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Review

Estimation of Thiol Compounds Cysteine and Homocysteine in Sources of Protein by Means of Electrochemical Techniques

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Thiols are compounds that contain one sulphanyl group (-SH) in their molecule. Due to this fact, many thiols play very specific and indispensable roles on which a given organism is very dependent. Regarding the redox properties of thiol groups practically all mercaptans can be detected very easily by means of electrochemical methods due to the fact that the oxidation of SH group provides great amount of electrons for the electrode reaction. Electrochemical techniques suitable for the detection of these compounds are still in progress. They can be used partly in stationary systems (where above all the methods of differential pulse polarography, voltamperometric techniques or catalytic signals are used) and partly in dynamic, flow-through and/or electromigration systems (where above all the methods of voltammetry, amperometry or coulometry are used). Development of miniaturized methods and their implementation into analytical system is a current aim. This study presents a survey of the most often used techniques and methods (as well of their modifications) used for the assessment of thiol compounds cysteine and homocysteine.

Keywords: electrochemical detection; thiol compounds; voltammetric and amperometric techniques, HPLC with electrochemical detection

1. INTRODUCTION

Electrochemical methods suitable for determination of thiol compounds are used because of their relative simplicity, cheapness and good sensitivity. Many electrochemical techniques and electrode materials enabling their estimation were suggested including the preparation of chemically modified electrodes and electrochemical detectors [1-4]. A predominating part of these compounds exist in the form of disulphides, which make them inaccessible for tagging agents so that it is necessary to split their disulphidic bonds with a suitable reducing agent [5]. Thiols are usually assessed

by means of chemiluminiscence [6] and fluorimetric [7] methods that are based either on a redox reaction or on the modification with chromophores and/or fluorophores. The final assay can be e.g. chromatographic [8-11] or electrochemical [12-21]. As far as the electrochemical methods are concerned, carbon materials are being used at most, above all because of their chemical inertness, wide potential and suitability for various types of analyses [22]. They can be used also for the preparation of modified electrodes including carbon nanotubes [23,24], fullerene c60 [25], and/or oxidated nanofibres [26,27], that enable an easier transfer of electrons and a better dispersion [28].

2. CHARACTERISTICS OF CYSTEINE

Cysteine (cys-L-2-amino-3-mercaptopropionic acid) belongs to the group of sulphur-containing biogenic amino acids. This compound contains a thiol group and it is very common in nature because it is an integral part of proteins. It is a result of transsulfuration reaction of L-methionine and L-serine. Cysteine is quite common in nature and plays an important role in many biological systems. It is a substrate that participates in the synthesis of proteins and formation of glutathione disulphide bonds. It is also a key extracellular reduction agent [29]. This amino acid participates in many important processes, among others also in the synthesis of proteins. The sulphhydryl group (-SH) plays a key role in biological activities of proteins and enzymes [30,31]. Cysteine is classified as a non-essential amino acid and this means that under normal physiological conditions, it is formed in sufficient amounts from the food. L-cysteine, exposed to atmospheric effects is oxidized to L-cystine, i.e. a dimer of two molecules of L-cysteine that contains a weak disulphidic bond. In plants, it participates in various metabolic pathways, including the transport of electrons and the photosynthetic phosphorylation [32]. Due to the reaction of electrons with Reactive Oxygen Species (ROS), cysteine functions as a detector of the redox status and subsequently its conformation is being changed. Mariotti et al. [33] reported that cysteine promotes glutathione levels in the liver of Wistar rats. Sulphur-containing antioxidants protect organisms against negative effects of heavy metals, modulate the radiosensitivity of cells, and protect DNA against damage [34].

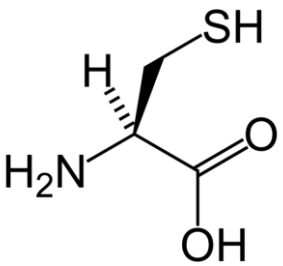

Figure 1. Structural formula of cysteine.

Cysteine is present in the majority of foodstuffs that contain high levels of proteins, e.g. quark, yoghurt, pork, wheat germs, oat flakes, and vegetables (garlic, onions, broccoli, and red peppers).

