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Determination of oxidized, reduced and protein-bound glutathione in eye lenses by high-performance liquid chromatography and electrochemical detection

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ABSTRACT

A method for the detection of oxidized, reduced and protein-bound glutathione in eye lenses has been developed. Homogenized lens samples are deproteinated with acetonitrile and perchloric acid. Protein-bound glutathione is reduced by 1,4-dithiothreitol. Separation of the different forms of glutathione and dithiothreitol is performed by ion-pair reversed-phase high-performance liquid chromatography with sodium octylsulphate as the ion-pairing agent. The compounds are detected amperometrically using on-line-generated bromine, which oxidizes thiols and disulphides. In this way two samples can be analysed in triplicate in a single day. The lower detection limits are 80 and 48 nmol per gram wet lens for reduced and oxidized glutathione, respectively. The amounts of free reduced and protein-bound glutathione in calf lenses, determined with this method, are 6.8 ± 0.4 and 0.96 ± 0.03 μmol per gram wet lens, respectively. That of oxidized glutathione is less than 0.048 μmol per gram wet lens.

INTRODUCTION

Since sulphydryls (thiols) and disulphides are involved in a number of biological processes, they are extensively studied. A range of detection techniques has been described for these types of compound [1]. Most are based on spectroscopic methods, which require pre- or post-column derivatization and detection limits are generally between 10 and 100 pmol/ml [1]. Few of these methods allow simultaneous detection of thiols and disulphides. One that does is enzymic recycling [2], but this is a time-consuming and expensive method. Some efforts have been made to determine thiol compounds by high-performance liquid chromatography (HPLC) combined with electrochemical detection [3–7]. In most of these approaches disulphides are electrochemically reduced on-line post-column to

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thiols, which are subsequently oxidized for detection. In order to achieve the required selectivity, electrochemically generated oxidizing agents can be used [8,9]. In our department, post-column on-line electrochemically generated bromine was used to oxidize both thiols and disulphides, and the amount of reduced bromine was detected amperometrically [10]. Thus glutathione in its reduced (GSH) and oxidized (GSSG) form was simultaneously determined in liver microsomes [11].

This paper describes an improvement of this method, an extension of it to include the determination of protein-bound glutathione (PSSG), and the application of the method to eye lenses. Since the amount of glutathione in the eye lens plays a role in cataract formation [12–14], a simple and accurate determination method could play an important role in the study of this disease.

EXPERIMENTAL

Materials

$KH_2PO_4 \cdot 2H_2O$, monochloroacetic acid, KOH, $LiNO_3$, acetonitrile, methanol and perchloric acid (70%) were all Baker grade (Deventer, The Netherlands). 1,4-Dithiothreitol (99%) and KBr (p.a.) were obtained from Merck (Darmstadt, Germany). $Na_2EDTA \cdot 2H_2O$ (p.a.), sodium dodecylsulphate and β-mercaptoethanol (98%) were purchased from Janssen Chimica (Beerse, Belgium), $NaBH_4$ (98%) from Aldrich (Brussels, Belgium), and sodium octylsulphate (HPLC grade) from Kodak (Rochester, NY, USA). GSH (98%) and GSSG (95%) were from Boehringer (Mannheim, Germany). Aqueous standard solutions were kept at 4°C and diluted when necessary. Na_2EDTA ($2 \cdot 10^{-3}$ *M*) and HNO_3 ($1 \cdot 10^{-3}$ *M*) were added to GSH solutions. Fresh stock solutions were prepared twice a week. Water was demineralized and distilled.

Sample preparation

Free glutathione. Bovine calf eyes (ten to eleven months), obtained from the local slaughterhouse, were kept on ice and processed within 2 h after slaughtering. Lenses were removed, decapsulated by tweezers as previously described [15] and homogenized by a PTFE-down homogenizer for 10 min. A 50-mg sample of this viscous lens fluid was collected in a 2.2-ml reaction vessel (Eppendorf 3812, Hamburg, Germany) containing 2 mm diameter glass beads. To this vessel, 100 μl of Na_2EDTA, 1.5 ml of acetonitrile and, after 1 min, 100 μl of 3.5 *M* perchloric acid were added. The mixture was whirled for 2 min, kept on ice for 10 min in order to precipitate the protein and centrifuged in an Eppendorf 5412 centrifuge at 10 000 *g* for 15 min at 5°C. The supernatant was collected and diluted with mobile phase to a final volume of 5.0 ml.

