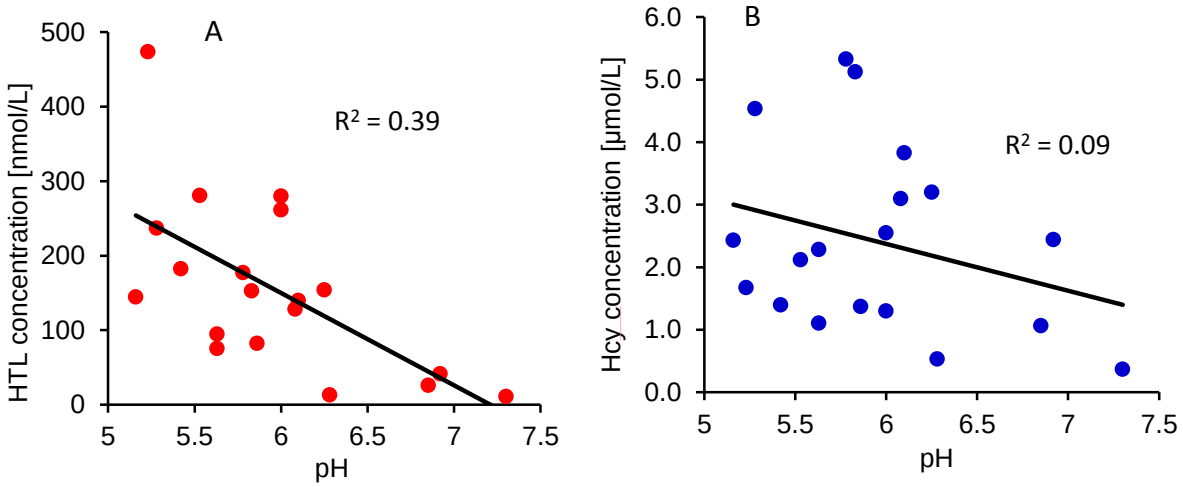


Fig. 14. Urinary concentrations of HTL (A) and Hcy (B) as a function of the pH, n = 19. Figure 3 in the article **H7**.

The urinary HTL pool is ~100-fold larger that the plasma HTL pool. This observation suggests that HTL is efficiently eliminated by urinary excretion, which is typical for the waste products of normal human metabolism. The confirmation of this thesis is the relative clearance of HTL and Hcy tested and calculated for 5 volunteers (Table 6). In this study, I found that the amounts of HTL and Hcy eliminated by urinary excretion were 286 – 415 nmol/day and 1.6 – 5.5 μmol/day, respectively. Calculations have indicated that 99% of filtered Hcy is reabsorbed while more than 95% of filtered HTL was excreted. These observations are consistent with a view that HTL is most likely a toxic metabolite in humans [21].

Table 6. Mean concentrations and relative clearances of HTL and Hcy in 5 volunteers [**H7**].

	Urine			Plasma			Relative clearance [b]	
	HTL nmol/L	Hcy μmol/L	Crn [a] mmol/L	HTL nmol/L	Hcy μmol/L	Crn [a] mmol/L	HTL	Hcy
1	82 ± 2	1.4 ± 0.03	7.1 ± 0.03	0.12 ± 0.03	3.5 ± 0.1	53 ± 1	4.74	0.003
2	389 ± 34	4.4 ± 1.2	27.4 ± 0.5	2.4 ± 0.1	4.4 ± 0.1	34 ± 0	0.21	0.001
3	280 ± 44	1.3 ± 0.2	9.3 ± 0.3	0.17 ± 0.03	5.6 ± 0.3	39 ± 1	6.96	0.001
4	95 ± 27	2.3 ± 0.3	12.6 ± 0.6	1.2 ± 1.0	5.3 ± 0.3	64 ± 0	0.40	0.002
5	183 ±15	1.4 ± 0.2	9.0 ± 0.5	0.32 ± 0.45	5.5 ± 0.2	47 ± 0.1	2.8	0.001

