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An HPLC-automated Derivatization for Glutathione and Related Thiols Analysis in *Brassica rapa* L.

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Abstract: The high content of glucosinolates and glutathione makes the Brassicaceae an important healthy food. Thiols and especially glutathione and γ-Glu-Cys-Gly tripeptide are involved in many fundamental cellular functions such as oxidative stress protection. Although several methods for sulphur compounds analysis in biological samples are actually used, the determination of glutathione and other sulphur derivatives in plant tissues is rather problematic due to their extreme susceptibility to oxidation, which can lead to their overestimation. The aim of this work was the improvement and validation of an automated method for determination of reduced and oxidised glutathione, cysteine and γ-glutamylcysteine in plant tissues. The method consists of a fully automated pre-column derivatization of thiols based on monobromobimane reagent, a high-performance liquid chromatography derivatives separation, and a fluorimetric detection and quantification. The method was successfully applied for determination of the oxidized and reduced forms of Cys, γ-GC and GSH content in leaves, petioles, inflorescences and roots of *Brassica rapa* L. subsp. Sylvestris. At harvest, in freshly cut plants, the average contents of GSH/2GSSG were 840/45, 345/70 and 150/70 nmol g^{-1} FW for the florets, leaf blades and stems, respectively; those of Cys/2Cys were 80/12, 29/12 and 24/6 nmol g-1 FW; while those of γ-GC/γ-GCCG-γ were 8.0/4.0, and 6.0/3.0, 3.0/2.0 nmol g^{-1} FW, respectively. Such amounts were lower in low-sulphur-grown plants at harvest. The very low coefficient of variation between repeated tests (maximum 1.6%), the high recovery of internal standard (>96%) and the linear correlation coefficient of the calibration ($R^2 > 0.99$) support the efficiency of this method that allowed analysing about 50 samples/die in a totally automated manner with no operator intervention. Our results show that the reported method integrations can significantly improve thiols detection via HPLC.

Keywords: *Brassica rapa* L. subsp. sylvestris; reduced and oxidized thiols determination; glutathione; γ-Glutamilcysteine; cysteine; HPLC analysis; monobromobimane reagent

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1. Introduction

Brassicaceae have a high health potential thanks to their high amounts of several nutraceutical compounds such as glucosinolates and glutathione [1]. The concentration of these and many other beneficial compounds depends on genetic and environmental factors such as climate, sun exposure and the concentration of nutrients, particularly sulphates, in the culture medium [2,3].

Glutathione, the γ-Glu-Cys-Gly tripeptide, is the most abundant thiol compound in plant and animal cells. It is involved in many fundamental cellular functions such as the homeostasis of cellular redox potential [4], the ascorbate–glutathione cycle that is the main route of detoxification of ROS thanks to its key role in reactions involving enzymes and metabolites with redox properties, avoiding thus oxidative damages in plants [5,6] and the

regulation of nuclear proteins and transcription factors by S-glutathionylation, affecting chromatin structure and condensation [7]. Thiols also have a pivotal role in defence and protection from biotic and abiotic stress [8,9]. In particular, glutathione can function as a substrate for the synthesis of phytochelatins [10–12] involved in the detoxification of heavy metals. It is the main compound involved in the transport and allocation of sulphur during plant growth [13] and can be used as a source of cysteine in sulphur deprivation [14]. In plants, glutathione synthesis occurs from cysteine, which is the first organic metabolite formed during sulphur assimilation, through a two-step pathway catalyzed by the γ-glutamylcysteine synthetase to form γ-glutamylcysteine from glutamate and cysteine and glutathione synthetase that add glicine to such compounds to form glutathione [15].

Glutathione analysis, as well as that of other thiol metabolites, is problematic because the reduced form rapidly oxidizes to the disulphide one during sample preparation [16], leading to an overestimation of oxidized form and determining a wrong evaluation of physiological cell status. The derivatization reactions, used for their determination, often form unstable products that may affect the analytical results [17]. In addition, because such compounds may be involved in different reactions, even the HPLC analysis may be difficult [18].