Protein-bound glutathione. The precipitate from above was dissolved in 1 ml of buffer (0.1 *M* KH_2PO_4 and 0.1 *M* KOH, 1:3, v/v, pH 12), and whirled for 2 min in an Eppendorf 3812 vessel containing glass beads in the presence of 0.1 ml of

0.03 *M* 1,4-dithiothreitol (DTT). The solution was kept at room temperature for 20 min to allow the total reduction of protein-bound glutathione. After the addition of 0.5 ml of acetonitrile and 0.1 ml of 3.5 *M* $HClO_4$, the mixture was whirled, kept on ice and centrifuged as above. Finally, the supernatant was diluted with mobile phase to 4.0 ml.

Total glutathione. The procedure for the determination of the total glutathione content is the same as that for PSSG, except that 50 mg of lens homogenate instead of the protein precipitate was used as the starting material.

Detection principle

Analytes are oxidized by bromine (Br_2), which is electrochemically produced on-line post-column from bromide (Br^-) present in the mobile phase. The amount of bromine generated is measured amperometrically. When no oxidizable agents are present in the system, the current in the detector (I_d) is at its maximum value, I_0, which in our case is 5 μA [10].

Instrumentation

The chromatographic system consisted of a Gilson 302 pump (Villiers-le-Bel, France), a laboratory-made HPLC injection valve, a precolumn (10 mm × 2 mm I.D.) packed with LiChroprep C_{18} (25–40 μm) (Merck), a stainless-steel analytical column (250 mm × 3.1 mm I.D.) packed with RoSil HL C_{18} (5 μm) (RSL, Eke, Belgium), a so-called KOBRA cell [10,11] (commercially available at the Free University of Amsterdam) to generate bromine, a reaction coil (PTFE tube, 1700 mm × 0.5 mm I.D.) and an amperometric detector. The latter comprised a Metrohm 1096/2 cell (Herisau, Switzerland) with a glassy carbon counter-electrode, a gold working electrode (5 mm diameter disk) and a reference electrode of Ag/AgCl/1 *M* LiCl in water–methanol (1:1, v/v). The potential difference between the working and reference eletrodes was kept at 300 mV by a laboratory-made potentiostat. Generally, the bromine generation current was 100 μA.

Chromatographic conditions

The mobile phase consisted of 6% (v/v) methanol in 0.1 *M* aqueous monochloroacetic acid, 0.1 m*M* Na_2EDTA (as antioxidant), 0.1 *M* $LiNO_3$ (as conducting electrolyte), 1.0 m*M* KBr (for bromine generation) and 1.5 m*M* sodium octylsulphate (as ion-pairing reagent). The pH of the mobile phase was set at 3.0 by the addition of KOH.

The flow-rate was 0.5 ml/min and 10- and 100-μl samples were injected. All measurements were performed at ambient temperature.

RESULTS AND DISCUSSION

Stability of GSH solutions

GSH in neutral or basic solutions is oxidized to GSSG, whereas it has been found to be stable in a solution of 0.02 *M* Na_2EDTA at a pH of 4.7 [16]. Such

solutions appeared to disturb the chromatography. When model solutions of 2 m*M* GSH in 2 m*M* Na_2EDTA and 1 m*M* HNO_3 (pH 3) were kept at 4°C, the GSH loss was less than 1% during 72 h. Using a GSH concentration of $2 \cdot 10^{-2}$ m*M*, a loss of 1.5% was found after 8 h. In untreated lens homogenate no GSH loss was observed after 24 h.

Chromatography of GSH and GSSG in model systems

Separation and reproducibility. A better than baseline separation of GSH and GSSG is obtained (see Fig. 1), with retention times of 6.5 min ($k' = 1.9$) and 9.3 ($k' = 2.7$) min, respectively. The reproducibility, determined from the average peak area of three measurements (10-μl injection of 20 μM glutathione) on five consecutive days, was found to be better than 1% for GSH and better than 1.4% for GSSG.

Linearity and detection limit. As shown in Fig. 2, linearity is obtained up to at least to 60% of total bromine consumption. Isaksson *et al.* [9] found non-linearity above 75% in a comparable device. Linear regression analysis of the peak area as a function of the amount of glutathione in the linear range (up to 2 nmol GSH or 1 nmol GSSG) yielded correlation coefficients larger than 0.9995 ($n=6$). Slopes were 29.59 (S.D. = 0.04) and 60.8 (S.D. = 0.1) μAs/nmol, and intercepts −0.04 (S.D. = 0.03) and 0.04 (S.D. = 0.05) μAs for GSH and GSSG, respectively.

