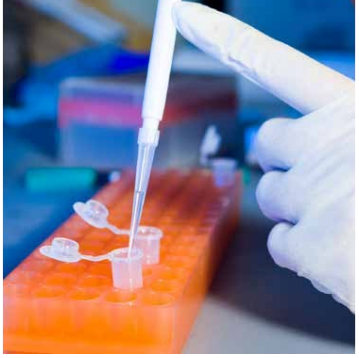
Application Note

Clinical & Diagnostics



The soundest LC-EC Applications for Clinical & Diagnostics analysis

Catecholamines Serotonin
Metanephrines VMA
HVA
5-HIAA Homocysteine
Glutathione
(di-)sulfides
Iodide
Vitamins A, C, D, E, and K
Q10
Ubiquinols

Glutathione, other thiols, and disulfides

- LC-ECD method with post-column in-line reduction of disulfide bonds
- Detection of components with thiol groups (incl. reduced disulfides)
- Reproducible and robust

Summary

In this note the analysis of glutathione (GSH), glutathione disulfide (GSSG) and several other thiols and disulfides is described using an LC-ECD method based on in-line conversion of disulfides before oxidative detection on a gold electrode.

Introduction

Glutathione (GSH) is a tripeptide composed of glutamate, cysteine and glycine (Fig. 1), which has numerous important functions within cells. Glutathione is among others involved in detoxification, amino acid transport across cell membranes (the γ-glutamyl cycle), it serves as a cofactor for some enzymatic reactions, and it aids the rearrangement of protein disulfide bonds. GSH also functions as an antioxidant in the cell.

The role of GSH as a reductant (antioxidant) is extremely important, particularly in the highly oxidizing environment of the erythrocyte. The sulfhydryl of GSH can be used to reduce peroxides formed during oxygen transport. The resulting oxidized form of GSH consists of two molecules bonded together to glutathione disulfide (GSSG). Therefore, the measurement of GSH/GSSG ratio (and other thiols and related disulfides) can be used as an indicator for the level of oxidative stress in vivo.

Thiols (like GSH) can be electrochemically detected in oxidative mode on a gold working electrode, but the disulfides are not detectable in this way (already oxidized). This can be overcome by adding a reactor cell in-line behind the HPLC column, and set it to reductive conditions. This cell will break the disulfide bonds and the resulting thiols are then detectable in the oxidative cell:

R'-S-S-R" + 2 H+ + 2 e- —> R'SH + R"SH

Thiols as well as the (unbound) disulfides can be quantified with this set-up with a single analysis. This is a major benefit over the alternative analytical method that is based on chemical reduction of the disulfide bonds in a split sample (requiring 2 analyses).

This note shows the simultaneous analysis of thiols and disulfides with the dedicated ALEXYS Disulfides Analyzer.

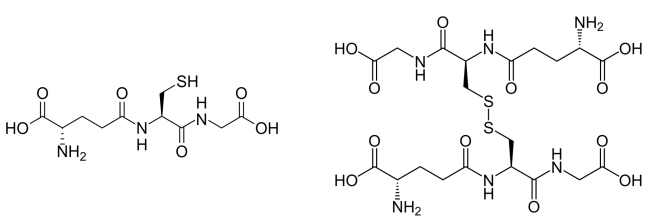


Figure 1: Molecular structures of (l) the thiol glutathione (GSH), and (r) glutathione disulfide (GSSG)

Method

The ALEXYS Disulfides Analyzer is an HPLC-ECD system with all parts optimized and dedicated to the simultaneous analysis of thiols and disulfides (Fig. 8). The DECADE Elite electrochemical detector of the ALEXYS system is outfitted with the Dual Cell Control (DCC) to facilitate the oxidative detection as well as the post-column reduction of disulfide bonds. Two FlexCells are in a serial arrangement behind the column. The FlexCell has a very small internal cell volume of less than 1 µL and therefore it should not contribute significantly to band broadening in the second cell. The first cell is equipped with a glassy carbon electrode, and the second cell is equipped with a gold electrode. The applied conditions are given in Table 1, unless stated otherwise.

Conversion in the post-column reductive cell

In serial flow cell configurations it is important that the first cell has a high conversion efficiency to favor detection in the second cell. The parameters that affect the conversion efficiency are working potential, flow cell dimensions (spacer thickness and working electrode area), and flow rate.

Working potential: The optimum working potential for most efficient conversion in the first cell was found to be in the range of -1.4 to -1.6 V. Without this reductive condition (Fig. 2), the disulfides are not detectable in the second oxidative cell.

Flow cell: The FlexCell has a relatively large working electrode surface, and was equipped with a thin 50 µm spacer. Using the thinner spacer in the flow cell will result in a smaller diffusion layer and this makes it easier for an analyte to reach the electrode surface.