In the literature, several methods for sulphur compounds analysis in biological samples are described. The first techniques used, which were based on enzymatic [19], fluorimetric [20] and colorimetric [21] assays, were inadequate due to their low sensitivity and low reproducibility.

Recently, several methods based on high-performance liquid chromatography (HPLC) were devised. These methods use: (i) UV absorbance measurement of sulphur compounds following derivatization with reagents that bind the amino group, such as 5,5 γ-dithiobis-(2-nitrobenzoic) acid (DTNB or Ellman's reagent) [22] or the sulfhydryl group such as 2,4-dinitrofluorobenzene [23]; (ii) fluorescence measurement following derivatization with monobromobimane [24], with *ortho*-phthalaldehyde (OPA) derivatives [25], 1-dimethylaminophthalene-5-sulfonyl chloride (dansyl chloride) [26], 7-fluorobenzo-2-oxa-1,3-diazol-4-sulfonate (SBD-F) [27]; and with or with derivatives of *N*-(1-pyrenyl) maleimide (NPM) [28]. There are also electrochemical methods that do not require derivatization [29,30].

Furthermore, many reagents used for analysis can support reactions with multiple functional groups or produce unstable derivatives, complicating the determination even via HPLC [18]. Most of the methods in the literature have complicated protocols, time-wasting procedures, low reproducibility and often insufficient sensitivity for poorly concentrated samples. Finally, most of them still have difficulty measuring the reduced and oxidised thiol forms.

For these reasons, the goal of this study was to develop a method for the determination of thiols that would overcome these problems. We report an improved automated HPLC method for the determination of cysteine (Cys/Cis), γ-glutamylcysteine (γ-GC: oxidised and reduced) and glutathione (GSH/GSSG) based on derivatization with monobromobimane (MBB) in plant samples compared to that previously described by Noctor and Foyer [31]. We have optimized the sample extraction and handling, the derivatization reaction, the chromatographic separation and the fluorimetric detection. It shows excellent reproducibility and high sensitivity. The method has been tested to determine simultaneously the concentration of cysteine, γ-glutamylcysteine and glutathione in plant extracts of broccoli (*Brassica rapa* L. subsp. Sylvestris var. esculenta).

2. Materials and Methods

2.1. Plant Material and Experimental Design

Seeds of ecotype of *Brassica rapa* L. c.v. sylvestris (Blumen company, Piacenza, Italy) were soaked in 2 mM calcium sulphate ($CaSO_4$) for thirty minutes to stimulate germination. Subsequently, seeds were placed on filter paper imbibed with deionized water in the dark at 22 ± 2 °C for four days. Seedling sprouts were transferred to hydroponics using plastic

dishes (15 × 25 × 12 h cm) filled with distilled water. Growth was carried out under the following conditions: photoperiod 16 h, PAR 500 µE, 16/19 °C day/ night, 60–70% RH. After 10 days of cultivation, the young plants were separated into 4 groups with different sulphur nutrition (K_2SO_4: 0.01, 0.05, 0.5 and 2 mM) in modified Hoagland medium (5 mM KNO_3, 0.5 mM KH_2PO_4, 0.5 mM K_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05 mM $FeCl_3$, trace elements solution: 10 mL/L). The nutrient solution was replaced every three days. After 45 days of hydroculture, leaves, petioles, inflorescences and roots were collected separately, frozen in liquid nitrogen and stored at −80 °C. Subsequently, plant material was powdered in a mortar and pestle with liquid nitrogen, and aliquots of 0.1 g of powdered material were stored at −80 °C for further analyses.

Experiments were performed in triplicate.

