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Journal of Chromatography A, 1031 (2004) 125–133
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Separation and identification by gel filtration and high-performance liquid chromatography with UV or electrochemical detection of the disulphides produced from cysteine and glutathione oxidation

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Abstract

Methods for quantification of oxidised and reduced forms of glutathione (GSSG and GSH) and cysteine (CSSC and CSH) and the disulphide glutathione–cysteine (GSSC) resulting from the oxidation of the mixture of CSH and GSH are performed by RP-HPLC with coulometric and UV detection after separation of these compounds by size-exclusion fast protein liquid chromatography. The fractionation of the disulphides (GSSG, GSSC and CSSC) was achieved by size exclusion using a Superdex peptide column coupled with an UV detection at 254 nm. The conditions of separation of these compounds by RP-HPLC were optimised using the response surface methodology. Optimal peak resolution and retention times were obtained on a C_{18} YMC ODS AQ column with 20 mM of ammonium phosphate at pH 2.5 and 2% of acetonitrile in the elution phase. In these experimental conditions, CSH, CSSC, GSH and GSSG were eluted within 20 min. Coulometric detection enabled a sensitivity 100 times higher for the disulphides than the UV detection at 220 nm. These methods were applied to follow the consumption of thiols and the disulphide formation by three oxidising systems, sulphydryl oxidase, glutathione dehydroascorbate oxidoreductase and potassium bromate. This study revealed that the relative proportions of the disulphides formed were similar for the three oxidising systems when the reactions are in their state of equilibrium.

Keywords: Response surface methodology; Resolution; Retention times; Glutathione; Cysteine; Organosulphur compounds

1. Introduction

Glutathione and cysteine are low-molecular-mass (LMW) thiols of major importance in the gluten network formation and influence the rheological properties of the dough during mixing [1–3]. The consumption of the LMW thiols by oxidative agents may prevent their participation in SH/SS exchange reactions resulting in the depolymerisation of the gluten proteins. In breadmaking, these LMW thiols are frequently the target of improving chemical agents ($KBrO_3$, ascorbic acid) [4,5]. $KBrO_3$ is known to react with thiols, like glutathione (GSH) and cysteine (CSH), producing the corresponding disulphides, GSSG and CSSC [6]. In the case of ascorbic acid supplementation, thiol oxidation is catalysed by the glutathione dehydroascorbate oxidoreductase (GSH-DHase) in the presence of dehydroascorbic acid (DHA) [7]. The latter is produced by ascorbic acid oxidation in the presence of oxygen and ascorbic acid oxidase. Nowadays, there is an increasing demand in the baking industry for alternatives to chemical additives such as potassium bromate due to the potential hazards [8]. Therefore, in the last decade, an active research is devoted to the use of oxidoreductases. Sulphydryl oxidase (SOX) isolated from *Aspergillus niger* oxidises GSH to its oxidised form (GSSG) by molecular oxygen producing hydrogen peroxide [9]. GSH-DHase and SOX were shown to oxidise CSH when GSH and CSH are added together into the reaction mixture although that CSH is not substrate of these enzymes [7,9]. These results suggested that both the symmetric CSSC and the asymmetric GSSC were produced in the mixture. Studies of these reactions require efficient analytical and preparative laboratory-scale methods for the thiols and their

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doi:10.1016/j.chroma.2003.10.136

corresponding disulphides. The main target of this work was to separate individual disulphide fractions, by developing size-exclusion chromatography (SEC) using a fast protein liquid chromatography (FPLC) system equipped with an UV detector. Jones and Carnegie [10] and Sarwin et al. [11] reported the fractionation of the symmetric disulphide GSSG from wheat flour proteins by size exclusion methods. To our knowledge, the separation by gel filtration of the two symmetric GSSG and CSSC and the asymmetric GSSC disulphide has never been described. HPLC methods were largely used to analyse thiols and disulphides using UV detection [12–14], fluorimetric detection [15] or electrochemical detection (ED). The latter included amperometric [16–18] as well as coulometric detection [19–21]. Few of these studies were devoted to the reduced and oxidised forms together. Some analyses were performed with too long running times [21] or with poor peak resolution [20]. In addition, the asymmetric disulphide GSSC was analysed only by Wang and Cynader [22] using derivatisation. In the present work, RP-HPLC was developed to analyse simultaneously the reduced (GSH, CSH) and oxidised (GSSG, CSSC and GSSC) forms of glutathione and cysteine. For this purpose, the optimisation of the separation of thiols and disulphides by HPLC analysis coupled with ED using the response surface methodology (RSM) was firstly achieved. The effects of pH, ammonium phosphate concentration and percentage of acetonitrile in the eluent were studied. The optimised HPLC conditions were also used with an UV detection at 220 nm.

The preparative laboratory-scale separation (SEC) and analytical (HPLC) techniques were then applied to study the action of SOX, GSH-DHase and $KBrO_3$, enabling to compare the behaviour of these oxidising agents towards mixtures of GSH and CSH.

2. Experimental

2.1. Chemicals

All chemicals were purchased from VWR (Fontenay S/Bois, France) and Sigma–Aldrich (St. Louis, MO, USA). Dehydroascorbic acid (2.5 mM) was prepared immediately before the assay according to the method of Kaïd et al. [7]. Eluents were prepared from deionised water. High purity deionised water with specific resistance of 16 MΩ/cm was obtained from a Milli-Q system (Infilco, Croissy Beaubourg, France). Ammonium phosphate and sodium acetate of Normapur grade were used.