In the food industry, it represents a basic component of various flavours that are used within the framework of the baking process [35,36]. It is also present in various cosmetics and pharmaceuticals [37] sanitary materials, and food additives [38]. The compound protects the brain and the liver in persons abusing alcohol and positively influences both flexibility and texture of the skin [39]. It shows a strong antioxidative effect and increases the level of glutathione as well. In conjunction with antioxidative enzymes, it inhibits oxidative damage caused by those reactive forms involved in the oxidative stress [40].

To satisfy a high demand existing in many branches of the national economy (e.g. in food industry, biochemistry, pharmaceutical industry and medicine), it is necessary to develop simple and quick methods of L-cysteine assessment [41,42]. The analysis of L-cysteine can be performed by means of electrophoresis [43-45], chromatography [46-48], electrochemistry [49], or spectrophotometry [50]. Various analytical methods are also used, e.g. HPLC, UV-spectrometry [51], capillary electrophoresis with amperometric detection or with laser-induced fluorescence [52], gas chromatography, mass spectrometry [53], or ionex chromatography [54].

3. CHARACTERISTICS OF HOMOCYSTEINE

D-homocysteine is another sulphur-containing amino acid that is a product of methionine demethylation. It is an intermediate product of methionine metabolism. In plasma, about 1 % of its (mostly) oxidized form is present. It plays a key role in the metabolism of sulphur-containing amino acids and reactions of carbon transfer. It is also a co-factor of some vitamins (e.g. folic acid, B_{12}, B_6, and B_2). It can be isolated from blood plasma [55] as well. Various forms of homocysteine can be mutually combined. Together with cysteine, homocysteine can also form mixed disulphites and proteins. The amino acid is not present directly in food but it is produced during the metabolism of methionine [56,57].

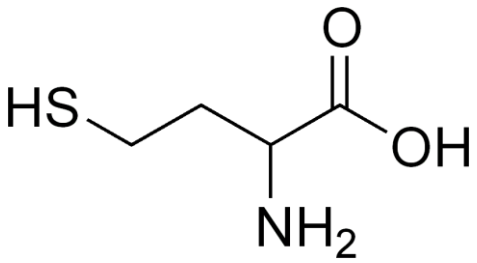

Figure 2. Structural formula of homocysteine.

In healthy individuals, the concentration of homocysteine in blood plasma ranges from 5 to 16 μM. Its increased concentrations may cause either hyperhomocysteinemia (with as much as 100 μM) or homocystinuria (about 500 μM) [58,59]. In humans, the total plasmatic concentration of homocysteine is defined as a sum of all kinds of this amino acid (i.e. its free, protein-bond, and

oxidized forms). Hyperhomocysteinemia is associated with a deficit of folic acid and cobalamine; this condition may cause complications during pregnancy and induce cognitive impairments, as well as formation of neoplastic tissues [60]. Its slightly increased plasmatic levels are associated with an increased risk of the occurrence of coronary and cerebrovascular problems [61-63], including atherosclerosis [64] and different types of thrombosis [65]. An increased level of homocysteine represents a risk factor for the occurrence of ischaemic heart disease, cardiovasculary diseases, and apoplexy [66,67] as well. In some works, homocysteine is revealed to be associated with some neurodegenerative processes (e.g. dementia and the Alzheimer disease) [68,69]. In the organism, homocysteine occurs in several forms: (i) free reduced homocysteine (1-2 %), (ii) oxidized form called homocystine (5 to 10 %) and (iii) mixed homocysteine disulphides (70-90 %). When estimating the free homocysteine, it is necessary to block free thiols; however, under normal conditions, it is rather difficult to fulfil this condition. For that reason, the total homocysteine (tHcy) is assayed, i.e. a sum of free and bond homocysteine [70]. In tissue cultures, a thioester of homocysteine called thiolactone [71,72] shows cytotoxic effects. An acute intravenous infusion of thiolactone is extremely neurotoxic and can induce spasms and it causes even death within several minutes after poisoning [73]. It also inhibits growth and causes developmental abnormalities. Thiolactone induces the growth of vascular endothelial and promyeloid cells and inhibits production of insulin [74] as well.