2.2. Chemicals and Reagents

GSH, GSSG, γ-GC, cysteine (Cys), cistine (cis), monobromobimane (MBB), 3-[4-(2-hydroxyethyl)-1- piperazinyl] propanesulfonic acid (EPPS), Diethylene triamine pentaacetic acid (DTPA), Dithiothreitol (DTT), hydrochloric acid (HCl) and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA), acetic acid from Carlo Erba Reagent SPA (Milan, Italy), methanol and acetonitrile (HPLC grade) from Romil Ltd. (Cambridge, UK). All HPLC buffers were filtered through 0.45 mm Millipore (Billerica, MA, USA) filters prior to use.

2.3. Standard Solutions

The stock solutions of GSH, GSSG, Cys, cis and γ-GC were prepared in 0.1 N HCl to minimize oxidation and stored at −20 °C for 1 month. Working standard solutions were prepared daily by dilution in 0.1 N HCl.

2.4. Derivatization Reagent

MBB stock solution was prepared under dim lighting by dissolving 25 mg of reagent-grade MBB in 614 µL of acetonitrile (final concentration 150 mM) and stored at −20 °C for 1 month. Working 30 mM solutions were prepared daily by dilution in acetonitrile and used within 3 days.

2.5. Chromatographic Conditions

A Hewlett & Packard HPLC system (model 1100; Agilent Technologies Cernusco sul Naviglio MI, Italia S.p.A, Italy), equipped with an autosampler provided the online derivatization of samples and the binary pump, allowing the construction of a discontinuous gradient (Table 1). The separation was performed using a Beckman Ultrasphere C-18 ODS (250 × 4.6 mm ID 5 µM particle size) protected by a C18 Security Guard pre-column (4 × 3 mm ID; Phenomenex Inc., Torrance, CA, USA). The column was kept at 25 °C. The fluorescence detection of MBB adducts was performed with excitation at 392 nm and emission at 480 nm. The control and data analyses were performed through the HP Chem-Station software version A.06.03 Agilent Technologies LC/MSD Cernusco sul Naviglio MI, Italia S.p.A, Italy.

Table 1. Chromatographic gradient. Buffer A was 40 mM sodium acetate and methanol 17% pH 3.9. Buffer B was a 100% methanol. The flow rate was 1 mL/min.

	Time (min)	A	B
Balancing	0	100	0
Analysis	12	94	6
	15	0	100
Wash	17	100	0
	20	100	0

2.6. Extract Preparation and Pre-Column Derivatization and Analysis

2.6.1. Extract Preparation

Frozen powdered samples (0.1 g) were suspended in 1 mL of cold 0.1 M HCl degassed solution, mixed and kept at 4 °C for 2 h. Then, they were centrifuged at 13,000× *g* for about 2.5 min (Microcentrifuge, 5424R, Eppendorf, Hamburg, Germany), and the resultant supernatants were analyzed by HPLC.

2.6.2. Pre-Column Derivatization Procedure

Briefly, after transferring sample extracts in plugged vials in the HPLC array, the autosampler program allowed us to do the following procedure: (i) keep 30 µL of extract or standard solution by the needle from the sample vial in the HPLC array; (ii) wash the outside of the needle five times in a vial containing a water/methanol 50/50 solution; (iii) keep 70 µL of 200 mM EPPS, 5 mM DTPA buffer pH 11.7 (to have a final pH of 9.0); (iv) wash the outside of the needle; (v) keep 6 µL of 30 mM MBB solutions from a selected vial; (vi) eject the whole solution in a clean reaction vial; vii) mix it five times; (viii) wait for 7.5 min; (xi) keep 70 µL of the derivatized sample and inject it into the column for the analysis.

The MBB reacts only with the reduced thiols (free-SH). In order to also determine the oxidized form, an automated reduction step with DTT was included in the software program before MBB reaction (point v). Therefore, after step iv, the autosampler program allowed us to (v') add 3.8 µL of 10 mM DTT (freshly daily prepared solution) to the solution vial; (vi') mix the whole solution five times; (vii') wait 2 min; (viii') wash the outside of the needle as above, in order to not contaminate the reagent solution; (xi') keep 6 µL of MBB 30 mM solution; (x') add it to the same reaction vial; (xi') mix five times for the derivatization procedure; (xii') wait for 7.5 min; (xiii') keep 70 µL of the derivatized sample and inject it into the column for the analysis.