2.2. Enzymes

Sulphydryl oxidase was purified from *A. niger* extract according to the method developed by Vignaud et al. [9] and used with a specific activity of 200 nkat/mg. Glutathione dehydroascorbate oxidoreductase was purified from wheat flour by the method described by Kaïd et al. [7]. Its specific activity was 300 nkat/mg.

2.3. Size-exclusion chromatography

The size exclusion was performed on a FPLC system (Amersham Biosciences, Uppsala, Sweden) equipped with a 24 ml Superdex peptide HR 10/30 (100–7000) column, an UV detector (UV-M II) fixed at 254 nm and a fraction collector (Frac−100). Data acquisition and processing were performed using the FPLC director software (Amersham Biosciences). Superdex peptide gel was calibrated by injecting different molecular mass markers being hydrogen peroxide, potassium bromate, cysteine, oxidised glutathione, peptide P (Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2) and insulin. The equation resulting from the calibration of the SEC gel was fitted by:

$$K_{av} = -0.317 \log M_r + 1.49 \quad (r^2 = 0.99).$$

K_{av} is the partition coefficient of the molecules and is defined as $(V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume, V_o the void volume and V_t the total column volume. M_r is the molecular mass of the molecules.

Half millilitre of samples were eluted at 0.8 ml/min isocratically using an eluent composed of 0.1 M sodium acetate buffer, pH 5.6. The collected fractions were 1.4 ml and were analysed by RP-HPLC.

2.4. RP-HPLC with coulometric detection

The HPLC apparatus was a Varian system (Les Ulis, France) equipped with a coulometric detector (Coulochem II, ESA) from Eurosep (Cergy, France). Data acquisition and processing were performed using a chromatography software (Star, Varian, France). Ten microlitres of samples were loaded onto a C_{18} YMC ODS AQ column (250 mm × 4.6 mm) (AIT, Le Mesnil le Roi, France) equipped with a guard column (17 mm × 4.6 mm). Elution was performed isocratically with a mobile phase containing 98% of 20 mM ammonium phosphate solution adjusted to pH 2.5 with orthophosphoric acid and 2% of acetonitrile. The flow rate was fixed at 0.7 ml/min. Potential conditions of detector were 1 V (versus Pd) on the guard cell (model 5020), 0.45 V (versus Pd) on the electrode 1 and 0.88 V (versus Pd) on the electrode 2 of the analytical cell (model 5010).

Table 1
Independent variables and respective levels in the experimental domain

	Level of the variables				
	−1.682	−1	0	+1	+1.682
X_1 (pH)	2	2.4	3	3.6	4
X_2 (ammonium phosphate)	5	13	25	37	45
X_3 (% CH_3CN)	0	0.8	2	3.2	4

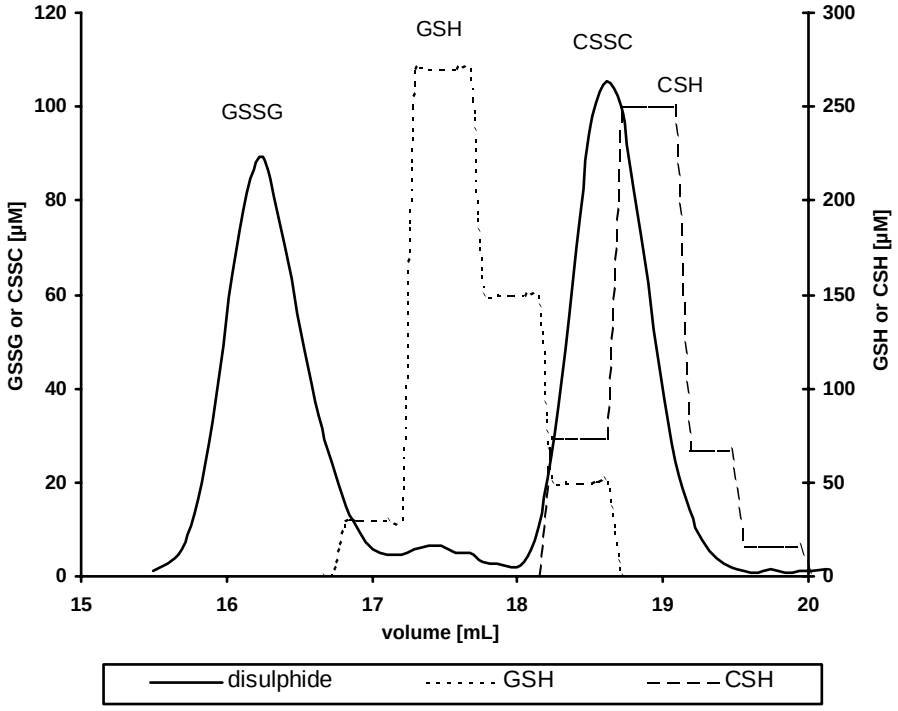

Fig. 1. Size-exclusion chromatogram of a standard mix of GSSG (0.25 mM), GSH (0.5 mM), CSSC (0.25 mM) and CSH (0.5 mM).

2.5. RP-HPLC with UV absorbance detection

The conditions of separation were similar to those given in Section 2.4, unless that an UV detector at 220 nm was used instead of the coulometric detector and that 20 μl of samples were loaded. The HPLC apparatus system was equipped with a photodiode array detection (DAD) system 996 Waters (Waters, Milford, MA, USA).

2.6. Reduction treatment with dithiothreitol

Fifty microlitres of the FPLC fraction was mixed with 250 μl of dithiothreitol (20 mM) and 500 μl of 0.2 M Tris buffer, pH 8.2. The mixture was incubated at 0 °C for 30 min. The excess of dithiothreitol was extracted three times with 2.5 ml of ethyl acetate. In these conditions, the extraction recovery was close to 95%. The resulting solution containing the CSH and GSH was properly diluted in the mobile phase before analysis by RP-HPLC.

