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DETERMINATION OF GLUTATHIONE IN SCOTS PINE NEEDLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AS ITS MONOBROMOBIMANE DERIVATIVE

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SUMMARY

A method for determination of glutathione in its reduced (GSH) and oxidized (GSSG) forms in Scots pine extracts by reversed-phase high-performance liquid chromatography utilizing monobromobimane as the derivatization reagent was developed. The recovery and the precision for GSH in pine needles was high, *ca.* 99 and 3.6%, respectively. The determination of GSSG showed lower recovery (*ca.* 80%) and poorer precision (13.3%). The identity of the putative GSH-bimane derivative was confirmed indirectly by gas chromatography–mass spectrometry. GSH comprised 77% of water-extractable thiols in pine needles, and the ratio GSSG/GSH was low (0.024).

INTRODUCTION

Glutathione (γ-L-glutamyl-L-cysteinylglycine) is thought to be the major low-molecular-weight thiol in most living eucaryotic cells. Although convincing evidence for its wide distribution has not been established in the plant kingdom[1], it is suggested to be involved in numerous processes in the cell[2] such as a component of the hydrogen-scavenging system in chloroplasts[3].

Traditionally, quantitative analyses of reduced glutathione (GSH) in plants have been performed by titration of the total amount of free thiols with the sulphhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[4]. A more reliable procedure for the determination of GSH and also the oxidized form of glutathione (GSSG) is based on the highly specific enzyme glutathione reductase (E.C. 1.6.4.2)[5]. In the presence of reduced nicotinamide–adenine dinucleotide phosphate (NADPH) this enzyme catalyses the reduction of GSSG to GSH and with the addition of DTNB to the reaction mixture, the stoichiometric formation of the 5-thio-2-nitrobenzoic acid (TNB) occurs.

Recently, the rapid development of high-performance liquid chromatography (HPLC) has rendered alternative procedures for analysis of thiols, and techniques for the separation, derivatization and detection of GSH have been described. Saetre and

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Rabenstein[6] presented a method based on a mercury electrochemical detection, and Reeve *et al.*[7] utilized Ellmans reagents to convert GSH and GSSG into the mixed disulphides of TNB which were separated by reversed-phase HPLC. An alternative HPLC technique for determining thiols is based on carboxymethylation of the thiol group to block thiol–disulphide exchange reactions[8]. Free amino groups are then converted into 2,4-dinitrophenyl derivatives by reaction with 1-fluoro-2,4-dinitrobenzene, providing a chromatophoric group that can be detected at the nanomole level. Another procedure has been described by Newton *et al.*[9] for determination of thiols at the picomole level. This method is based on the conversion of thiols into fluorescent derivatives with monobromobimane (mBBr) and separation of the bimane derivatives by reversed-phase HPLC.

Glutathione, as well as other thiols, is considered to be an important metabolite in relation to the phytotoxicity of air pollutants to plants. In experiments with Scots pine[10] and *Picea abies*[11] it has been shown that GSH can serve as an indicator of the phytotoxic effects of compounds such as SO_2 and O_3. However, the analysis of glutathione in conifer extracts requires special precautions, both during the initial extraction and the subsequent analysis. GSH oxidizes readily[12] and conifers are known to contain a number of compounds including high levels of phenolic compounds and terpenes[13], that might interfere in the analysis. Adaption of an analytical method for quantitative analysis of glutathione in conifer tissues therefore necessitates careful examination to optimize the procedure.

The aim of the present study was to investigate possibilities of adapting the method by Newton *et al.*[9] for the analysis of GSH and GSSG in extracts from conifer needles. Quantitative analysis by reversed-phase HPLC with fluorescence detection of GSH-bimane derivatives in pine extracts was examined in terms of accuracy and precision. The identity of the putative GSH-bimane HPLC peak was subsequently confirmed by gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Millipore water was used in all steps of analysis.

Extraction of plant material

Extraction was performed in a cold room (4°C) by grinding 200 mg needles of Scots pine (*Pinus sylvestris* L.) in liquid nitrogen in a mortar. The plant material was then transferred to a glass column and eluted twice with a total of 5 ml of an ice cold medium consisting of water or 0.1 m*M* dithiothreitol (DTT) and 1 m*M* EDTA. The whole procedure was completed within 10 min. An aliquot of the extract was used for determination of GSH or GSSG.