The software program repeated the procedures of derivatization, separation and analysis for each sample extract. For each sample, a new clean reaction vial for each analysis was programmed in the array, and the selected vials containing reagents and the washing solution were substituted and refilled daily. In addition, to prevent false results due to the eventuality that the needle could be partially clogged by some sample particles that remained in the decanted extract, each analysis was repeated three times.

2.6.3. Chromatographic Conditions and Analysis

The separation and analysis were performed by using the cromatograpic apparatus described in Section 2.5 (chromatographic conditions). The quantification was performed under the binary elution gradient elution (mobile phase: A = 40 mM sodium acetate and methanol 17% pH 3.9; B = methanol (100%)), with the gradient reported in Table 1. During the derivatization procedure, the column was furtherly balanced with the eluent A.

The fluorescence detection of MBB adducts was performed with excitation at 392 nm and emission at 480 nm. The control and data analyses were performed through the HP Chemstation software.

2.7. Validation by Recovery Experiments

Authentic GSH, GSSG, Cys and CSSC were added before analysis to the different tissue samples from plants grown with different sulphur nutrition (K_2SO_4: 0.01, 0.05, 0.5 and 2 mM) or from different plant parts. The added internal standards did not mask endogenous levels because their concentrations were about the same recorded in the samples. Control samples without internal standard addition were also run at the same time. The analytes were analyzed alone and together to verify that there was not any interference in order to validate the method.

2.8. Statistical Methods

Results for oxidized and reduced content of Cys, γ-GC and GSH were expressed as mmol/100 g fresh weight. Data were elaborated and analysed using SigmaPlot v. 14.5, Systat Software Inc., San Jose, CA, USA.

3. Results

3.1. Samples Preparation

An accurate determination of GSH and GSSG levels depends on prevention of GSH oxidation during sample preparation. To reduce this phenomenon, the contact with oxidizing agents was minimized. In order to remove oxygen from the solutions, nitrogen was bubbled into the extraction and assay mixes for 10 min prior to use and nitrogen was flushed onto the samples during their preparation. DTPA was used as a chelant for heavy metal ions. Finally, the oxidation of the thiols during extraction was minimized by using 0.1 N HCl as extracting solution.

3.2. Optimization of Fluorometric Detection

The optimal excitation and detection wavelengths of MBB derivatives were determined by performing a fluorescence spectrum in which the excitation wavelength was set in the range 300–430 nm for excitation and 440–600 nm for emission detection (Figure 1). The maximum fluorescence value was obtained at 392 nm for the excitation and 480 nm for the emission (Figure 1).