2.7. Experimental design

The experimental design was a modified central composite design with three variables at five levels and eight replicates at the centre point (Table 1) [23].

The 22 experiments were conducted by varying pH (between 2 and 4), the ammonium phosphate concentration (between 5 and 45 mM) and the acetonitrile percentage content

Table 2
Coefficients b_i, b_{ij} and b_{ii} (parameter estimates) obtained, calculated r^2 values between theoretical and experimental responses and experimental variance

	$t_{R(CSSC)}$	$t_{R(CSH)}$	$t_{R(GSH)}$	$t_{R(GSSG)}$	$PR_{CSSC/CSH}$	$PR_{CSH/GSH}$	$PR_{GSH/GSSG}$
b_0	**4.23**	**4.64**	**7.18**	**10.25**	**2.53**	**14.88**	**13.56**
b_1	−0.02	−0.06	**−0.70**	**−3.27**	**−0.33**	**−3.39**	**−6.55**
b_2	0.09	0.09	−0.02	−0.34	−0.17	−0.63	−0.37
b_3	−0.07	−0.11	**−1.22**	**−5.77**	**−0.24**	**−4.79**	**−9.92**
b_{12}	0.01	0.02	0.09	0.48	0.13	0.79	1.23
b_{13}	0.003	0.006	**0.28**	**2.10**	0.01	0.60	**1.63**
b_{23}	0.005	0.005	0.04	0.37	−0.02	0.09	0.71
b_{11}	−0.09	−0.09	−0.12	0.07	−0.02	−0.54	−0.14
b_{22}	**−0.15**	**−0.15**	0.04	0.40	**0.27**	**1.15**	0.14
b_{33}	−0.06	−0.05	**0.30**	**2.41**	**0.19**	**1.73**	**2.73**
r^2	0.51	0.51	**0.94**	**0.98**	0.72	**0.96**	**0.98**
Experimental variance (%)	5.61	5.57	5.57	6.14	5.85	6.96	6.28

Significant coefficients are indicated in bold ($P \leq 0.05$).

(between 0 and 4%) of the mobile phase. The eluent was constituted with an ammonium phosphate solution adjusted at different pH and supplemented or not with acetonitrile with a flow rate fixed at 0.7 ml/min. Each injection was repeated twice. Thiols and disulphides were simultaneously detected by the second electrode of the coulometric detector. The retention times (t_R) and the peak resolution (PR) were the studied responses. Peak resolution (PR) was defined as the ratio between the difference of the t_R and the average value of the half width of two adjacent peaks. For each response, a polynomial equation was established in order to quantify the influence of each variable. Within the limits of the experimental domain, a second order polynomial model was fitted to the dependent variables using the following equation:

$$Y = b_0 + \sum_i b_i X_i + \sum_{ij} b_{ij} X_j X_i + \sum_{ii} b_{ii} X_i^2$$

where Y is the estimated response, b_0, b_i, b_{ij} and b_{ii} the parameter estimates corresponding to the constant, linear, interactive and quadratic effects, respectively, using the least squares method, and X_i and X_j the independent variables in coded values. Student's t-test was used to check the reliability of the polynomial and the significance of parameters. The level of statistical significance for the process variables was defined at $P \leq 0.05$.

2.8. Reaction mixtures

GSH and CSH (0.25–2 mM) were mixed in 0.1 M sodium acetate buffer solution at pH 5.6 with SOX (30 nkat) or $KBrO_3$ (2 mM), or in 0.1 M sodium phosphate buffer solution at pH 6.2 with GSH-DHase (30 nkat) for a final volume of 2 ml. Dehydroascorbic acid (2 mM) was added to the solution in the case of reaction performed in the presence of GSH-DHase. After 30 min of incubation at 25 °C, 0.5 ml of the reaction mixtures was injected onto the SE column.

2.9. Enzyme assays

SOX activity was estimated polarographically at 30 °C in 3 mM of glutathione air-saturated solution at pH 5.6 [9]. GSH-DHase activity was determined by the spectrophotometric assay of the formation of ascorbic acid at 266 nm. DHA (2.5 mM) and GSH (3 mM) prepared at pH 6.2 were used for routine assays [7].

3. Results and discussion

3.1. Analysis by the size-exclusion FPLC method

3.1.1. Standardisation of the size-exclusion FPLC method

Different standard solutions of GSSG and CSSC (0.25 mM) were injected onto the SEC column. The resulting peak areas obtained for each compound were expressed