Flow rate: At flow rates associated with standard-bore HPLC (about 1 mL/min), the conversion efficiency is typically 5 -10%. The conversion efficiency can increase considerably up to 30 - 80% when lowering the flow rate (even up to 100% at flow rates < 10 µL/min). As column dimensions dictate the applied flow rate, a decrease in flow rate can only be realized by using a smaller bore HPLC column. The use of microbore HPLC with 1 mm ID columns was thus chosen for this application, which gives a few additional benefits: it is compatible with small sample volumes and does not compromise detection sensitivity, and the lower flow rate also reduces the volume of chemical waste produced by the method

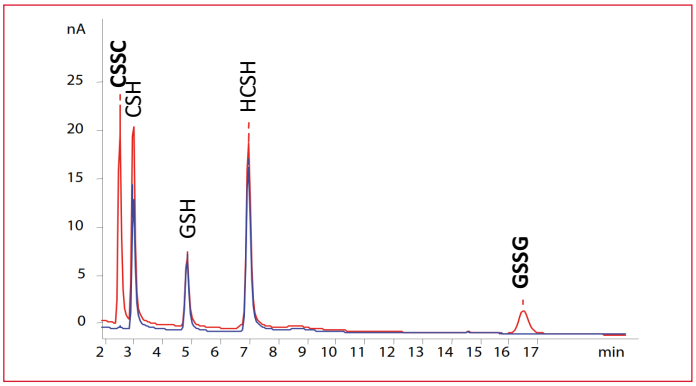


Figure 2: Analysis of 2 µL 1 µM cystine (CSSC), cysteine (CSH), glutathione (GSH), homocysteine (HCSH) and glutathione disulfide (GSSG) in mobile phase with reductive reactor cell on (red) or off (blue).

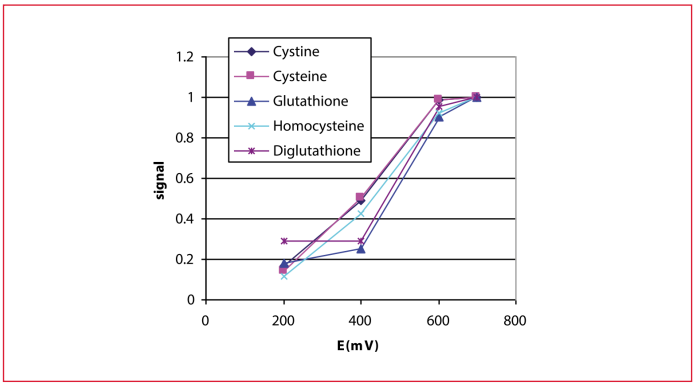


Figure 3: Normalized hydrodynamic voltammograms for oxidative detection of thiols and the indirect detection of disulfides after preceding reduction to their constituting thiols. The reference electrode that had been applied during construction was an ISAAC/2 mM Cl^-.

Electrochemical detection

A hydrodynamic voltammogram was constructed for different thiols and their associated disulfides. The disulfides were reduced to thiols before detection. The optimum electrode potential for oxidative detection of GSH, cysteine (CSH) and homocysteine (HCSH) on a gold electrode was 600 mV vs. ISAAC/2 mM Cl^- (Fig. 3), which corresponded to about 250 mV vs. HyREF on a FlexCell. For detection of thiols a FlexCell with gold electrode was applied as it is very easy to polish the gold electrode when it needs maintenance.

Table 1

Conditions	
LC system#	ALEXYS Disulfides Analyzer, Au including DECADE Elite DCC for red-ox application
Column	HyPURITY™ AQUASTAR™ HPLC column, 150 x 1.0 mm ID, 3 µm (Thermo Scientific™)
Mobile phase	50 mM phosphate buffer set to pH 3.0 with NaOH solution, 500 mg/L octane sulfonic acid (sodium salt hydrate)
Flow rate	50 µL/min
Backpressure	About 90 bar
Injection	2 µL
Temperature	35 °C (separation and detection)
Flow cell 1 (reduction)	GC FlexCell, HyREF, 50 µm spacer
$E_{cell\ 1}$	-1.4 V
$I_{cell\ 1}$	about -30 µA
Flow cell 2 (detection)	Au FlexCell, HyREF, 50 µm spacer
$E_{cell\ 2}$	+ 0.25 V
$I_{cell\ 2}$	about 20 nA
$ADF_{cell\ 2}$	0.5 Hz
$Range_{cell\ 2}$	100 nA/V

Original work done with an older but equivalent version of the new ALEXYS system

Cleaning pulse for the gold electrode

The electrochemical detection of thiols on a gold electrode involves the formation of covalent bonds with the surface (making it insensitive). Therefore, the gold electrode surface has to be regenerated regularly. For best reproducibility, each run is started with a short cleaning pulse. A working potential of +1 V and -1 V is applied for only 3 s, which is programmed in the time table of the method. After about 10 minutes the baseline has stabilized and the method automatically continues with a sample injection (Fig. 4).