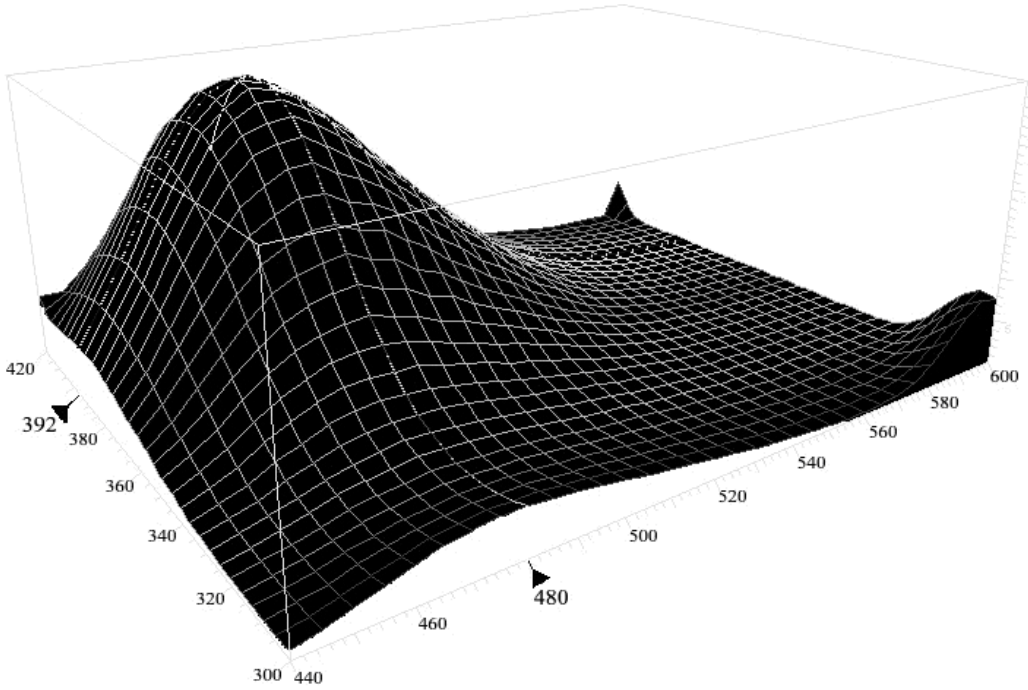

Figure 1. Luminance 3D graph of the GSH-MBB adduct obtained by means of an HPLC 1100 device equipped with a fluorimetric detector and ChemStation software version **A.06.03** Agilent Technologies LC/MSD Cernusco sul Naviglio MI, Italia S.p.A, Italy.

In this view, the excitation for further analysis was set at 392 nm and emission at 480 nm. These conditions were optimal also for cysteine and γ glutamylcysteine adducts.

3.3. Chromatographic Gradient Optimization

The chromatogram (Figure 2) was obtained using the chromatographic gradient described in Table 1.

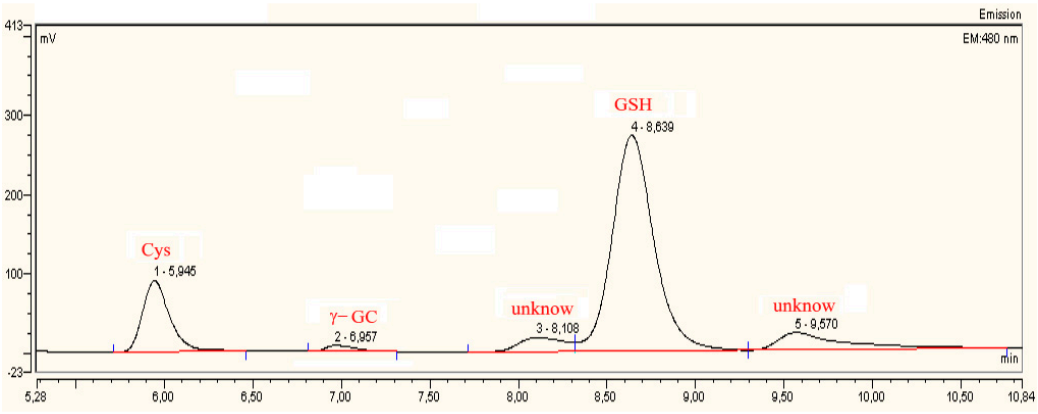

Figure 2. HPLC-FLD profile of Cys, γ-GC and GSH from acid extract of *Brassica rapa* L. separated using a discontinuous gradient of sodium acetate-methanol on Beckman UltraSphere C-18 ODS reverse-phase column. Excitation wavelength 392 nm; detection wavelength 480 nm.

The gradient was initially very slow (between 2 and 28 min) to resolve the peaks of the tested compounds. Under such conditions, no interference of reagent or adduct peaks were evidenced even at low sample concentrations, which differed from previously reported data [32]. The elution times for Cys, γ-GC, and GSH were 5.95, 6.96 and 8.64 min, respectively. The identification was made by direct comparison with the pure standards as well as in biological samples in which the internal standards were added.