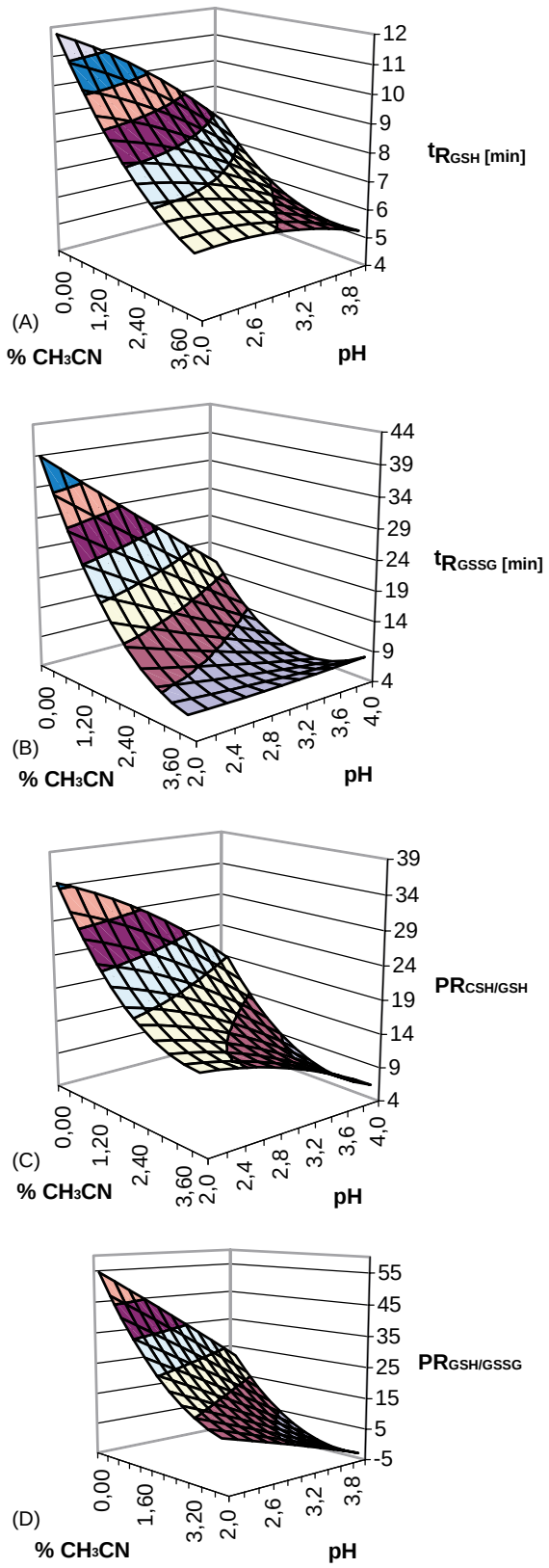

Fig. 2. Response surfaces of $t_{R(GSH)}$ (A), $t_{R(GSSG)}$ (B), $PR_{CSH/GSH}$ (C) and $PR_{GSH/GSSG}$ (D) obtained for an ammonium phosphate concentration of 20 mM.

in AU ml. A linear response of the detector was obtained for injected disulphide amounts up to 0.6 μmol. The sensitivity of the detector signal was 5 nmol of GSSG and CSSC loaded on the column. The molar extinction coefficient at 254 nm was deduced from the slope of the plot, and was determined as being 292 M^{-1} cm^{-1} for both compounds, with a R.S.D. of 5 and 6% ($n = 8$) for GSSG and CSSC, respectively.

Absorbance measurements obtained at 254 nm using the diode array spectrophotometer gave molar extinction coefficient values closed to those determined by FPLC detector, being 305 and 295 M^{-1} cm^{-1} (±2 and 4%, $n = 6$) for GSSG and CSSC, respectively. These values are in accordance with those already described [24].

3.1.2. Quantification of the size exclusion fractionated disulphides

A standard mix of oxidised (0.25 mM) and reduced (0.5 mM) forms of glutathione and cysteine was injected on the SEC column. Since GSH and CSH were not detected at 254 nm, the SEC profile exhibited only two peaks eluted at 16.3 and 18.6 ml, corresponding to the GSSG and CSSC, respectively (Fig. 1). The collected fractions were analysed by RP-HPLC equipped with a coulometric detector. The composition of each collected fraction is given in Fig. 1. All the thiol and disulphide compounds were collected between 15 and 20 ml since the yield was close to 100% for each molecule, meaning that no specific interaction occurred between the gel matrix and the thiol and disulphide compounds in our elution conditions. GSH and CSH were eluted at 17.5 and 19.1 ml, respectively. The peak of CSSC was largely polluted by CSH and partially by GSH.

3.2. Development of the disulphide and thiol separation by RP-HPLC with coulometric detection

3.2.1. Choice of the variables of the experimental design

The main target for the optimisation was to obtain an elution time for the studied molecules as short as possible and an elution order of the molecules that remains constant. Preliminary studies have shown that increased acetonitrile percentage decreased the GSSG retention time, while a slight variation of the percentage in the eluent may in some cases modify the elution order of the other studied molecules. Moreover, concentrations above 4% of acetonitrile in the mobile phase lead to a low peak resolution.

The upper limit of pH was set at pH 4 to avoid the possible thiol oxidation in disulphide compounds and the lower limit was set at pH 2 since the YMC ODS AQ gel must be used with mobile phase at pH above 2. The ammonium phosphate concentration range results from a compromise between the reproducibility of the chromatographic separation (better for higher salt concentrations) and the sensitivity of the coulometric detection (better for lower salt concentrations).

3.2.2. Validity of the polynomial models

Standard errors were calculated from the responses in the centre point of the design (eight replications). Validity of the model was estimated with the correlation coefficient (r^2) between theoretical and experimental data. Thus, the validity of the model is poor for the separation parameters concerning CSH and CSSC ($t_{R(CSSC)}$, $t_{R(CSH)}$ and $PR_{CSSC/CSH}$) since the r^2 values are largely less than 0.90 (Table 2). There-