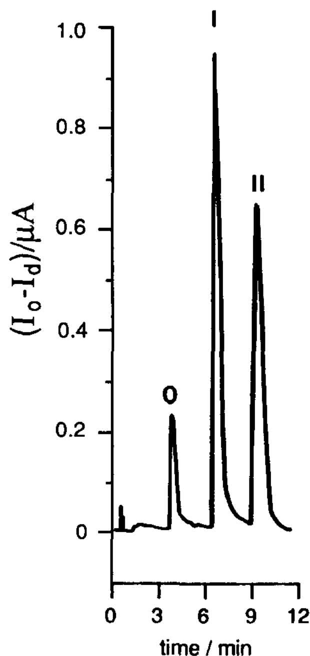

Fig. 1. Separation of a standard solution of 30 μM GSH (I) and 15 μM GSSG (II) in 10^{-3} *M* HNO_3–$2 \cdot 10^{-3}$ *M* Na_2EDTA; peak 0 = injection peak; injection volume, 10 μl.

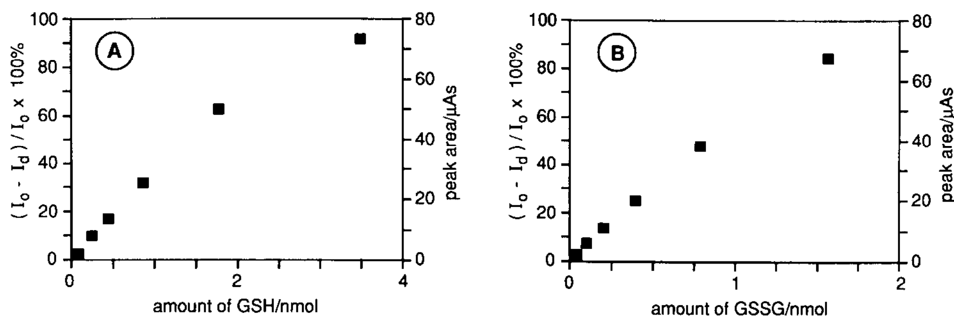

Fig. 2. Concentration of glutathione *versus* peak height as percentage of I_0 (detector current, I_d, in absence of analyte) consumed and *versus* peak area, for GSH (A) and GSSG (B).

Lower detection limits (signal-to-noise ratio = 3) were 7.8 and 4.8 pmol, respectively. Hence, our linear range covers more than two orders of magnitude, which is an improvement of about one order of magnitude over earlier studies [9,11], with a comparable lower detection limit.

The noise level can be decreased by lowering the bromine concentration. When a bromine generation current, I_g, of 10 μA instead of 100 μA was used, the detection limit of GSSG (100-μl injection) was 2.1 pmol. However, a lower I_g results in a smaller linear detection range [10], and stabilization of the bromine concentration takes longer. Since in biological systems generally [17], and in eye lenses in particular [18,19], GSSG/GSH ratios of *ca.* 1:100 have been reported, we decided to proceed with I_g = 100 μA.

Deproteination of eye lens samples and recovery of glutathione

Although $HClO_4$ is generally accepted as a useful deproteination agent, we could not use it in this case because, when it is added at high concentrations, GSH is oxidized to GSSG. Dilute $HClO_4$ solutions gave a colloidal precipitate that was difficult to process. Moreover, occlusion of the analytes may occur. When methanol was used for deproteination, the GSH recovery in test systems was poor. Pure acetonitrile yielded a restricted precipitation. Therefore we tested a combination of $HClO_4$ with either acetonitrile or methanol. We first used a model system consisting of 56 mg/ml eye lens proteins (the isolated but unseparated water-soluble fraction [15]) and 9.66 μmol/mg GSH in 0.06 *M* Na_2HPO_4–KH_2PO_4 buffer (pH 7.0). The GSH concentration used is close to reported values for lenses [19]. Lenticular protein concentrations range up to more than 300 mg/ml [15]. GSH isolation was performed as described in the Experimental section, but 200 mg of the model solution were used instead of 50 mg of real sample, and $HClO_4$ and organic solvent were added simultaneously. The GSH recovery was 60 ± 7% for methanol and 98 ± 3% for acetonitrile. Therefore we proceeded with perchloric acid–acetonitrile for real samples.