After the elution of compounds of interest, the gradient was kept at 100% methanol for 5 min to wash the column and then rebalanced to the initial elution buffer gradient with 40 mM sodium acetate in 10% methanol for 10 min. Under these conditions, no significant displacement of the elution times in subsequent analyzes occurred. After analysis, the column was washed with 100% methanol for 30 min.

3.4. Monobromobimane Solution Stability

In order to analyse many samples loaded by the autosampler HPLC system at room temperature, the MBB solution stability over time was tested. Figure 3 shows the result of the derivatization of a fresh solution of GSH 20 µM by a solution of MBB 30 mM in acetonitrile kept at 25 °C in the dark for up to eight days. MBB solution still retained over 90% reactivity towards GSH after 3 days of storage.

3.5. Derivatization Reaction in Dependence on pH

As shown in Figure 4, the MBB derivatization reaction occurred at basic pH, and the reaction rate increased up to pH 11.00. However, at higher pH, the MBB reactivity with other functional groups different from thiols increased, leading to the appearance of other peaks that complicated the analyses. In this context, the best condition for a clear chromatogram was to stay at pH 9.00, at which the reaction rate was still 90% of the maximum.

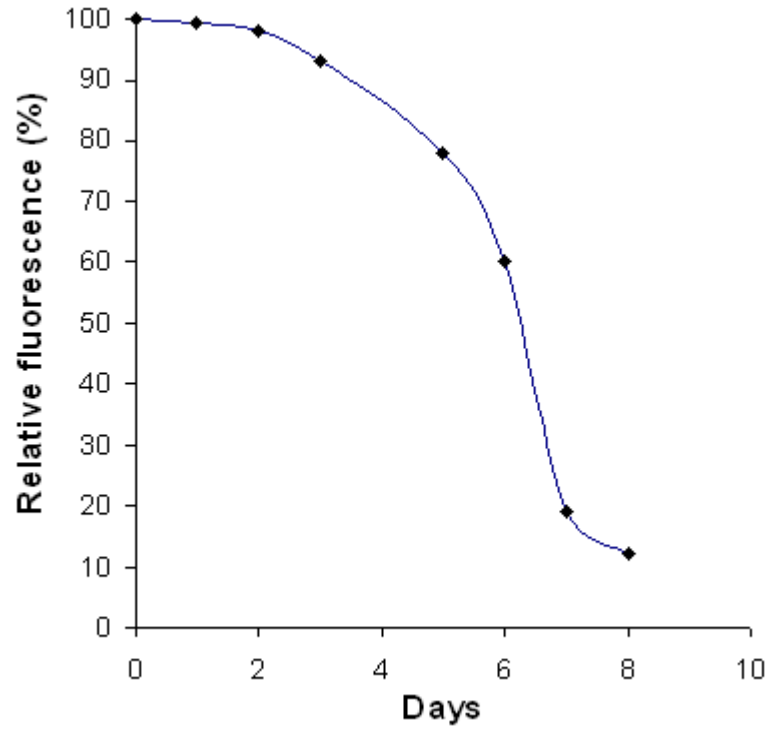

Figure 3. Effect of storage time on the stability of a 30 mM MBB solution in aceonitrile stored at room temperature up to 8 days and used to derivatize a 20 μM GSH solution. The relative fluorescence for the MBB solution immediately after preparation was set at 100.