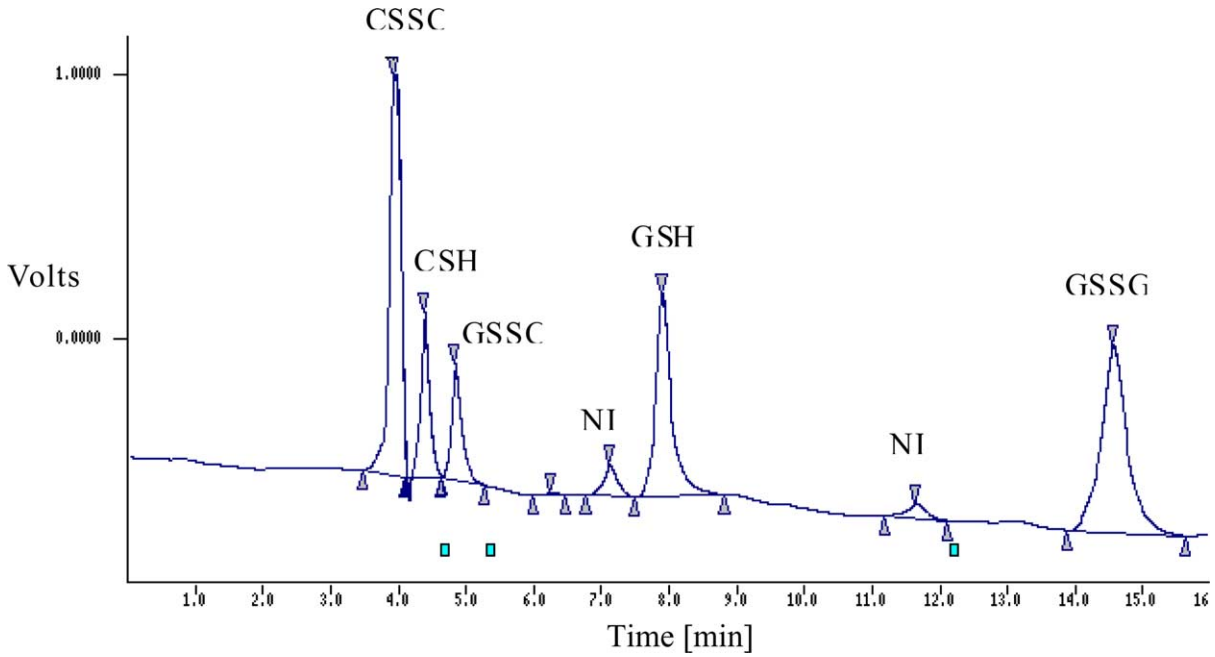

Fig. 3. Coulometric HPLC chromatogram of a standard mixture of CSH (50 μM), GSH (40 μM), GSSC (50 μM), CSSC (50 μM) and GSSG (30 μM). NI: non identified peak.

fore, only the responses $t_{R(GSH)}$, $t_{R(GSSG)}$, $PR_{CSH/GSH}$ and $PR_{GSH/GSSG}$ will be discussed further.

3.2.3. Main effects of variables

The coefficients b_2, b_{12} and b_{23} linked to the ammonium phosphate concentration were low and not significant meaning that, in the range studied, the salt concentration has a weak effect on the separation parameters. Consequently, ammonium phosphate concentration was fixed at 20 mM and only response surfaces corresponding to the effects of pH and acetonitrile concentration on $t_{R(GSH)}$, $t_{R(GSSG)}$, $PR_{CSH/GSH}$ and $PR_{GSH/GSSG}$ were examined (Fig. 2). The results show that $t_{R(GSH)}$ and $t_{R(GSSG)}$ decrease rapidly when the acetonitrile percentage and the pH of the mobile phase are increased. A statistically significant interaction b_{13} (positive value) was observed between pH and acetonitrile percentage meaning that for high values of acetonitrile percentage, $t_{R(GSH)}$ and $t_{R(GSSG)}$ are short and almost not affected by pH variation between pH 2 and 4. Similarly $PR_{CSH/GSH}$ and $PR_{GSH/GSSG}$ decrease with increased acetonitrile percentage and pH of the mobile phase (Fig. 2C and D). Optimal elution conditions were then deduced from a compromise between a t_R as short as possible and a high PR given by the corresponding response