Ion pairing separation

The thiols and disulfides of interest not only contain sulfide groups, but also amino and carboxyl groups as part of the molecular structure. Therefore, ion-pairing separation with acidic mobile phase on a reversed phase C_{18} micro-bore HPLC column was the method of choice for separation.

The retention of amino acids is affected by pH, the concentration of ion pairing reagent and percentage modifier.

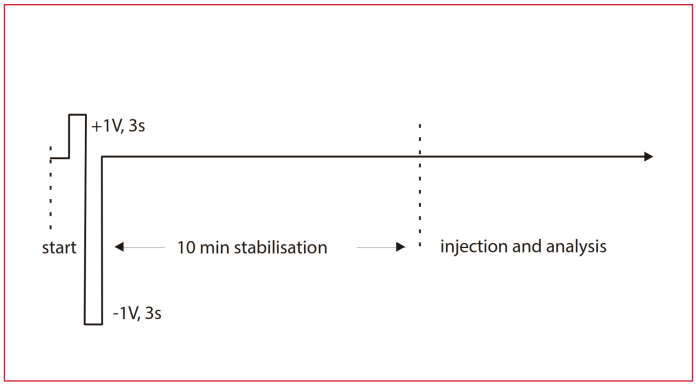


Figure 4: Schematic representation of the method timing

Decreasing the mobile phase pH below 4 (which is the pKa of carboxyl groups), will result in an increase of retention times as the carboxyl groups become neutral (Fig. 5 upper two traces). On the other hand, the free amine groups will have a positive charge (protonated) at acid pH. This can be counteracted by adding negative charged ion pairing agents to the mobile phase (Fig. 5). When multiple functional groups are involved, the retention behavior becomes less predictable. For example, at higher ion-pairing concentrations, the retention of glutathione disulfide decreases (Fig. 4, top and third trace).

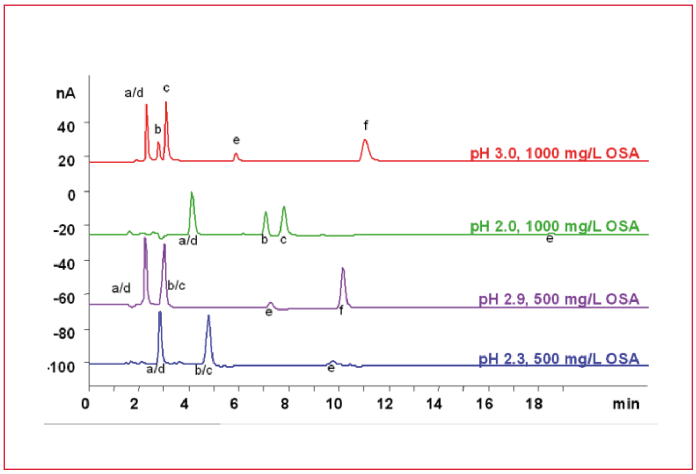


Figure 5: Chromatograms of CSH (a), GSH (b), HCSH (c), CSSC (d), GSSG (e) and HCSSCH (f) analyzed with different mobile phase pH and octane sulfonic acid sodium salt (OSA) concentrations. All other parameters were kept the same (see Table 1).

Results

The method was evaluated for reproducibility, linearity and detection limits.

Reproducibility

Reproducibility was investigated by analysis of standards (n=10). Before each run a cleaning pulse was applied as described in the method section. The RSD was between 2 - 7 % for peak heights and 2 - 6 % for peak areas. In case of strongly tailing peaks, the reproducibility of peak area is influenced by the automated integration parameters, and manual integration is advised in such cases

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Linearity and LOD

The linearity was investigated for a mix of thiols and disulfides in the range of 0.1 - 1 µmol/L (Fig. 7). Correlation coefficients better than 0.998 were found for peak heights. Linear regression data is given in Table 3.

Detection limits were calculated as the concentration resulting in a signal that is 3 times the peak-to-peak noise of the baseline. For CSSC, GSH and HCSH, a detection limit of 10 nM was found. If necessary, this can be further improved (by adjusting ADF, range, and the use of the new SenCell).

Table 2

Averages and % RSD of retention time, area and height, based on analysis of 1 µM thiols and disulfides (2 µL, n=10). Chromatograms shown in Fig. 7.