Biological thiols (including homocysteine) play a key role in the preservation of an internal redox homeostasis, mainly due to its high reactivity [75]. Their deficiency is also associated with growth inhibition, hair depigmentation, swellings, liver damages, and skin lesions [76]. The assessment of homocysteine is important not only for various biochemical studies but also for the diagnosis of associated diseases. Its increased level in blood plasma is also a factor that indicates the risk of heart attack [77] and damage of peripheral veins [78]. Homocysteine plays an important role in protection of cells against reactive forms of oxygen and some reactive intermediate products [79]. In human blood serum, homocysteine is the most abundant thiol. An exact assessment of homocysteine in blood plasma is very important for clinical diagnoses and it is also known that it plays an important role in diagnostics of congenital metabolic defects of sulphur-containing amino acids.

There are many different methods of homocysteine assessment. Chromatographic methods are rather elaborative and the both time and reagents consuming. For that reason, they cannot be routinely used in laboratories for high number of samples processing. Specifications of individual methods were published in number of papers [80-83]. Their advantage consists of the opportunity to assess disparate fractions in few steps. Capillary electrophoresis enables to assess a group of thiols, including homocysteine, in the course of only one analyse. In biological materials, homocysteine can be assessed by means of enzymatic methods [84], high performance liquid chromatography (HPLC) combined with electrochemical [85-88], UV [89], or fluorimetric detection [90], capillary electrophoresis [91-94], gas chromatography [95,96], immunochemical assays, and electrochemical methods [97].

4. ELECTROCHEMICAL ASSESSMENT OF CYSTEINE AND HOMOCYSTEINE

Basing on redox parameters of thiol compounds, it is possible to assess them by means of voltammetry and/or amperometry [98]. These two electrochemical methods can be used either directly or in combination with some separation methods, e.g. HPLC or capillary electrophoresis (CE) [99].

4.1 Voltammetric methods

Most frequently, a direct electrochemical assessment of thiol compounds is performed in stationary systems using electrodes containing mercury, glassy carbon, copper, gold, and platinum [98-106]. The sensitivity of individual methods is increased by means of thin films of various elements, compounds and nanoparticles that are immobilised on the surface of the electrodes. In many cases, the electrochemical analysis of thiol compounds is limited due to the fact that their electrocatalytic activity decreases with time [14,107-109].

Various authors assessed L-cysteine by means of voltammetric methods [110-115]. Although many of these methods have a good detection limit, they are not fully selective for L-cysteine and/or have a narrow linear dynamic scope, e.g. on glassy carbon electrodes. A glassy carbon electrode modified by Nile blue (NBA) can be used as a mediator of a quick, sensitive, and highly selective voltammetric assessment of L-cysteine [112,113]. The NBA seems to be a method that is suitable for the oxidation of L-cysteine. In presence of L-cysteine, the NBA anode peak current increases very steeply. The position of the NBA peak is more negative than that of L-cysteine. On the other hand, however, no obvious cathodic peak for L-cysteine can be observed in the system. This indicates that the process is irreversible, probably due to a weak interaction of sulphur atoms with graphite. The anodic NBA current increases with the increasing concentration of L-cysteine. The oxidized form of NBA reacts with L-cysteine. The intensity of L-cysteine catalytic oxidation is several times higher than that of NBA. The overall mechanism of this reaction consists of two steps. In the first one, NBA passes through a two-electron oxidation and loses one proton in the form of a positively charged ion. After that, an electrocatalytic reaction between the oxidized form of NBA and cysteine takes place [116].

In quoted reference [117] a method based on the chemisorption reaction of sulphur moiety of the cysteine molecule on the semi-crystallic gold electrode is described together with its subsequent reductive desorption. Electrochemical measurements can be performed using the differential pulse voltammetry combined with flow injection analysis for electrochemical detection. Electroactive species can be absorbed at the potential level 0.1 V versus SCE. In some other assays, their decreasing desorption takes place, at level -0.6 V versus SCE while the catalytic current runs simultaneously and the third potential step is applied with the objective to reach a complex regeneration of the gold electrode surface (-1.3 V versus SCE). A linear response with a good reproducibility was observed within the range of 1 to 6×10^{-6} M. Relative standard deviation is quite low (> 3.2 %) so good reproducibility of the method can be anticipated.