[a] Crn - creatinine

[b] Relative clearance = $(Analyte_{urine} / Analyte_{plasma})/(Creatinine_{urine} / Creatinine_{plasma})$

Maternal transfer of methimazole

Methimazole (MMI) is a thyreostatic drug used in the treatment of chronic hyperthyroidism. MMI has also found its way into the agricultural industry being illegally applied to animals to obtain a higher live weight gain (enhancing water retention in tissue) for economic interest. The introduction of MMI into the human food chain can not only lead to meat quality reduction, but can also have serious implications for human health. The HPLC method for determination of reduced and total MMI [**H1**] was used to determine the maternal transfer of MMI in hens [22]. The total and reduced MMI was determined in eggs and embryos of the MMI-treated hens and in brain, liver, thyroid gland and plasma of these hens at the end of the treatment. The results are shown in Table 7 and 8.

Table 7. Levels of reduced and total MMI in plasma and tissues of adult hens, n=3 [**H1**].

Brain		Liver		Thyroid		Plasma	
Mean ± SD µg/g tissue	RSD %	Mean ± SD µg/g tissue	RSD %	Mean ± SD µg/g tissue	RSD %	Mean ± SD µg/mL	RSD %
Reduced MMI							
1.3 ± 0.1	6.3	2.2 ± 0.1	5.0	20.8 ± 0.8	3.7	12.5 ± 0.6	4.7
1.2 ± 0.1	6.0	5.2 ± 0.5	8.7	15.5 ± 1.0	6.1	4.8 ± 0.2	4.3
1.2 ± 0.1	5.1	4.5 ± 0.3	5.8	22.3 ± 0.6	2.5	7.2 ± 0.3	3.4
1.1 ± 0.1	7.5	1.9 ± 0.1	7.5	24.9 ± 0.7	2.7	4.0 ± 0.1	2.5
Total MMI							
2.4 ± 0.1	3.7	2.6 ± 0.2	7.8	21.1 ± 1.1	5.3	13.5 ± 0.5	3.7
1.9 ± 0.1	5.2	5.9 ± 0.5	8.6	16.4 ± 0.8	4.6	5.1 ± 0.2	4.0
2.0 ± 0.1	6.5	4.9 ± 0.3	6.7	27.4 ± 2.1	7.7	7.8 ± 0.3	4.0
1.8 ± 0.1	7.7	2.4 ± 0.2	6.6	26.5 ± 1.9	7.1	4.3 ± 0.1	3.3

Table 8. Levels of reduced and total MMI in eggs and 6-day-old embryos, n=3 [**H1**].

White		Yolk		Head		Trunk	
Mean ± SD µg/g tissue	RSD %	Mean ± SD µg/g tissue	RSD %	Mean ± SD µg/g tissue	RSD %	Mean ± SD µg/g tissue	RSD %
Reduced MMI							
9.5 ± 0.1	1.0	6.9 ± 0.2	2.2	13.3 ± 1.3	9.5	16.9 ± 1.6	9.5
14.3 ± 0.2	1.2	10.8 ± 0.2	1.5	12.9 ± 1.1	8.5	15.4 ± 1.2	7.6
16.9 ± 0.1	0.5	9.9 ± 0.5	5.3	11.6 ± 0.7	6.2	14.7 ± 1.4	9.2
28.2 ± 0.2	0.8	19.3 ± 0.6	3.1	20.3 ± 1.1	5.4	22.2 ± 1.4	9.3
8.8 ± 0.7	7.5	7.3 ± 0.2	3.1	8.9 ± 0.6	6.3	8.4 ± 0.5	5.3
16.2 ± 0.2	1.0	14.4 ± 0.2	1.2	6.4 ± 0.6	9.4	4.6 ± 0.4	9.3
14.3 ± 0.1	0.6	10.9 ± 0.1	0.8	8.9 ± 0.8	9.3	8.6 ± 0.7	8.2
13.6 ± 0.2	1.5	12.4 ± 0.1	1.1	4.3 ± 0.4	9.2	6.3 ± 0.5	8.3
Total MMI							
10.1 ± 0.1	0.5	8.8 ± 0.2	2.1	14.7 ± 0.9	5.9	19.3 ± 1.3	6.7
14.9 ± 0.3	2.3	14.1 ± 0.3	2.0	14.4 ± 0.9	6.4	17.0 ± 1.5	8.7
17.7 ± 0.3	1.5	10.9 ± 0.4	4.0	11.9 ± 1.2	9.7	15.7 ± 1.1	7.1
29.2 ± 0.2	0.5	20.1 ± 0.5	2.3	22.6 ± 0.7	3.0	23.5 ± 1.4	5.9
9.2 ± 0.1	1.0	7.4 ± 0.4	5.1	9.2 ± 0.9	9.6	8.8 ± 0.5	5.7
17.1 ± 0.2	1.3	18.7 ± 0.3	2.0	7.6 ± 0.6	7.9	4.3 ± 0.4	9.4
13.9 ± 0.1	0.8	14.4 ± 0.3	2.0	9.4 ± 0.8	8.5	8.6 ± 0.8	9.3
14.4 ± 0.1	0.7	15.7 ± 0.1	0.6	4.4 ± 0.2	4.1	6.3 ± 0.3	4.8