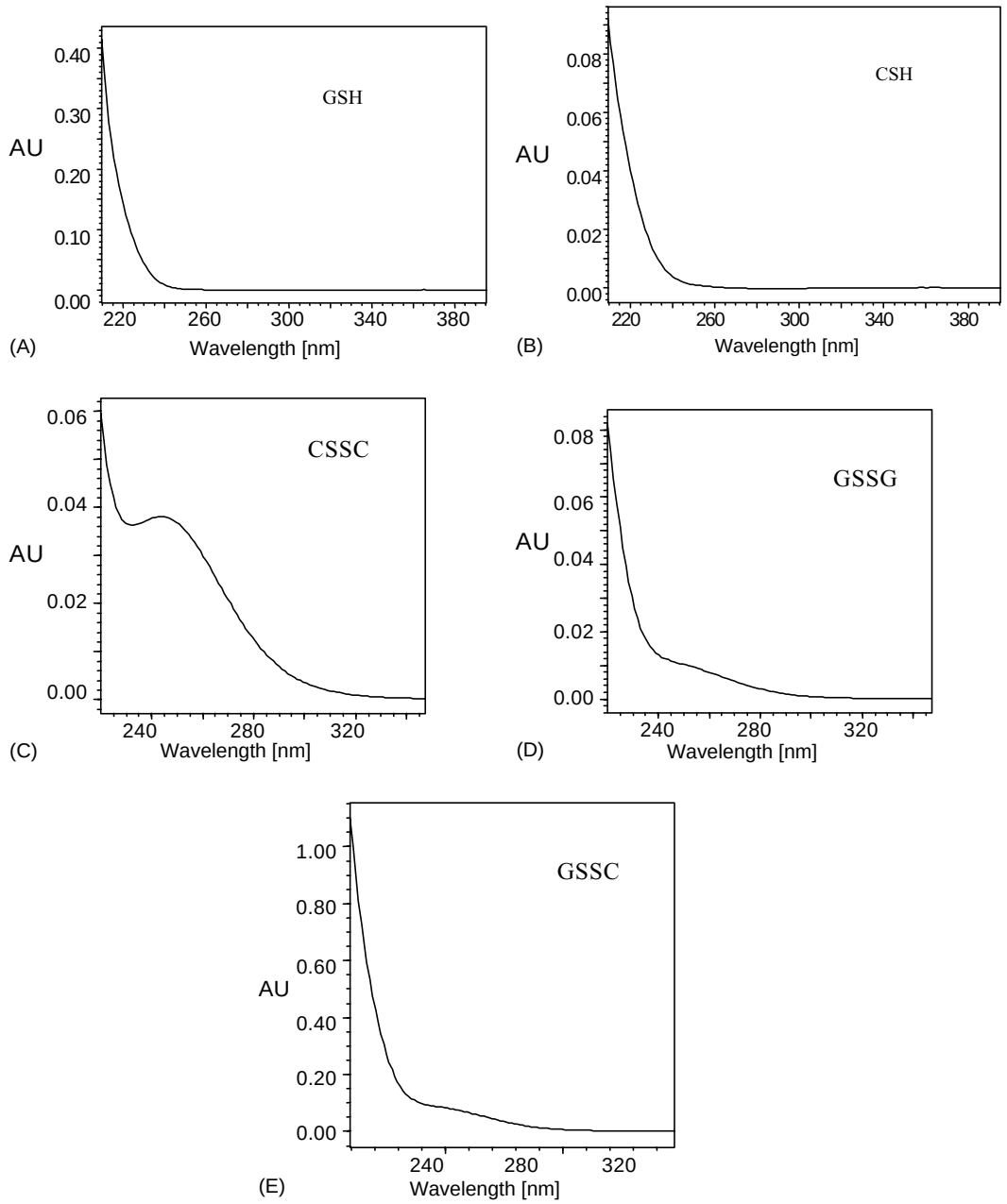

Fig. 4. UV spectra measured by HPLC–DAD with standard compounds of GSH (A), CSH (B), CSSC (C), GSSG (D) and the asymmetric disulphide GSSC (E).

surfaces. Therefore, efficient separation for an analysis run below 20 min was obtained when elution conditions were set at pH 2.5, ammonium phosphate concentration 20 mM and acetonitrile 2%.

In these experimental conditions and at a flow rate of 0.7 ml/min, CSSC, CSH, GSH and GSSG were eluted at 3.9, 4.4, 7.8 and 14.5 min, respectively (Fig. 3), with a $PR_{CSH/GSSG}$ and $PR_{GSH/GSSG}$ of 21.5 and 24.5, respectively. In these conditions, GSSC was eluted at 4.8 min. The sensitivity of this method was 10 pmol for the thiols (CSH and GSH) and 20 pmol for the disulphides (CSSC and GSSG), with a R.S.D. close to 10% ($n = 8$).

3.3. Analysis by RP-HPLC with UV absorbance detection

By using the optimised elution conditions determined above with the coulometric detection, disulphides and thiols were also analysed by RP-HPLC with UV detection. The spectra obtained with a photodiode array detector (Fig. 4) showed that thiols and disulphides absorb at 220 nm, a wavelength which can be selected for the detection and the quantification of these compounds provided there was no interfering substances in the analysed sample. Moreover, the three disulphides showed a maximum of absorbance at 254 nm whereas CSH and GSH did not absorb at this wavelength. For the three disulphides, the values of the absorbance ratio (A_{254}/A_{220}) were 0.59, 0.25 and 0.125 for CSSC, GSSC and GSSG, respectively. Compared to the coulometric detection, the UV detection at 220 nm is less sensitive (e.g. the detection limit is 100 times higher for the disulphide compounds) and more susceptible to interfering substances. Nevertheless, the UV detector is more stable and easier to handle. Therefore, RP-HPLC with UV detection is well suited for analysis of thiols and disulphides mixtures of this study. Calibration plots were built-up over the concentration range of 0.5–25 nmol for GSSG, GSH, CSSC and CSH. A linear relationship between the peak area and the injected amount up to 40 nmol was GSSG ($r^2 = 0.998$), GSH ($r^2 = 0.993$), CSSC ($r^2 = 0.958$) and CSH ($r^2 = 0.908$). The detection limits obtained were 2.5 nmol for CSSC and GSSG and 5 nmol for GSH and CSH.

3.4. Applications of the size-exclusion chromatography to the analysis of model mixtures containing GSH, CSH and an oxidising system

Reaction mixtures containing disulphides produced from GSH and CSH in the presence of an oxidoreducing enzyme have been analysed by size-exclusion FPLC. For this purpose, sulphydryl oxidase was mixed with GSH and CSH for 30 min in air-saturated buffer solution at pH 5.6. Half millilitre of the reaction mixture was fractionated by size-exclusion FPLC. At 254 nm, three major peaks were detected at 16.3, 17.5 and 18.6 ml (Fig. 5). In addition, one peak is eluted at the exclusion volume of the column which can be identified as the enzyme protein. The relative composition in thiols of all the collected fractions (1.4 ml) was determined by RP-HPLC after reduction. It revealed that the disulphide GSSC resulting from the oxidation of GSH and CSH was eluted at 17.5 ml and confirmed that GSSG and CSSC were eluted at 16.3 and 18.6 ml, respectively. Assuming a similar value of the molar extinction coefficient for GSSC, GSSG and CSSC (292 $M^{-1}\,cm^{-1}$), the disulphide amounts were calculated from the resulting size-exclusion FPLC peak areas. The thiol and disulphide amounts in all the size-exclusion FPLC fractions have been determined by RP-HPLC. For all the mixtures tested, similar amounts of disulphides have been obtained by RP-HPLC and size-exclusion FPLC meaning that peak areas responses