Derivatization

Typically, 500 μl of sample or standard were added to a premixed solution of 200 μl 0.1 *M* Tris–HCl (pH 8), 100 μl 4 m*M* mBBr in a 3-ml test-tube. The samples were mixed vigorously and the reaction continued for 30 min in darkness at 25°C. A 150-μl volume of glacial acetic acid was then added to stop the reaction, and the samples were diluted to a final volume of 1 ml in water. Prior to HPLC analysis the samples were centrifuged at 7000 *g* (Labofuge 6000, Heraeus) for 10 min. Samples

eluted with 5-sulphosalicylic acid (5%, v/v) were derivatized with mBBr according to Anderson and Meister[12].

GSSG was determined in extracts or standard samples after alkylation of GSH by derivatization with the sulphhydryl reagent 2-vinylpyridine[14]. This was achieved by vigorously mixing 2-ml samples with 100 μl 0.1 *M* Tris–HCl (pH 6.5) and 40 μl 9.3 *M* 2-vinylpyridine in test-tubes. After 60 min at 25°C, excess of 2-vinylpyridine was removed by partitioning the sample three times against 150-μl volumes of diethyl ether. Then the GSH content of one part of the sample was determined and used as a blank and the other part was used for the determination of GSH after GSSG had been reduced to GSH. This was carried out by adding 50 μl 4 m*M* NADPH and 2 μl glutathione reductase from spinach (*ca.* 59 units ml^{-1}, Sigma) to the derivatization mixture with mBBr as described above.

Derivatization of GSH and GSSG utilizing 9-fluorenylmethyl chloroformate (FMOC) was performed according to Näsholm *et al.*[15].

High-performance liquid chromatography

Solvents were delivered at a flow-rate of 1 ml min^{-1} by a Waters liquid chromatographic system consisting of two M 45 pumps, a dynamic solvent mixer and a M 680 gradient master. Samples were introduced off-column via a Valco loop injector fitted with a 25-μl loop. Reversed-phase separations were carried out on a 250 mm × 4.6 mm I.D. ODS-Hypersil (5 μm) column eluted from 0 to 20 min with an isocratic system using 85% of solution A [10% methanol (v/v) and 0.25% glacial acetic acid (v/v), adjusted to pH 3.9 with NaOH] and 15% of solution B [90% methanol (v/v) and 0.25% glacial acetic acid (v/v), adjusted to pH 3.9 with NaOH], followed by 100% of solution B to regenerate the column. The column effluent was directed to a Shimadzu Model RF fluorimeter (excitation at 379 nm, emission at 475 nm).

The separation and detection of GSH and GSSG-FMOC derivatives by reversed-phase HPLC was performed according to procedures described for amino acids by Näsholm *et al.*[15], with a slight modification of the solvent programme. The separation was carried out with the following percentages of methanol in buffer (7 ml glacial acetic acid and 1 ml triethylamine to 1 l water, adjusted to pH 4.2 with NaOH): 0–15 min, 55%; 15–20 min, 55–65%; 20–40 min, 65%; 40–50 min, 100% and 50–60 min, 55%.

GC–MS analysis

Qualitative analysis of GSH in an extract from pine needles was carried out using the procedure outlined in Fig. 1. Approximately 90 nmol of the putative GSH-

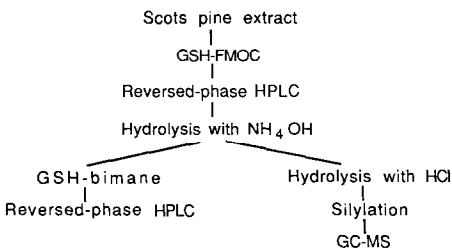

Fig. 1. Qualitative analysis of reduced glutathione in Scots pine.

