

Low molecular mass thiols represent an essential factor of human metabolism and cellular homeostasis. As a result the imbalance in their physiological levels is often accompanied by severe pathological conditions and is closely related to some human disorders. Nowadays, accumulating evidences indicate that plasma homocysteine (Hcy) and metabolically related compounds, including cysteinyl-glycine (Cys-Gly), γ -glutamyl-cysteine (γ -GluCys), cysteine (Cys) and glutathione (GSH) are associated with these disorders and in some cases can act as a strong predictors of mortality in cardiovascular and cancer patients. Moreover, it is well established that their levels are related to other civilization diseases, including neurodegenerative diseases, obesity, chronic kidney disease, chronic renal failure, etc. Thus, significant efforts have been made over past decades to develop accurate and precise methods for determination of Hcy and other aminorhiols in different matrices.

Nowadays, the biological fluids most commonly analyzed for diagnostic purposes are blood (plasma) and urine but there is an emphasis on finding alternatives to them for clinical, pharmacological and pharmacokinetics studies. The latest publications dealing with this problem indicate saliva as an encouraging diagnostic specimen which exhibit many distinct advantages over blood (plasma) and urine. Contrary to them saliva offers a fast, easy and non-invasive sampling matrix for determination of different species. Moreover, its collection is possible everywhere regardless religious beliefs and avoids intrusion of privacy as well as discomfort associated with occasional inability to collect urine under supervision. Importantly, saliva is a highly complex fluid harboring biological molecules from a variety of sources including the genome, transcriptome, proteome, metabolome and oral microbiome which sampling is safe for operator. Interestingly, it has been shown that approximately 40% of biomarkers (or correlated compounds) of cancer and cardiovascular diseases is present in saliva, so far. Latest studies have also revealed that various drugs, metabolites as well as biomarkers can be found in saliva with high correlation to plasma and urine levels. Nevertheless, the problem concerning the possibility of replacement of blood (plasma) by easy accessible oral fluid in term of biologically relevant sulfur-containing compounds determination has not received any attention as yet.

So far, several procedures for the determination of low molecular mass thiols in biological specimens have been published in a large number of original papers and reviews. In particular, most of them depend on derivatization followed by

chromatographic separation in reversed phase mode (RP-HPLC). Indeed, these HPLC based methods offer a complementary tools for the determination of aminothiols but in most cases the goal is the analysis of the only one matrix. Moreover, majority of chromatographic assays dedicated to hydrophilic thiols measurement suffer from a relatively long sample preparation and analysis time as well as exploit buffered eluents containing harmful organic solvents, eg. acetonitrile (MeCN) in the mixture with ion-pairing reagents such as trifluoroacetic acid (TFA) or trichloroacetic acid (TFA). Unfortunately, such approach requires a long equilibration time of a chromatographic column, significantly increases the total costs of analysis and leads to negative influence on the environment because most of mobile phase constituents exhibit corrosive or cytotoxic properties and are persistent in the environment. Therefore, taking under consideration environmental conservation, human health and economy, it is reasonable to drive shift toward the development more green assays.

According to this, the main aim of this work was focused on elaboration of a new, precise, accurate and adhering to green chemistry rules HPLC based methods which will enable to determine low molecular mass thiols in different biological fluids. As a result, three simple and fast assays for the determination and quantification of urinary, salivary and plasma aminothiols in the form of their UV-absorbing 2-S-quinolinium, 2-S-lepidinium as well as tetrahydrothiazine and thiazolidine derivatives by high performance liquid chromatography have been developed. In particular, their environmental impact was minimized by application of user-friendly derivatizing reagent, significant reduction the use of hazardous mobile phase constituents and shortening analysis time without compromising separation. A major advantage that also evidently arises is the development of a single HPLC methods that are applicable to different matrices and enable to determine two or six aminothiols in one analysis run. Moreover, streamlined sample preparation procedures, involving reduction of disulfide bonds, derivatization and deproteinization, combined with the use of modern and easy accessible in clinical laboratories equipment guarantees that elaborated methodologies can be a valuable analytical tools to routine clinical analysis. These proposed high-throughput methods enable to analyze up to 570 samples per 24 hours. Importantly, the estimated validation parameters were more than sufficient to allow all of analytical methods to be used for monitoring of low molecular mass

thiols. The calibration lines performed with human plasma, urine and saliva spiked with thiol disulfides, within the practical concentration ranges, showed linear response of the detector with the coefficient regression of 0,99 or more. The unprecision of these methods, expressed as a relative standard deviations, ranged from 0,01 to 12,70% (intra-day) and 0,71 - 14,80% (inter-day) while the analytical recovery varied from 89,7% to 114,3% (intra-day) and 90,6% to 114,3% (inter-day). Finally, the utility of elaborated assays was confirmed by their successful implementation to analysis of the samples donated by 15 apparently healthy individuals as well as 10 anonymous patients suffering from breast cancer. Importantly, obtained results were consistent with earlier findings concerning thiol levels in biological fluids. Interestingly, these studies have also revealed correlation between saliva and plasma thiol levels. As a result, it has been concluded that saliva appears to be a valuable alternative to plasma regarding biological thiols determination. Nevertheless, in order to get more credible results a larger population should be undoubtedly investigated.

In conclusion, obtained results significantly enriched knowledge concerning sulfur-containing compounds analysis. Importantly, elaborated methodologies have some considerable advantages over other HPLC based methods what makes them an attractive choice for the determination of thiols in human plasma, saliva as well as urine. These experiments have also led to new insights concerning the potential replacement of plasma by saliva for diagnostic purposes. Moreover, it has been demonstrated that pyridoxal 5'-phosphate (PLP), primarily biologically active form of vitamin B6, is an effective Hcy and Cys derivatization reagent in human plasma, for the first time. Interestingly, it can be reasonably expected that the products of these reactions should be present in plasma as PLP reacts with Cys and Hcy under physiological conditions. These statements remains to be thoroughly examined. I strongly believe that the application of elaborated methodologies will help to verify these assumptions near future.