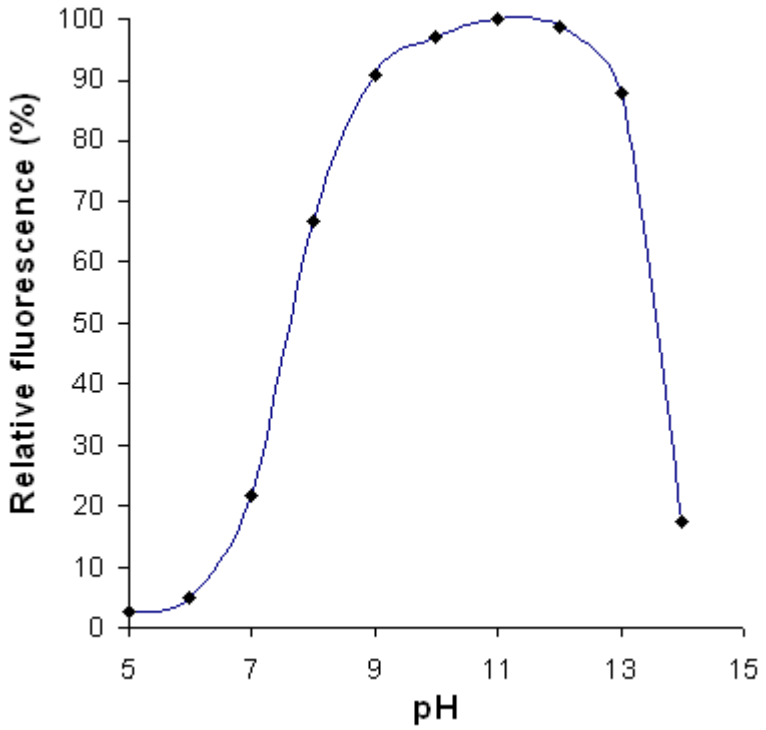

Figure 4. pH effects on GSH-MBB adduct formation. A 20 μM GSH solution was incubated for 7.5 min with MBB and reaction buffer at the indicated pH. Relative fluorescence was set at 100 for pH 11.

3.6. Reaction Time Course

The long reaction time was responsible for increasing the number of interfering peaks due to the reaction of the MBB with other functional groups or with di-sulphides, tri-sulphides and other compounds generated by multiple functional group thiols.

The reaction time indicated in the literature is 10–15 min [16,31]. Figure 5 shows tests carried out by reacting a solution of GSH 20 μM with MBB at different times.

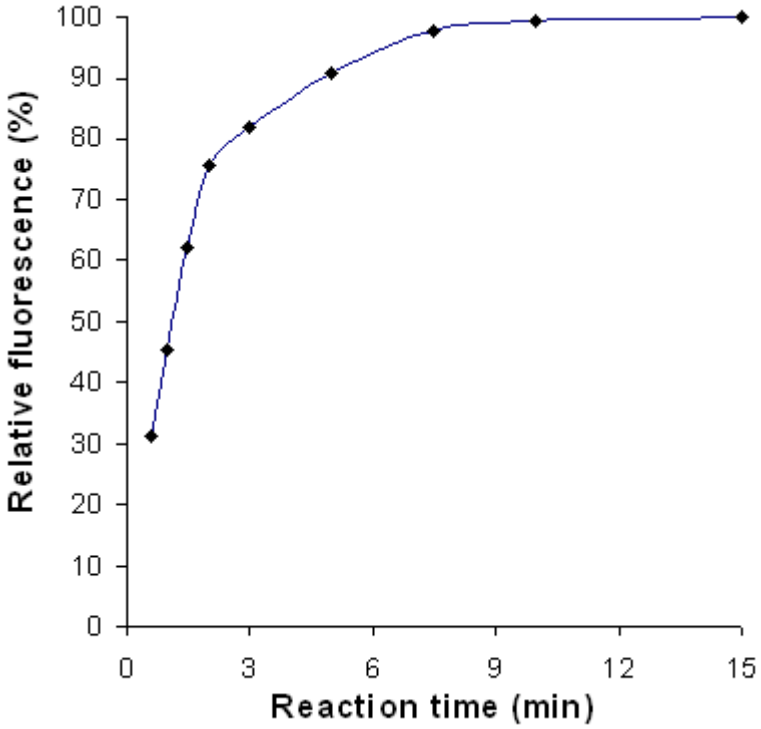

Figure 5. MBB-GSH adduct as a function of time. The GSH 20 μM solution was derivatized at pH 9 with MBB. The relative fluorescence for the reaction time of 15 min was fixed at 100.