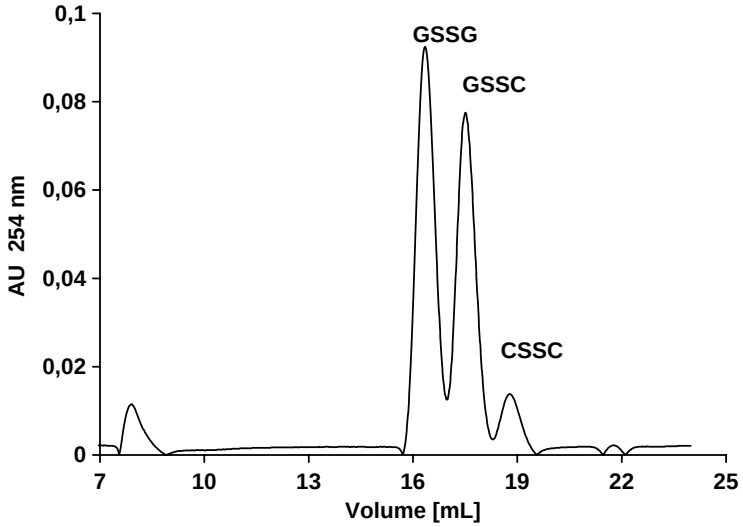

Fig. 5. Separation by size-exclusion FPLC on a Superdex peptide column of the disulphides. Mobile phase: 0.1 M sodium acetate buffer, pH 5.6. Sample: 0.5 ml of a mixture containing GSH (2 mM), CSH (1 mM) and SOX (30 nkat) after 30 min of reaction at pH 5.6.

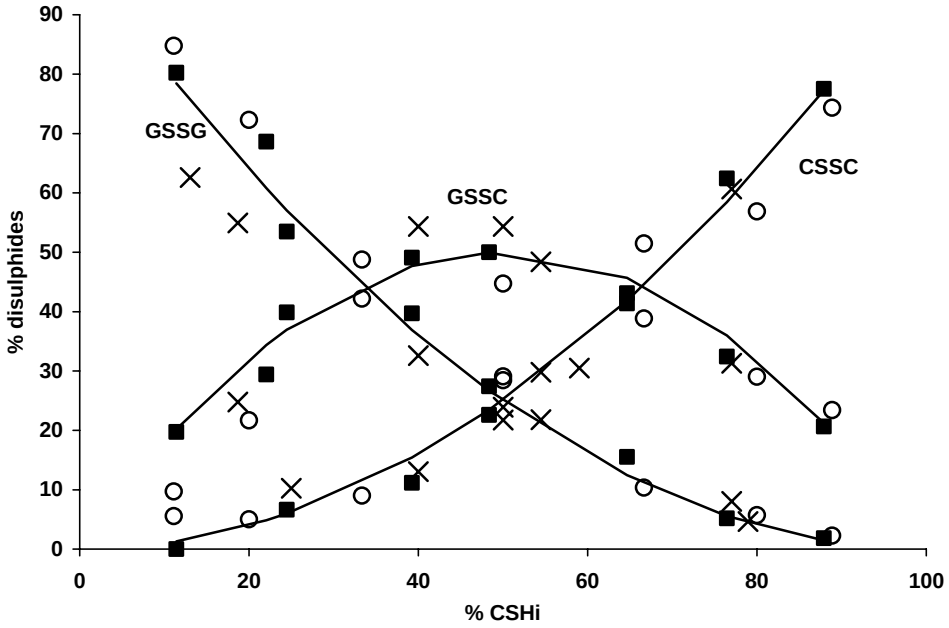

Fig. 6. Relative proportions of the disulphides formed after complete oxidation of different initial concentrations of GSH (GSH_i) and CSH (CSH_i) mixtures, in the presence of 30 nkat SOX (○), 30 nkat GSH-DHase (×), and 2 mM $KBrO_3$ (■).

are reliable to assay the GSSG, GSSC and CSSC amounts in model mixtures.

In order to compare the oxidising ability of SOX, GSH-DHase and $KBrO_3$, different mixtures of GSH and CSH were prepared and treated by these oxidising systems. In almost all cases, GSH and CSH were undetectable and the amounts of disulphides corresponded to the amounts of thiols initially introduced in the mixtures. Size-exclusion FPLC results showed that GSSG, GSSC and CSSC were the unique products obtained in our conditions whatever the oxidising system. The influence of the ratio of the initial concentrations of CSH and GSH, respectively, CSH_i and GSH_i, on the disulphide formation was examined. The relative disulphide amounts expressed as a percentage of the total disulphide amount were plotted (Fig. 6). Similar curves were obtained when the effects of SOX, GSH-DHase and $KBrO_3$ were compared. Therefore, for a given initial mixture of GSH and CSH, the three oxidising systems led to approximately the same final composition in disulphides. However, slight differences appeared which may be attributed to the kinetic characteristics of the oxidising system, since SOX and GSH-DHase catalyse preferentially the GSH oxidation whereas $KBrO_3$ oxidises more rapidly CSH. It can be assumed that the systems were probably not systematically in their state of equilibrium. Nevertheless, the result is in agreement with the thermodynamical principle stating that the final state of equilibrium of a system is not dependent on the type of catalyst but it depends on the amount of species initially present. It must be stressed that $KBrO_3$ is not a true catalyst since it acts as an oxidant in the formation of disulphides. However, since the amount of $KBrO_3$ consumed during the reaction was very low compared to its initial amount, it can be considered as a catalyst in our reaction conditions.