Lens homogenate was treated as described in the Experimental section but acetonitrile and $HClO_4$ were added simultaneously. In order to get an impresssion

about the recovery of glutathione, the precipitate was retreated in the same way. When 200 mg of homogenate was used, no more glutathione was detected after three consecutive cycles. With 50 mg of homogenate two cycles were needed. Matrix effects, higher viscosity and occlusion could be responsible for the lower extraction rate in real samples. Therefore, we decreased the precipitation rate by waiting for 1 min between addition of acetonitrile and $HClO_4$. In this way no more glutathione was detected after a single cycle.

From the actual detection limits (DL) for GSH and GSSG given above, one can calculate the theoretical detection limits in lenses as $DL \cdot V_s/m_h$, where DL is given in concentration units, V_s is the sample volume (5.0 ml) and m_h is the mass of the lens homogenate (50 mg). Thus, when an injection volume of 100 μl is used, detection ranges of 7.8 to $2 \cdot 10^3$ and 4.7 to $1.2 \cdot 10^3$ nmol per gram of lens are found for GSH and GSSG, respectively. With a 10-μl injection volume these values are ten-fold greater.

Glutathione reduction

In order to determine the protein-bound fraction of glutathione (PSSG), it has to be reduced to free GSH. When this is done before the separation of free glutathione, GSSG is also reduced. We studied several reducing agents. β-Mercaptoethanol has, in addition to its unpleasent smell and toxic effects, the disadvantage that it is also detected by our system, and its retention time coincides with that of oxidized glutathione. $NaBH_4$ does not dissolve well in acetonitrile, and it reacts with methanol to hydrogen gas, which disturbs the chromatography and detection. Therefore, we selected DTT as reducing agent. Since DTT is a thiol compound itself, it is also detected. Retention times of the oxidized and reduced form of DTT were 12.2 and 23.1 min, respectively, so no interference with GSH and GSSG occurred. Various conditions and reaction rates have been reported for the reduction of disulphides by this compound [17,20]. We tested the reduction of glutathiones by DTT with GSSG model solutions. Several DTT/GSSG ratios were studied in 1.675 ml aqueous KOH solutions (pH 12.0). The reaction was stopped at different times by addition of 100 μl of 3 *M* HNO_3, and GSH and GSSG concentrations were measured. Even when the DTT/GSSG ratio was 4.5, complete conversion of GSSG into GSH was observed within 20 min. Concentrations of DTT over 10^{-2} *M* resulted in column overload and blocked detection for several hours. With $2.7 \cdot 10^{-3}$ *M* no problems were met. Since PSSG concentrations in lenses are assumed to be a few micromoles per gram of lens at most [18,19,21], this concentration seems to be sufficient for a total reduction of PSSG. This is confirmed by two observations. (i) When PSSG was determined in real samples, only a small part of the DTT was converted into its oxidized form, and the amount of reduced DTT left (peak off-scale) was significantly more than 4.5 times the amount of PSSG found (see Fig. 3). (ii) When the precipitate of the PSSG determination was retreated with DTT, no more GSH or oxidized DTT was found.

Glutathione in calf lenses

Content. Ten lenses were homogenized together, and the amounts of GSH, GSSG, PSSG and total glutathione were determined in triplicate. Chromatograms are shown in Figs. 3 and 4. No GSSG is observed at a signal-to-noise level of 3, which means the presence of less than 48 nmol per gram of lens. The peaks at retention times below 6 min are probably cystine, cysteine and methionine, which are also detected by our system. Concentrations averaged over ten independent