The highest level of MMI was found in the thyroid gland of adult hens but in the brain and liver remained relatively low. A different result was found in embryos where MMI levels were similar in the all organisms (head and trunk). Relatively high concentrations of MMI was found in eggs of hens that have received MMI in the drinking water. The results of this experiment suggests, that reduced MMI and/or its oxidized metabolites may have cytotoxic effects on a developing embryos [22].

Pharmacokinetic study

Lipoic acid (LA) is a naturally occurring in plants and animals compound, that exists in oxidized and reduced form. Both forms together constitute the universal antioxidant redox couple of many biological systems [23, 24]. LA is widely used as a dietary supplement [25]. The HPLC method [**H5**] was successfully applied for monitoring of LA urinary excretion. Urine was received from 4 apparently healthy women in the age 23 – 41 years who received a single 600 mg oral dose of LA after an overnight fast of 8 hours. The urine samples were collected for 4 hours after administration of LA. Results of LA urinary excretion, recalculated against creatinine are shown in Fig. 15.

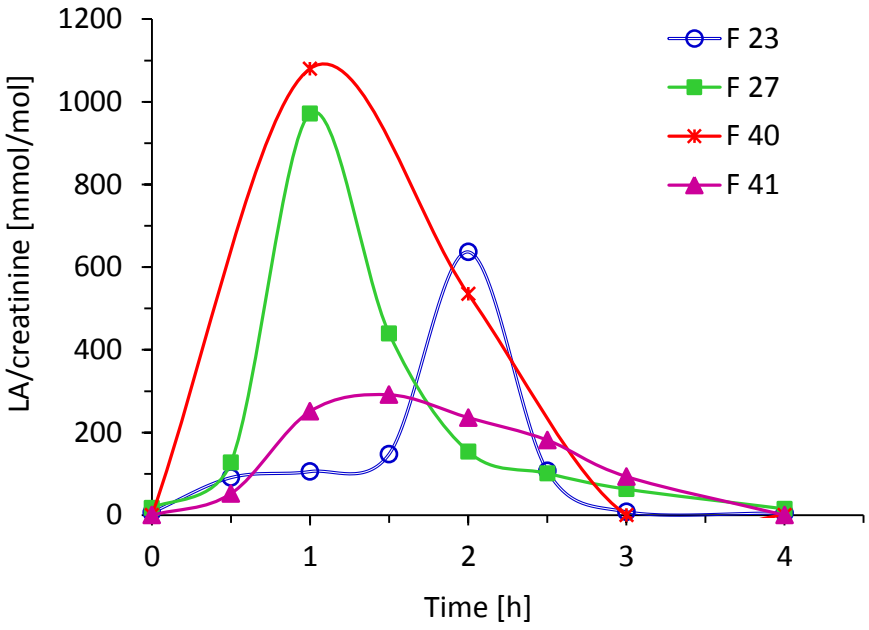


Fig. 15. Urine LA concentration-time profiles for different healthy volunteers after single oral administration of 600 mg of LA. Figure 5 in the article **H5**.