An electrode with immobilised film consisting from Prussian blue can be also used for the estimation of L-cysteine. In case that the redox status is changed, colour of the electrochromic material

alters as well. The Prussian Blue (KFe_3 [Fe_2 $(CN)_6$]) is a standard blue pigment that is often used for experimental purposes [118]. Different electrode materials, e.g. glassy carbon [119], graphite [120], gold [121] and platinum [122] were immobilized in the form of thin film layers. Some of them are based on the function of a selective competition of ions while others use direct electrocatalytic strategies, e.g. detection of hydrogen peroxide [123], cysteine [124] or morphine [125].

The solid silver amalgam electrode was used for the assessment of cysteine and cysteine-containing peptides by means of differential pulse voltammetry in presence of cobalt ions [126]. A modified electrode enabling the estimation of cysteine was made of a carbon electrode by means of a mechanic immobilisation with $CuFe(CN)_6$ [127]. Electrochemical behaviour of glutathione, cysteine, homocysteine, and acetylcysteine at different pH were studied using fluorine doped tin oxide electrode (FTO). At optimum pH values (2.4; 2.8 and 3.4), voltammetric studies on the electrochemical oxidation of glutathione, cysteine, and homocysteine had their peaks at 0.35; 0.29, and 0.28 V, respectively. As far as homocysteine and glutathione were concerned, experimental square wave voltammetry showed a linear course of current dependence at concentrations ranging from 0.1 to 1.0 mM [128]. When using the differential pulse voltammetry, anodic oxidation signals of cysteine and homocysteine were differentiated by means of a gold electrode modified with a fluorosurfactant (i.e. Zonyl (R) FSO) [129]. An electrochemical adaptation of the conventional Ellman reaction represents a new approach to the detection of thiol compounds. The electrochemical assessment of cysteine, N-acetylcysteine, homocysteine, glutathione, and captopril was performed using a glassy carbon electrode. The linear range of the cysteine assessment method was 2-120 μM [130]. The possible application of this method for detection of thiols in both acidic and alkaline environments was also tested [131].

The application of nanoparticles immobilised on electrodes represents the latest group of direct electrochemical assessment of thiols. A copper electrode was coated with vertically aligned carbon nanotubes. The electrochemical behaviour of cysteine was monitored by means of cyclic voltammetry, differential pulse voltammetry, and chronoamperometry. In this case, the electrode showed a higher electrocatalytic activity of cysteine oxidation than other comparable electrodes. Within the range of 1 and 500 μM and with applied potential +0.45 V, its linear dependence on cysteine concentration had coefficient of determination $R_2 = 0.998$ [132]. Nanoparticles were also used in studies dealing with an improvement of voltammetric assessment of N-acetyl-L-cysteine. A modified electrode was made as follows: at first, a poly (2,6-pyridinedicarboxylic acid) layer was applied on the surface of a glassy carbon electrode and thereafter, it was coated with nanoparticles of gold. N-acetyl-L-cysteine was assessed by means of a method of differential pulse voltammetry. The linear range of the method was 4-130 μM [133].

Lawrence et al. [134] used catechol difenylamin 4-sulfonic acid and octacyanomolybdate as a mediator in their study on the assessment of homocysteine while Siangproh et al. [135] worked with a diamond electrode. Further, electrodes with modified sulfhydryl oxidase [136], fluorosurfactants [137], gold nanoparticles [138] and carbon nanotube [139] were used as well. Because the oxidation potential of ascorbic acid is very similar to that of homocysteine, it is very difficult to estimate this thiol in presence of ascorbic acid. A good stability and reproducibility cannot be reached due to a surface contamination caused by the absorption of oxidized products on the electrode surface. There

were also some studies on the possible use of electropolymerised film of 2amino-1,3,4-thiadiazole, which enabled to assess homocysteine in presnce of high concentrations of ascorbic acid. The electrochemical method based on the application of Au-Hg dual electrodes is highly specific [140]. Frequent changes in the sites of Hg disposal the Au surface is quite necessary for the accomplishment of an optimum electrochemical reaction.