FMOC peak from reversed-phase HPLC of a Scots pine extract were collected and 200 μl concentrated ammonium hydroxide were added for 12 h at 100°C to perform base hydrolysis of the FMOC derivative. An aliquot of the sample was thereafter reduced to dryness with a stream of nitrogen and dissolved in 200 μl medium (0.1 m*M* DTT and 1 m*M* EDTA). This sample was taken for determination of GSH by derivatization with mBBr and analysis by reversed-phase HPLC to confirm the peak identity. The other part of the base hydrolysed putative GSH-FMOC fraction was reduced to a dryness with a stream of nitrogen and hydrolysed with 6 *M* HCl under vacuum for 24 h at 100°C. Thereafter the sample was again reduced to dryness, dissolved in 100 μl acetonitrile and silylated with bis(trimethylsilyl)trifluoroacetamide (100 μl, 80°C for 12 h). Mass spectrometric analysis of concentrated aliquots was performed with a HP 5890 gas chromatograph linked via a direct capillary inlet to an HP 5970B mass selective detector equipped with an HP 9000 computer system. Samples were introduced in the splitless mode (splitless time 2 min) at 225°C onto a 25 m × 0.31 mm I.D. cross-linked methyl silicone capillary column with a 0.52-μm film. The column temperature was initially held at 60°C for 3 min, then raised at 30°C min^{-1} to 130°C than at 7°C min^{-1} to 235°C. The interface temperature was maintained at 250°C. The retention time for air was 63 s.

GSH standards and the total water extractable thiol concentration in Scots pine needles were estimated by the method described by Grill *et al.*[16].

RESULTS

Extraction

The maximum yield of GSH was achieved by grinding approximately 200 mg needles in liquid nitrogen in a mortar and extracting the material with 5 ml ice-cold medium, consisting of 0.1 m*M* DTT and 1 m*M* EDTA or water, 1.103 and 1.146 μmol GSH (g fresh weight)$^{-1}$, respectively (Table I). Extraction of the plant material with additional medium, or by using extended extraction times, did not improve the yield. In addition, hot ethanol also gave low yields of GSH[17]. The stability of GSH during the extraction was tested after 0, 15 and 60 min after initial extraction in the presence of 0.1 m*M* DTT and 1 m*M* EDTA or 5-sulphosalicylic acid (results not shown). It was found that GSH is stable within the time used for the assay. A slight

TABLE I

COMPARISON OF DIFFERENT EXTRACTION MEDIA AS REGARDS THE YIELD OF GLUTATHIONE EXTRACTED FROM PINE NEEDLES

The samples were derivatized with monobromobimane and the values are the averages of three separate extractions, except for 5-sulphosalicylic acid (n = 2). Data are mean ± S.D.

Extraction	*GSH (μmol/g f.wt.)*	*Yield (%)*
Water	1.146 ± 0.033	100 ± 2.9
0.1 m*M* DTT/1 m*M* EDTA	1.103 ± 0.024	96.3 ± 2.2
Ascorbate (0.15%)	0.884 ± 0.008	77.1 ± 0.9
5-Sulphosalicylic acid (5%)	0.924 ± 0.007	80.6 ± 0.7

reduction of GSSG standards by 0.1 m*M* DTT and 1 m*M* EDTA was however detected (3.1%) when compared to samples dissolved in water.

Derivatization

Samples and standards showed maximum derivatization efficiency at a buffer pH of 8–9 (Fig. 2). The derivatization efficiency with time indicated that a derivatization time of 15 min was sufficient.

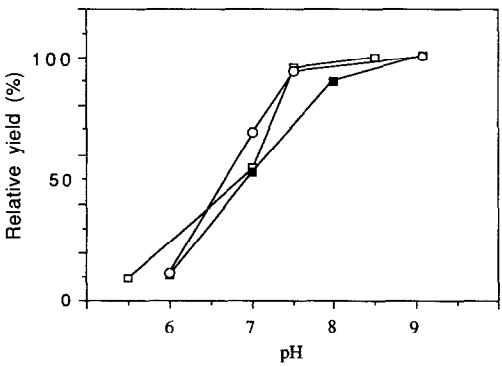

Fig. 2. pH dependence of the yield of reduced glutathione from a standard and an extract of Scots pine. 0.1 *M* sodium phosphate buffer was used in the derivatization mixture in the range pH 5–7, and 0.1 *M* Tris–HCl in the range pH 8–9. Standards were dissolved in 0.1 m*M* DTT/1 m*M* EDTA and needles were eluted in 0.1 m*M* DDT/1 m*M* EDTA or 0.15% ascorbate. ○ = standard; □ = extract in 0.1 m*M* DTT/1 m*M* EDTA; ■ = extract in 0.15% ascorbate.