	Retention		Height		Area	
	tr (min)	RSD (%)	H (nA)	RSD (%)	A (nA.s)	RSD (%)
CSSC	2.48	0.2	22.8	3.2	168.9	2.7
CSH	2.95	0.2	24.7	3.1	195.4	3.6
GSH	4.80	0.1	6.8	6.5	69.6	6.0
HCSH	6.92	0.1	18.8	2.7	233.4	2.8
GSSG	16.42	0.1	2.2	5.2	55.5	5.0

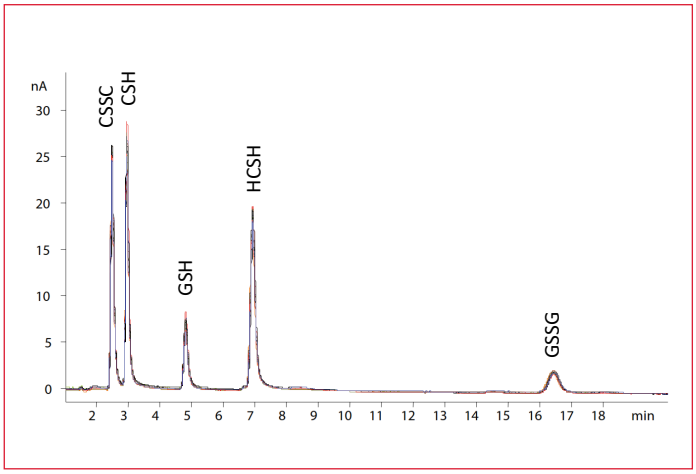

Figure 6: Overlay of 10 chromatograms of 1 uM CSSC, CSH, GSH, HCSH and GSSG dissolved in mobile phase. Injection volume 2 µL

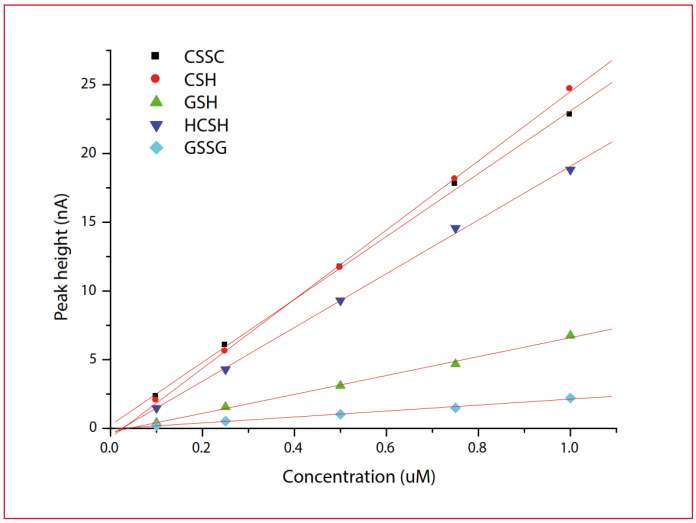

Figure 7: Calibration plots for various thiols and disulfides

Table 3

Linear regression (Y = a + bX) and correlation coefficients (r)

	Intercept a	Slope b	r
CSSC	0.23 ± 0.3	22.8 ± 0.4	0.99949
CSH	-0.67 ± 0.2	25.1 ± 0.3	0.99980
GSH	-0.27 ± 0.1	6.88 ± 0.2	0.99836
HCSH	-0.47 ± 0.2	19.5 ± 0.4	0.99943
GSSG	-0.03 ± 0.06	2.16 ± 0.1	0.99661

References

1. Johnson, D. C., & LaCourse, W. R. (1990). Liquid chromatography with pulsed electrochemical detection at gold and platinum electrodes. Analytical Chemistry, 62(10), 589A-597A.

Conclusion

Thiols as well as the oxidised free disulfides can be quantified using the ALEXYS Disulfides Analyzer. A cleaning step for the gold working electrode improves the reproducibility. A detection limit down to 10 nmole/L was achieved.

Figure 8: Recommended instrument configuration for this application: the ALEXYS Disulfides Analyzer, Au
The system consists of a P6.1L pump with integrated degasser, an AS6.1L autosampler, and the DECADE Elite electrochemical detector with Dual Cell Control (DCC).

Ordering information

Recommended ALEXYS analyzer + parts	
180.0068W	ALEXYS Disulfides Analyzer, Au
250.1045	Flattening/polishing kit for metal WE
Flow cell and software covered by the ALEXYS analyzer	
102.4305	Flexcell GC HyREF
102.4325	FlexCell Au HyREF
195.0035#	Clarity CDS single instr. incl LC, AS module

#) optional: The ALEXYS Disulfides Analyzer can also be controlled with Thermo Fisher Scientific™ Chromeleon™ CDS. Please contact Antec for more details.

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