4.2 Methods using chromatographic techniques

A combination of electrochemical techniques and liquid chromatography represents another group of methods enabling to assess thiol compounds. The major part of these analytical methods is based on a combination of the chromatographic separation and pre- or post-column derivatization [141,142]. The most frequently used derivatization reagents are ninhydrin [143,144], phenylisothiocyanate [145] and 9 fluorenylmethoxycarbonyl chloride [146,147]. After derivatization, analyts can be measured due to these substances. Means of a method that combines high performance liquid chromatography with either electrochemical or other type of detection is suitable for the purposes [83,148-150].

Assessments by means of HPLC very often involve pre- or post-column derivatization of analytes, so that the results of chromatographic assessment are improved (as well as the detection limit). The chemical instability of glutamyl-S-ethenyl-cysteine (that occurs at pH differing from the neutral value) inhibits the progress of derivatization. However, glutathione can be assessed also without a preliminary derivatization step, only on the base of its peptide structure that enables a direct detection at 215 nm. As compared with other methods that necessitate the derivatization, this makes the analysis shorter. In another study [151] Zhang et al. combined the capillary-column liquid chromatography with the amperometric detection (CLC) and estimated thiols by means of a carbon electrode. In order to achieve a better separation and detection performance for analytes, several operational parameters were investigated: the working potentials, pH, and flow rate. Under the optimum conditions, the method could effectively separate and determine cysteine (Cys), glutathione (GSH), dopamine (DA) and 6-thiopurine (6-TP). The linear range covered over three orders of magnitude and the limits of detection were 8 fM for cysteine, 20 fM for glutathione, 8 fM for dopamine and 20 fM for 6-thiopurine, respectively.

L-cysteine was assessed by means of a combination of chromatography with electrochemical detection using the gold electrode and/or an amalgam-containing electrode in 0.01 M 2-N-morcobaltphtalocyanine on a carbon paste electrode (CPE) in the milieu of ethansulphonic acid at pH 5.5. Deoxygenation argon reacts with mercury already at a low oxidation potential [142].

A new HPLC-ECD method was developed and thereafter optimised for analyses of thiols (cystine, cysteine, homocysteine, methionine), reduced (GSH) and oxidised (GSSG) glutathione, and ascorbic acid present in human blood plasma and erythrocytes using dopamine as an internal standard [152]. To estimate thiols in urine, the method of CLC was used in combination with an amperometric detection on the carbon electrode [153]. Under optimum conditions, this method enables an effective separation and detection of cysteine, glutathione, dopamine, and 6-thiopurine [151].

Recently, a combination of electrochemical detection with capillary electrophoresis is being used very often. The main reason of this is a possibility of miniaturisation of both electrochemical detection systems and separating capillary electrophoresis down to the level of chips (Lab-on-chip) [154,155]. A method of homocysteine detection based on capillary electrophoresis combined with electrochemical detection was developed as well. The detection limit for the assessment of homocysteine was 500 nM and the course of the reaction was linear within the range of 1 to 100 μM. Methods of assessment of total (tHcy) and protein-bound (pbHcy) homocysteine in human blood plasma were studied and the method was optimized for the purpose. It was found out that, in healthy individuals, plasma concentrations of pbHcy and tHcy were 2.79 + / - 0.31 (n = 4) μM and 3.37 + / - 0.15 (n = 3) μM, respectively. This method was thereafter transferred into the CE-ES format microchips and tested when detecting homocysteine and reduced glutathione [156]. A method of HPLC combined with electrochemical detection was developed to assess total and free homocysteine in blood plasma. It is based on the reduction with 2-mercaptoethanole, carboxymethylation of free thiol and derivatization with ortho phthalaldehyde. The assessment of homocysteine in blood plasma is time-consuming. In biological systems, thiols easily pass through the oxidation and both the pre-centrifugation temperature and anticoagulants used show a marked effect on concentrations of homocysteine [157].