HPLC separation

The chromatographic conditions used give a separation of the GSH-bimane derivative peak from other components in the pine needle extract (Fig. 3). There was no significant background interference with the analysis and when GSH was masked with 2-vinylpyridine prior to derivatization no GSH-bimane peak was detectable (data not shown). Using FMOC as a chromatographic group it was possible to get a separation of both GSSG and GSH from pine needle extract (Fig. 4). The GSSG-FMOC peak was identified by spiking the sample with standard GSSG-FMOC (Fig. 4b).

Identification of GSH from a pine extract by GC–MS analysis

The putative GSH-bimane derivative peak from HPLC was identified by using the procedure outlined in Fig. 1.

Silylated amino acids produce characteristic ions at $M^+ - 15$, $M^+ - 43$ and $M^+ - 117$ corresponding to loss of $-CH_3$, $-COCH_3$ and $-COO-TMS$ respectively[18], but also ions with *m/z* 73 [$Si(CH_3)_3$] and 147 [$(CH_3)_3SiOSi(CH_3)_2$] are diagnostic for silylated amino acids.

Retention times in the GC system for the putative N-trimethylsilyl esters of Gly, CySH and Glu of a hydrolysed GSH fraction from HPLC were 9.11, 12.70 and 13.64 min. In all GC peaks the base fragments for the corresponding amino acid were present [$M^+ - 117$, *m/z* 174, 220 and 246 for Gly, CySH and Glu, respectively (Table

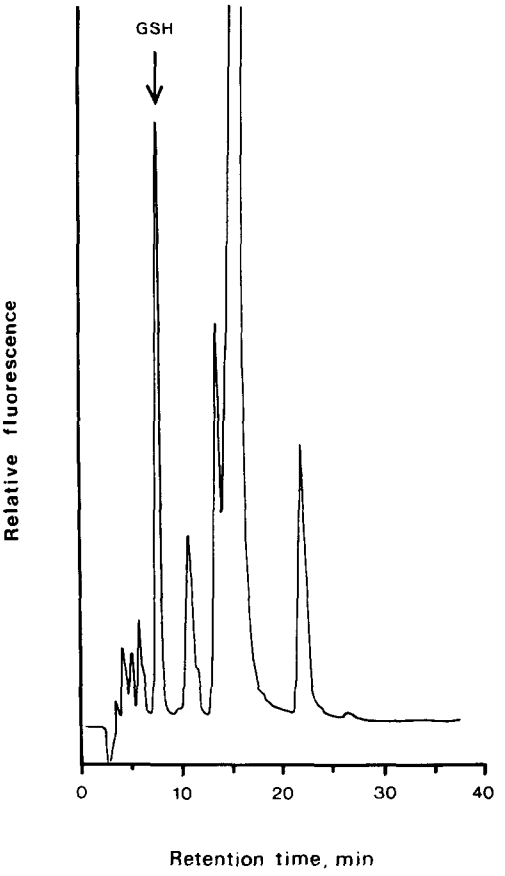

Fig. 3. Representative chromatogram of reduced glutathione (GSH) from an extract of Scots pine needles derivatized with monobromobimane.

III)], together with *m/z* 73 and 143. Only the putative Glu peak produced a detectable M^+ [Glu(TMS)$_3$], together with *m/z* 84 and 156, which were specific for the Glu and Gln derivatives. The largest fragment of the putative CySH was *m/z* 322 corresponding to $M^+ - 15$ (bis-TMS). In addition, a fragment at *m/z* 218 (TMS–NHCHCOO–TMS) was present, which represents loss of the side chain from the molecular ion of CySH[18]. Ions at *m/z* 174 and 86 are diagnostic for the structure RCH_2NH_2, and both were present in the putative Gly peak [*m/z* 174, $(TMS)_2N^+=CH_2$; 86, $(CH_3)_2Si^+=CH_2$][18]. In addition, a characteristic ion for CySH at *m/z* 248 was also observed in this spectrum.