After oral ingestion of LA (600mg), its concentration in urine was in the range 0.1–28.3 μmol/L. In urine collected at the morning, before swallowing the drug, there was no presence of LA. Concentration of LA in the urine reaches maximum value at 1 hour after oral administration of tablets for 27 and 40 years old women and at 1.5 and 2 hours for 41 and 23 years old women, respectively. In all examined cases the contents of LA returns to the initial value (0 mmol/mol creatinine) after 4 hours.

Main achievements

1. I have developed new sample preparation methods that involve various processes, such as homogenization, derivatization and reduction reaction, extraction, and deproteinization. These procedures allowed to analyze:
   - the thyroid gland, liver and brain of adult hens, chicken embryos and chicken eggs for MMI level [**H1**],
   - the plasma samples for MMI [**H1**] and HTL [**H6**] levels,
   - the urine samples for HTL [**H7**], LA [**H5**], thiosulfate [**H2**], CSH and CGSH [**H2**, **H3**], and sum of total thiols [**H4**].
2. I have developed a simple, sensitive and rapid methods for the routine determination of metabolically related sulfur compounds and sulfur pharmaceutical products in biological samples:
   - the method for the determination of MMI in animal tissues [**H1**],
   - the method for the determination of thiosulfate and thiols in urine [**H2, H3**],
   - the method for the determination of HTL in urine [**H7**],
   - the method for the determination of HTL in plasma [**H6**],
   - the method for the determination of LA in urine [**H5**],
   - the method for the determination of sum of total thiols in urine [**H4**].
3. I have applied the developed methods for clinical and pharmacokinetic studies, which allowed to:
   - estimate the urinary thiosulfate levels [**H2**],
   - evaluate the urinary excretion of the compounds containing the –SH group [**H2-H4**],
   - discover a previously unknown pool of HTL in human urine [**H7**],
   - determine HTL levels in urine and plasma, which allowed to study HTL role in mammals [**H6**, **H7**],
   - study the relationships between HTL and Hcy in human organism [**H6**, **H7**],
   - measure a reduced-to-oxidized ratio of main sulfur amino acids, excreted in the urine [**H3**],
   - evaluate the maternal transfer of MMI [**H1**],
   - conduct pharmacokinetic study of LA [**H5**].

References (without the publications which are the scientific achievement)

[1] Y. Ingenbleek, H. Kimura, *Nutritional essentiality of sulfur in health and disease*. Nutr Rev. 71 (2013) 413-432.

[2] H. Jakubowski, R. Głowacki, *Chemical biology of homocysteine thiolactone and related metabolites*. Adv. Clin. Chem. 55 (2011) 81-103.

[3] A. Hulanicki, *Współczesna chemia analityczna*. 2011, PWN, Warszawa.

[4] H. Jakubowski, *The determination of homocysteine-thiolactone in biological samples*. Anal. Biochem. 308 (2002) 112-119.

[5] K. Kuśmierek, G. Chwatko, R. Głowacki, P. Kubalczyk, E. Bald, *Ultraviolet derivatization of low-molecular-mass thiols for high performance liquid chromatography and capillary electrophoresis analysis*. J. Chromatogr. B, 879 (2011) 1290-1307.

[6] K. Kuśmierek, G. Chwatko, R. Głowacki, E. Bald, *Determination of endogenous thiols and thiol drugs in urine by HPLC with ultraviolet detection*. J. Chromatogr. B, 877 (2009) 3300-3308.

[7] E. Bald, R. Głowacki, *2-Chloro-1-methylquinolinium tetrafluoroborate as an effective and thiol specific UV-tagging reagent for liquid chromatography*. J. Liq. Chrom. Rel. Technol. 24 (2001) 1323-1339.

[8] Y. Mukai, T. Togawa, T. Suzuki, K. Ohata, S. Tanabe, *Determination of homocysteine thiolactone and homocysteine in cell cultures using high-performance liquid chromatography with fluorescence detection*. J. Chromatogr. B, 767 (2002) 263-268.