4.3 Methods of biosensoric detection of thiols

Another prospective group of methods for detecting of thiols is based on the application of electrochemical biosensors. Because enzymes are highly selective and they react quickly with individual specific substrates, the enzymatic electrodes represent a group of most frequently studied biosensors. A biosensor can be defined as an analytical device that combines biological materials (e.g. enzymes, cells, microorganisms, tissues etc.) with corresponding convertors (e.g. optic, electrochemical, calorimetric, and/or piezoelectric ones) that can supply selective and quantitative analytical data [4,158,159]. The application of many of these methods is limited, namely due to the fact that they lack specificity and/or that they are not adequately comparable with the other techniques.

For the assessment of cysteine, a biosensor was developed based on selective oxidase-peroxidase activities of copper and zinc superoxide dismutase (SOD) [160]. The biosensor consisting of SOD immobilised together with horse radish peroxidase (HRP) on polypyrrole (PPy)-platinum (Pt) electrode was characterised by means of cyclic voltammetry. Because of a bicarbonate-dependent peroxidase activity stimulated by thioloxidase activity SOD, the bienzymatic polypyrrole (PPy)-platinum (Pt) electrode showed an electrochemical response with cysteine. Of three thiols under study (i.e. cysteine, homocysteine, a glutathione), a marked current amplification was observed only in case of cysteine, so that it could be concluded that this biosensor was highly selective for cysteine. The response on the electrode was linear up to the concentration of 500 μM and the limit of detection was 10 μM. The cysteine biosensor is very stable, selective, and reproducible so that it is suitable for analytical purposes. This biosensor was tested in experiments with the assessment of cysteine in cysteine-containing food additives and in human blood serum and urine.

Inhibition effects of thiol organic compounds and heavy metals were studied as well [161]. A biosensor consisting from an electrode with enzymes from *Aspergillus niger* and *Agaricus bisporus* was developed for the assessment of L-cysteine in samples of human blood plasma. A biosensor constructed of carbon paste electrode modified with laccase separated from the fungus *Aspergillus oryzae* was suggested for the assessment of L cysteine in pharmaceutical compounds. It was characterised with regard to phenolic substances (caffeic acid, catechol, dopamine, hydrochinone, L-DOPA and methyl-L-DOPA), inhibitive effects (ascorbic acid, benzoic acid, L-cysteine, sodium sulphite, thiourea, and Ag^{+}, Zn^{2+}, Hg^{2+} ions. Methods based on the enzyme inhibition offer a high sensitivity and selectivity.

There are many biosensors constructed on the base of enzymatic inhibition [162]. A team of Chines scientists [163] assessed a low concentration of L-cysteine using a tyrosinase amperometric biosensor with catechol as a substrate. In their experiment, tyrosinase was immobilized on surface of a polyamide coated graphite electrode. A method based on immobilisation of peroxidase on the surface of a gold electrode using L-cysteine as a binding agent was developed and successfully performed as well. When the gold electrode comes into contact with L-cysteine, a bond between Au and thiol is formed [164,165]. The immersion of the electrode activates L-cysteine and hydrogen ions are separated from the hydroxyl group. Glutaraldehyde induces formation of Schiff bases cross-linking aldehyde groups with amino groups of molecules so that the sensitivity of the electrode is increased [159,166].

Other methods are based on a combination with redox enzymes glucosooxidase [167], alcohol oxidase [119], and cholesterol oxidase [168]. Optic sensors are used as well [169]. A thin film layer coating a transparent dielectric base can be used as a reducing agent and this mean can initiate optical changes [170].

Mayer et al. [171] who used a modified electrode, described the analysis of L-cysteine in cucumber leaves. In their experiments, the enzyme cysteine-desulfhydrase was immobilized on the electrode surface [172]. This enzyme catalysed the hydrolysis of L cysteine to pyruvate, hydrogen, hydrogen sulphide, and ammonia and the detected concentration of NH_3 was proportional to the concentration of L-cysteine in the sample.

5. CONCLUSION

This paper presents a survey and a comparison of various electrochemical techniques used for the assessment of thiol compounds cysteine and homocysteine. Above all in the domain of clinical diagnostics, there are high requirements concerning quick and accurate analytical methods. Because of their unique properties, they are also used as markers of the environmental pollution, various oncological diseases and/or oxidation stresses. Electrochemical techniques are both sensitive and selective and for that reason they represent an ideal tool how to satisfy these needs and requirements

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