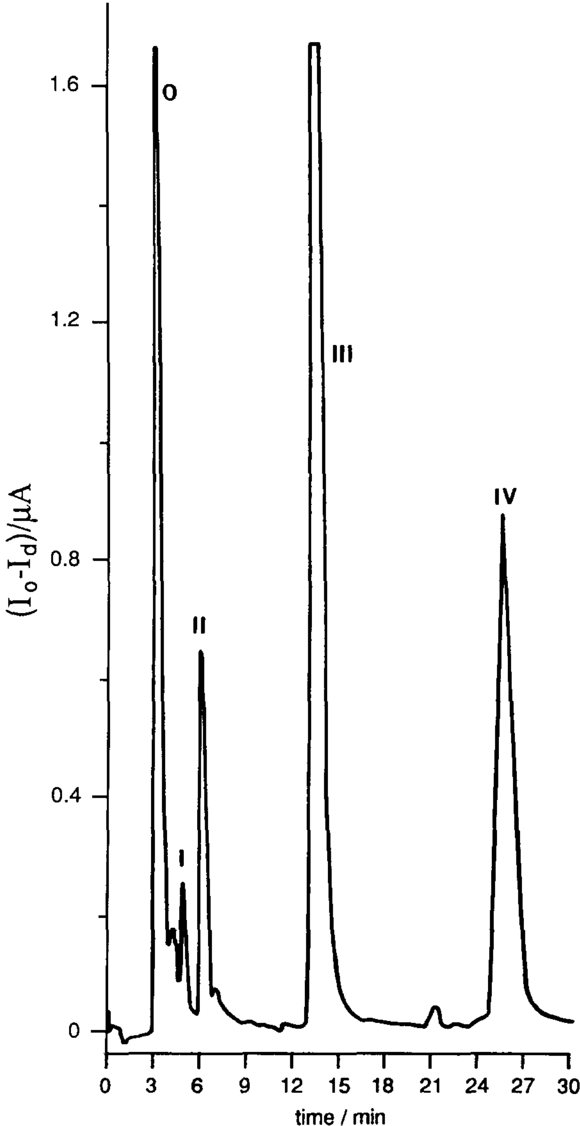

Fig. 3. Detection of protein-bound glutathione in calf lenses. Peaks: 0 = injection peak; I = cysteine?; II = reduced PSSG; III = DTT (reduced); IV = DTT (oxidized). Injection volume, 10 μl.

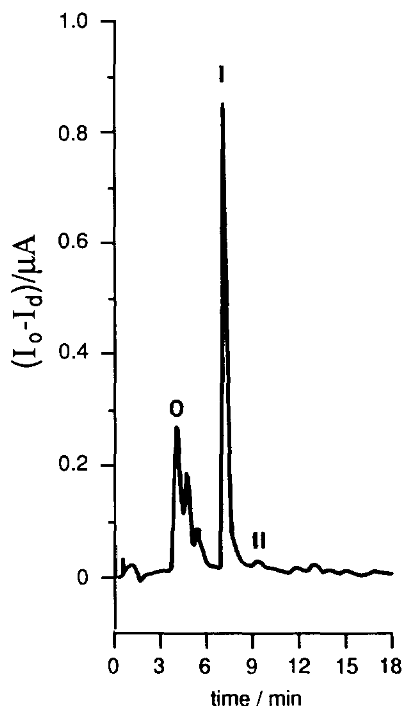

Fig. 4. Detection of GSH (I) and GSSG (II) in calf lenses. Peak 0 = injection peak. Injection volume, 10 μl.

measurements are given in Table I. Our values for GSH compare well with literature values [21–24], but our GSSG values are considerably lower [21–23]. This may be attributed to the instability of GSH. When no precautions are taken, GSH oxidizes easily to GSSG. The determined PSSG values are significantly higher than those found by others [19,21]. Possibly no total reduction of PSSG to GSH was obtained in those studies. In the present study both the oxidation-protection of GSH and the reduction of PSSG were carefully checked.

TABLE I

GLUTATHIONE CONTENT IN CALF LENSES

Type	Content (μmol/g of lens)
GSH	6.79 ± 0.38
GSSG	<0.048
PSSG	0.96 ± 0.03
Total	7.75 ± 0.41

TABLE II

EFFECT OF STORAGE ON GLUTATHIONE CONTENT

Storage conditions	Glutathione content (μmol/g of lens)			PSSG (%)
	GSH	PSSG	Total	
Lenses				
Fresh	6.8	0.96	7.8	13
24 h, 4°C	6.8	–	–	–
24 h, −20°C	6.8	0.99	7.8	13
7 days, −20°C	7.5	1.08	8.6	13
14 days, −20°C	9.6	1.87	11.4	16
Homogenate				
Fresh	6.1	1.2	7.2	16
13 days, −20°C	6.8	1.5	8.2	18

Distribution of glutathione contents in single lenses. Our final aim is the study of glutathione in lenses at different ages. For such studies it is essential to know the variation of glutathione content between single lenses of equal age. The GSH content in single calf lenses was determined, and values between 6.5 and 7.1 μmol/g lens were obtained. This corresponds to a maximum deviation of *ca.* 5%, which is comparable with the error made during sample preparation.