Recovery and precision

The accuracy of the method was determined by comparing standards with standards of different concentrations mixed with subsamples from pine needle extract. The amount of GSH-bimane derivatives detected was linear (tested by least squares linear regression) in the concentration range tested. The recovery of 50 and

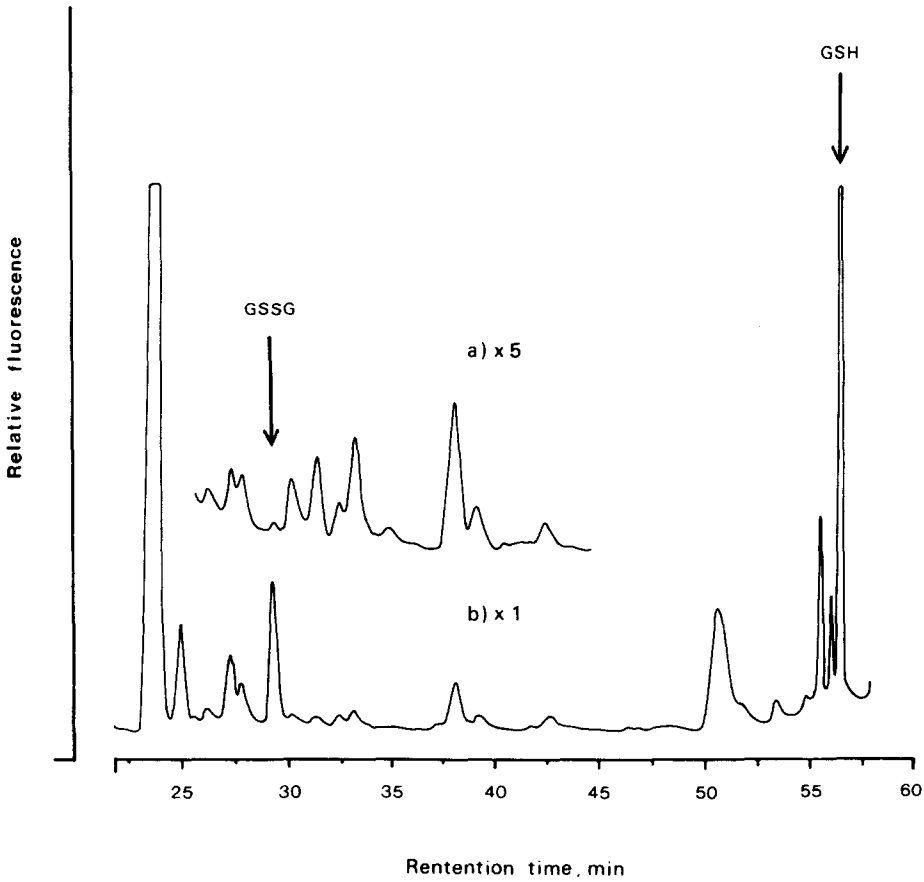

Fig. 4. (a) Chromatogram of Scots pine needles extract derivatized with 9-fluorenylmethyl chloroformate (FMOC). (b) As in (a) but spiked with 200 pmol GSSG derivatized with FMOC.

250 nmol GSH added to extracts was 99 and 100.3%, respectively (Table II). Recoveries from extracts spiked with 1 and 2 nmol GSSG were 80 and 86.7%, respectively (Table II).

The precision of the method was calculated as the standard deviation of the amount of GSH and GSSG determined in subsamples of three individual pine extracts. The precision was 3.6% for the determination of GSH and 13.3% for GSSG (Table II).

Analysis of GSH, GSSG and water-extractable thiols from a pine extract

Pine needles collected outdoors in April (1987) in Umeå were analysed for GSH, GSSG and water-extractable thiols and the averages from two samples were 0.201, 0.0045 and 0.26 μmol (g fresh weight)$^{-1}$, respectively.

TABLE II

RECORDER RESPONSES FROM STANDARDS AND STANDARDS OF DIFFERENT CONCENTRATIONS MIXED WITH SUBSAMPLES FROM A PINE NEEDLE EXTRACT

Samples were derivatized with monobromobimane and prepared for analysis as described in the text. Data are mean ± S.D. of three samples.