[9] R.F. Anderson, J.E. Packer, *The radiolysis of aqueous solutions of homocysteine thiolactone*. Int. J. Radiat. Phys. Chem. 6 (1974) 33-46.

[10] V. Ravichandran, S. Shalini, K.M. Sundram, H. Rajak, *Validation of analytical methods - Strategies & importance*. Int. J. Pharmacy and Pharm. Sci. 2 (2010) 18-22.

[11] T. Toyo'oka, *Recent advances in separation and detection methods for thiol compounds in biological samples*. J. Chromatogr. B, 877 (2009) 3318-3330.

[12] M.P. Murphy, *Mitochondrial thiols in antioxidant protection and redox signaling: distinct roles for glutathionylation and other thiol modifications*. Antioxid. Redox. Signal, 16 (2012) 476-495.

[13] K. Kuśmierek, R. Głowacki, E. Bald, *Analysis of urine for cysteine, cysteinylglycine, and homocysteine by high-performance liquid chromatography*. Anal. Bioanal. Chem. 385 (2006) 855-860.

[14] A. Pastore, R. Massoud, C. Motti, A. Lo Russo, G. Gucci, C. Cortese, G. Federici, *Fully automated assay for total homocysteine, cysteine, cysteinylglycine, glutathione, cysteamine, and 2-mercaptopropionylglycine in plasma and urine*. Clin. Chem. 44 (1998) 825-832.

[15] I. Fermo, C. Arcelloni, R. Paroni, *High-performance liquid chromatographic method to quantify total cysteine excretion in urine*. Anal. Biochem. 307 (2002) 181-183.

[16] P. Lochman, T. Adam, D. Friedecky, E. Hlidkova, Z. Skopkova, *High-throughput capillary electrophoretic method for determination of total aminothiols in plasma and urine*. Electrophoresis, 24 (2003) 1200-1207.

[17] G. Chwatko, E. Forma, J. Wilkosz, R. Głowacki, P. Jóźwiak, W. Różański, M. Bryś, A. Krześlak, *Thiosulfate in urine as a facilitator in the diagnosis of prostate cancer for patients with prostate-specific antigen less or equal 10 ng/mL*, Clin. Chem. Lab. Med. 51 (2013) 1825-1831.

[18] M.C. Belardinelli, A. Chabli, B. Chadefaux-Vekemans, P. Kamoun. *Urinary Sulfur Compounds in Down Syndrome*. Clin. Chem. 47 (2001) 1500-1501.

[19] H. Jakubowski, *Molecular basis of homocysteine toxicity in humans*. Cell. Mol. Life Sci. 61 (2004) 470–87.

[20] H. Jakubowski, *Translational accuracy of aminoacyl-tRNA synthetases: implications for atherosclerosis*. J. Nutr. 131 (Suppl.), (2001) 2983-2987.

[21] H. Jakubowski, *Homocysteine in Protein Structure/Function and Human Disease*. Springer 2013.

[22] S.L. Van Herck, S. Geysens, E. Bald, G. Chwatko, E. Delezie, E. Dianati, A. Ahmed, V.M. Darras, *Maternal transfer of methimazole and effects on thyroid hormone availability in embryonic tissues*. J. Endocrinol. 218 (2013) 105-115.

[23] L. Packer, E. Cadenas, *Lipoic acid: energy metabolism and redox regulation of transcription and cell signaling*. J. Clin. Biochem. Nutr. 48 (2011) 26-32.

[24] A. Bilska, L. Włodek, *Lipoic acid – the drug of the future?* Pharmacol. Rep. 57 (2005) 570-577.

[25] K.P. Shay, R.F. Moreau, E.J. Smith, A.R. Smith, T.M. Hagen, *Alpha-lipoic acid as a dietary supplement: Molecular mechanisms and therapeutic potential*. Biochim. Biophys. Acta, 1790 (2009) 1149-1160.

Grazyna Chwatko