Conservation of lenses. Since lenses cannot always been treated directly, the effect of storage on the glutathione content of lenses was also studied (Table II). This was done with intact lenses and with homogenate. The latter was from calves four to five months old. Storage at 4°C or −20°C for 24 h did not have any effect. When lenses were stored for a week, the GSH and PSSG content was substantially increased, but the GSH/PSSG ratio did not change significantly and no GSSG was found. Moreover, the lens volume was decreased. Apparently, no reduction or oxidation takes place, but rather evaporation of water from the lens. When lens homogenate was stored instead of intact lenses the same phenomenon was encountered (even at −20°C and in Eppendorf vessels), though less than in intact lenses.

CONCLUSION

Deproteination by $HClO_4$–acetonitrile followed by HPLC and electrochemical detection with on-line-generated bromine appears to be a relatively easy, fast and reliable method for the determination of the different forms of glutathione in eye lenses. In this way, glutathione amounts can be detected down to a few picomoles. The linear dynamic range of the detection system allows for the determination of GSH and GSSG (concentration ratio 100:1) in a single run. After

reduction with DTT, the protein-bound fraction can also be detected. Two samples can be analysed in triplicate in a day. For reliable determinations of GSH, GSSG and PSSG in eye lenses, attention has to be paid to the instability of GSH, proper reduction of PSSG and the risk of occlusion during protein precipitation. Our method offers a solution to these problems, and accurate determinations are possible.

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REFERENCES

1 W. B. Jakoby and O. W. Griffith (Editors), *Methods in Enzymology*, Vol. 143, Academic Press, Orlando, FL, 1987, Ch. 11–21, pp. 44–140.
2 A. J. Alpert and H. F. Gilbert, *Anal. Chem.*, 144 (1985) 553.
3 L. M. Shaw, H. S. Bonner, A. Turrisi, A. L. Norfleet and M. Kligerman, *J. Liq. Chromatogr.*, 9 (1986) 845.
4 K. Iriyama, M. Yoshiura and T. Iwamoto, *J. Liq. Chromatogr.*, 9 (1986) 2955.
5 D. Dupuy and S. Szabo, *J. Liq. Chromatogr.*, 10 (1987) 107.
6 S. M. Lunte and P. T. Kissinger, *J. Liq. Chromatogr.*, 8 (1985) 691.
7 L. A. Allison and R. E. Shoup, *Anal. Chim.*, 55 (1983) 8.
8 T. J. Pastor, V. J. Vajgand and V. V. Antonijevic, *Microchim. Acta*, III (1983) 203.
9 K. Isaksson, J. Lindquist and K. Lundstrom, *J. Chromatogr.*, 324 (1985) 333.
10 W. Th. Kok, U. A. Th. Brinkman and R. W. Frei, *Anal. Chim. Acta*, 162 (1984) 19.
11 A. J. J. Debets, R. van de Straat, W. H. Voogt, H. Vos, N. P. Vermeulen and R. W. Frei, *J. Pharm. Biomed. Anal.*, 6 (1988) 329.
12 K. R. Hightower, *Curr. Eye Res.*, 4 (1985) 857.
13 M. F. Lou, J. E. Dickerson, Jr. and R. Garadi, *Exp. Eye, Res.*, 50 (1990) 819.
14 H. Pau, P. Graf and H. Sies, *Exp. Eye Res.*, 50 (1990) 17.
15 H. Bloemendal (Editor), *Molecular and Cellular Biology of the Eye Lens*, Wiley, New York, 1981, pp. 1–47.
16 J. Sedlak and R. Lindsay, *Anal. Biochem.*, 25 (1968) 192.
17 A. Russo, in D. Glick (Editor), *Methods of Biochemical Analysis*, Wiley, New York, 1988, Vol. 33, pp. 165–241.
18 J. Harding, *Biochem. J.*, 114 (1970) 88.
19 V. N. Reddy and R. F. Han, *Doc. Ophthalmol. Proc. Ser.*, 8 (1976) 153.
20 M. Johansson and D. Westerland, *J. Chromatogr.*, 385 (1987) 343.
21 K. N. Gelatt, M. Bruss and E. Daniel, *Am. J. Vet. Res.*, 43 (1982) 1215.
22 J. H. Kinoshita and T. Masurat, *Ophthalmology*, 57 (1957) 266.
23 N. Hata and O. Hockwin, *Ophthal. Res.*, 9 (1977) 256.
24 W. G. Chaney and A. Spector, *Curr. Eye Res.*, 3 (1984) 345.