Amount added (μM)	*Recorder response (mV, relative unit)*		*Recovery (%)*
	Standard	*Standard + extract*	
GSH			
0.00	–	13.0 ± 0.5	–
0.05	69.0 ± 0.9	81.2 ± 1.4	99
0.25	354.0 ± 1.2	368.0 ± 4.2	100.3
GSSG			
0.000	–	9.8 ± 1.3	–
0.001	20.2 ± 2.3	24.0 ± 4.2	80
0.002	37.6 ± 2.7	41.1 ± 2.0	86.7

TABLE III

CHARACTERISTIC AND ABUNDANT IONS IN THE 70 eV ELECTRON IMPACT MASS SPECTRA OF AMINO ACIDS FROM A HYDROLYZATE OF GLUTATHIONE FROM PINE NEEDLES

Amino acid	M^+ *[m/z (%)]*	$M^+ - 15$ *[m/z (%)]*	*Base peak (m/z)*	*Other characteristic ions [m/z (%)]*	
CySH	–	322(3)	220	218(88)	100(22)
Glu	363(2)	348(5)	246	156(16)	84(8)
Gly	–	276(6)	174	248(17)	86(15)

DISCUSSION

In the present study the method of Newton *et al.*[9], using mBBr as a chromatophore, originally devised for determining thiols in red blood cells, was applied to extracts from Scots pine needles. The method developed for extraction of GSH from pine needles and detection of the GSH-bimane derivative by reversed-phase HPLC was shown to be acceptable both with regard to recovery (*ca.* 99%) and precision (3.6%) (see Table II). The recovery of GSH was similar to the value reported by Fahey *et al.*[19] for red blood cells (97%). After grinding the needles in liquid nitrogen, the extraction with 0.1 m*M* DTT and 1 m*M* EDTA or water both showed high yields of GSH. Usually extraction of GSH from biological samples has shown a need for rapid acidification, because of rapid autooxidation at pH > 7 and the effect of γ-glutamyl transpeptidase[20]. However, acidification was not necessary when GSH was analysed in pine needles. This may be due to the acidic nature of the unbuffered pine extract which was typically pH 4.5–5.5. Oxidation of GSH did not occur in the pine extract. This was indicated by the amount of GSH remaining unchanged when derivatized up to 45 min after extraction. Further evidence of the stability of GSH is the

fact that yields were not enhanced when tissue was extracted with 5-sulphosalicylic acid. This indicates that both extraction with a low concentration of DTT (0.1 m*M*) or water can be used successfully when GSH is extracted from pine needles. However, since a small amount of the GSSG (3.1%) in standard solution is reduced to GSH, water is preferable when GSSG is analysed. The DTT would not interfere with the GSH analysis when the GSSG concentration or any other reducible GSH conjugate is low compared to the concentration of GSH. This is obviously the case when pine needles were extracted by 0.1 m*M* DTT since no increase in the yield of GSH was detected when 0.1 m*M* DTT was used compared to water alone (Table I). Reproducible results were obtained when samples were derivatized with 0.5 m*M* mBBr in 25 m*M* Tris–HCl at pH 8 for 15 min. The reaction of bromobimanes with GSH are second order and dependent on pH, the active nucleophile being the thiolate anion, GS-(ref. 21). Although mBBr reacts preferentially with thiols, it also reacts with amines, phosphate, carboxylates and other nucleophiles and thus may interfere with the analysis[22]. Kosower and Kosower[23] also showed that amine buffers give an higher background if used above pH 8 due to the amine acting as a nucleophile. It is thus preferable to derivatize at a pH that does not enhance nucleophilic reactions that could interfere with the analysis. Tests of the stability of GSH in pine needles during storage at −80°C for up to 3 weeks indicated no change in endogenous GSH levels.

The analyses of GSSG from pine extracts showed lower recoveries (*ca.* 80%) and poorer precision (13.3%) than obtained with GSH (see Table II). The procedure for measuring GSSG, after the conversion of GSSG into GSH by glutatione reductase, is more complicated than the determination of GSH and this may account for the lower recoveries and less precise estimates. One possible reason for the low recovery is that phenolic and tannin compounds interacted with the enzyme and decreased the yield of GSH.