4. Conclusions

Using response surface methodology, the optimal elution parameters have been determined for a rapid separation by RP-HPLC of the reduced and oxidised forms of glutathione and cysteine without any derivatisation. In these conditions, the asymmetric disulphide GSSC can also be analysed. The identification and the quantification of these molecules were achieved using UV and coulometric detection. The sensitivity obtained by coulometric detection is much greater compared to the results described by Reed et al. [12]. The choice between the coulometric and UV detection relies on a compromise between the sensitivity and the ease to handle. Besides these analytical techniques, we succeeded to separate the symmetric (GSSG and CSSC) and asymmetric (GSSC) disulphides by a SE chromatographic method. The disulphides produced from GSH and CSH in the presence of SOX were identified as GSSG, GSSC and CSSC after size-exclusion FPLC fractionation of the resulting mixture and RP-HPLC analysis of the collected fractions. Using these coupled methods, kinetic studies of two oxidoreducing enzymes and potassium bromate were carried out in model mixtures with variable amounts of GSH and CSH. For a given mixture of thiols, the three oxidising systems led to a quasi similar relative composition of the three disulphides meaning that in our experimental conditions the reactions were close to their state of equilibrium.

Since in wheat flour samples the average levels of CSH, GSH and GSSG are 10, 50 and 20 nmol/g of flour, respectively [10,11,25–27] the reported methods could be applied to determine the thiols and disulphides contents in wheat flour. Studies concerning this application are carried out in our laboratory and will be the subject of a next publication.

References

[1] A.H. Bloksma, Cereal Chem. 49 (1972) 104.
[2] I.K. Jones, J.W. Phillips, F.J.R. Hird, J. Sci. Food Agric. 25 (1974) 1.
[3] W. Dong, R.C. Hoseney, Cereal Chem. 72 (1995) 58.
[4] C.S. Fitchett, P.J. Frazier, in: J.M.V. Blanchard, P.J. Frazier, T. Gaillard (Eds.), Chemistry and Physics of Baking, Royal Society of Chemistry, London, 1986, p. 179.
[5] X. Chen, J.D. Schofield, Z. Lebensm. Unters. Forsch. 203 (1996) 255.
[6] L. Louarme, C. Vignaud, L. Rakotozafy, J. Potus, J. Nicolas, in: C.M. Courtin, W.S. Veraverbeke, J.A. Delcour (Eds.), Proceedings of the Third European Symposium on Enzymes in Grain Processing, Katholieke Universiteit Publishers, Leuven, 2003, p. 311.
[7] N. Kaïd, L. Rakotozafy, J. Potus, J. Nicolas, Cereal Chem. 74 (1997) 605.
[8] B. Dupuis, Cereal Foods World 42 (1997) 171.
[9] C. Vignaud, N. Kaïd, L. Rakotozafy, S. Davidou, J. Nicolas, J. Food Sci. 67 (2002) 2016.
[10] I.K. Jones, P.R. Carnegie, J. Sci. Food Agric. 22 (1971) 358.
[11] R. Sarwin, G. Laskawy, W. Grosch, Cereal Chem. 70 (1993) 553.
[12] D.J. Reed, J.R. Babson, P.W. Beatty, A.E. Brodie, W.W. Ellis, D.W. Potter, Anal. Biochem. 106 (1980) 55.
[13] M.B. Sliwkowski, M.X. Sliwkowski, H.E. Swaisgood, H.R. Horton, Arch. Biochem. Biophys. 211 (1981) 731.
[14] M.W. Davey, E. Dekempeneer, J. Keulemans, Anal. Biochem. 316 (2003) 74.
[15] H. Zielinski, J. Honke, A. Troszynska, H. Kozlowska, Cereal Chem. 76 (1999) 944.
[16] L.A. Allison, R.E. Shoup, Anal. Chem. 55 (1983) 8.
[17] J.P. Richie, C.A. Lang, Anal. Biochem. 163 (1987) 9.
[18] N. Kaïd, J. Potus, J. Nicolas, Sci. Aliments 20 (2000) 237.
[19] P.R.C. Harvey, R.G. Ilson, S.M. Strasberg, Clin. Chim. Acta 180 (1989) 203.
[20] N.C. Smith, M. Dunnett, P.C. Mills, J. Chromatogr. B 673 (1995) 35.
[21] S. Melnyk, M. Pogribna, I. Pogribny, R. Jean Hine, S. Jill James, J. Nutr. Biochem. 10 (1999) 490.
[22] X.F. Wang, M.S. Cynader, J. Neurochem. 74 (2000) 1434.
[23] J. Goupy, Plans d'Expériences pour Surfaces de Réponse, Dunod, Paris, 1999.
[24] W.B. Gratzer, in: H.A. Sober (Ed.), Handbook of Biochemistry, Chemical Rubber Co., Cleveland, 1970, p. B72.
[25] B. Hahn, R. Sarwin, W. Grosch, in: Proceedings of the International Conference: Wheat Structure, Biochemistry and Functionality, Royal Society of Chemistry, London, 2000.
[26] J.D. Schofield, X. Chen, J. Cereal Sci. 21 (1995) 127.
[27] F.R.J. Hird, I.W.D. Croker, W.L. Jones, J. Sci. Food Agric. 19 (1968) 602.