The reversed-phase HPLC separation of the GSH-bimane derivative was performed with a slight modification of the isocratic system described by Anderson and Meister[12]. This eluted the GSH-bimane derivative as a single peak. It was not possible to characterize this peak by GC–MS, although its identity was confirmed by GC–MS of rederivatized amino acid components of the GSH-FMOC peak. Lower amounts of N-trimethylsilyl esters of CySH were detected compared to esters of Glu and Gln. However, CySH is not stable to strong acid[24]. Using FMOC as a chromatophoric group it was possible to make qualitative determinations of both GSH and GSSG in an extract of Scots pine needles by HPLC.

A comparison of the amount of GSH to the total water-extractable thiols shows that GSH made up 77% of the low-molecular-weight thiols in Scots pine needles collected outdoors in April. This value is lower than the 96% reported for *Picae abies* by Grill *et al.*[16]. However, it is in agreement with values reported for non-glutathione thiols from hardened citrus leaves[17]. The ratio GSSG/GSH was low (0.024), in agreement with published data on other species[17,25].

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REFERENCES

1 T. Kassai and P. O. Larson, in W. Herz, H. Grisebach and G. W. Kirby (Editors), *Progress in the Chemistry of Organic Products*, Vol. 39, Springer, Vienna 1980, p. 243.
2 H. Rennenberg, *Phytochemistry*, 21 (1982) 2771.
3 K. Asada and M. Takahashi, in D. J. Kyle, C. B. Osmond and C. J. Arntzen (Editors), *Photoinhibition*, Elsevier, Amsterdam, 1987, Ch. 10, p. 227.
4 G. L. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70.
5 F. Tietze, *Anal. Biochem.*, 27 (1969) 502.
6 R. Saetre and D. L. Rabenstein, *J. Agric. Food Chem.*, 26 (1978) 982.
7 J. Reeve, J. Kuhlenkamp and N. Kaplowitz, *J. Chromatogr.*, 194 (1980) 424.
8 D. J. Reed, J. R. Babson, P. W. Betty, A. E. Brodie, W. W. Ellis and D. W. Potter, *Anal. Biochem.*, 106 (1980) 55.
9 G. L. Newton, R. Dorian and R. C. Fahey, *Anal. Biochem.*, 114 (1981) 383.
10 G. Wingsle, T. Näsholm, T. Lundmark, A. Ericsson and J. E. Hällgren, in S. Schulte-hostede (Editor), *2nd International Symposium on Air Pollution and Plant Metabolism, April 6–9, 1987, Workshops and Poster Presentations*, GSF-Bericht 9/87, München, 1988, p. 62.
11 H. Mehlhorn, G. Seufert, A. Schmidt and K. J. Kunert, *Plant Physiol.*, 82 (1986) 36.
12 M. E. Anderson and A. Meister, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 707.
13 B. Nyman, *Phytochemistry*, 24 (1985) 2939.
14 O. W. Griffith, *Anal. Biochem.*, 106 (1980) 207.
15 T. Näsholm, G. S. Sandberg and A. Ericsson, *J. Chromatogr.*, 396 (1987) 225.
16 D. Grill, H. Esterbauer and U. Klösch, *Environ Pollut*, 17 (1979) 187.
17 C. L. Guy, J. V. Carter, G. Yelenosky and C. T. Guy, *Cryobiology*, 21 (1984) 443.
18 H. Iwase, Y. Takeuchi and A. Murai, *Chem. Pharm. Bull.*, 27 (1979) 1307.
19 R. C. Fahey, G. L. Newton, R. Dorian and E. M. Kosower, *Anal. Biochem.*, 111 (1981) 357.
20 M. E. Anderson, *Methods Enzymol.*, 113 (1985) 548.
21 A. E. Radkowsky and E. M. Kosower, *J. Am. Chem. Soc.*, 108 (1986) 4527.
22 R. C. Fahey and G. L. Newton, *Methods Enzymol.*, 147 (1987) 85.
23 N. S. Kosower and E. M. Kosower, *Methods Enzymol.*, 147 (1987) 76.
24 D. C. Carpenter, *J. Am. Chem. Soc.*, 53 (1931) 1812.
25 W. Bielawski and K. Joy, *Phytochemistry*, 25 (1986) 2261.