The best compromise to enhance chromatogram quality, thiol selectivity and limit sensitivity was 7.5 min. In these conditions, in fact, more than 98% of GSH reacted, resulting in a clear chromatogram more than that produced in longer reaction times (not shown). In this time, cys and γ-GC also largely reacted.

3.7. Data Analysis

To verify the linearity of the fluorescence response in the overall analysis, serial dilutions of pure standards of GSH, Cys, and γ-GC were injected in sequence. Figure 6 shows the calibration curves for Cys, γ-GC and GSH. The linear correlation coefficient $R^2 = 0.9980$, $R^2 = 0.9981$ and $R^2 = 0.9984$, for Cys, γ-GC and GSH respectively, ensured a linear response from 0 to 1.2 nmol injected. The high linear correlation coefficient R^2 (greater than 0.9980) provided a linear response at least up to 1.2 nmol of injected adduct (Figure 6). The fluorescence coefficient for pmoles of injected compounds was 3.2760 for Cys, 3.1685 for γ-GC and 3.7316 for GSH. Method reproducibility was verified by injecting 5 times the same leaf extract of *Brassica rapa* L. derivatized with MBB. The per cent coefficient of variation was 1.1 for Cys, 1.6 for γ-GC and 1.4 for GSH.

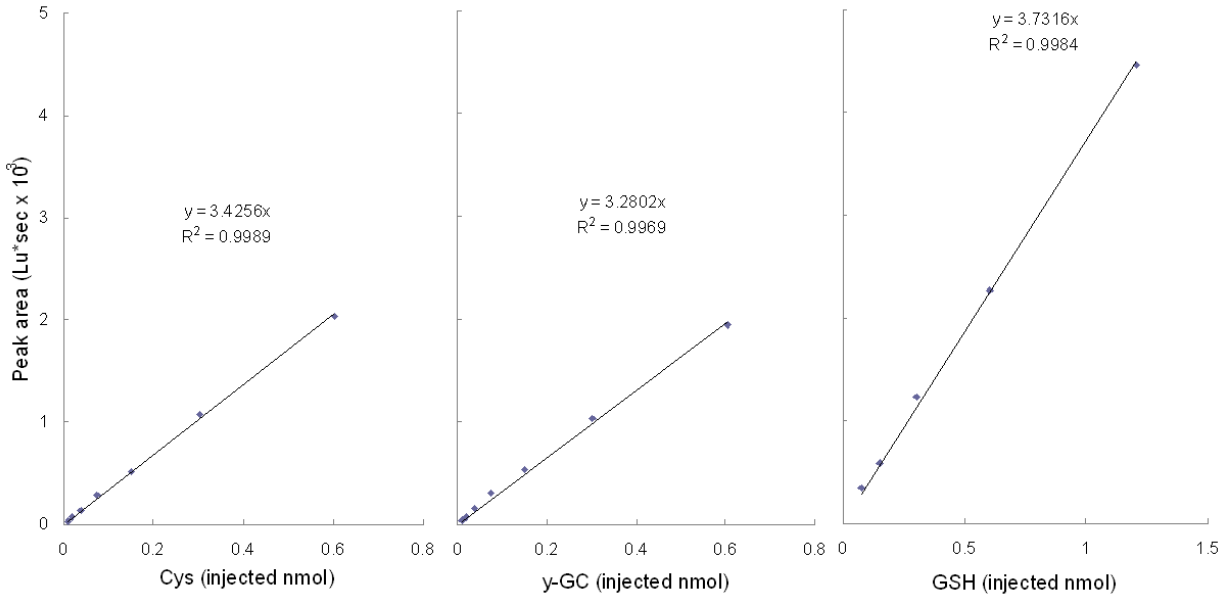

Figure 6. Calibration curves for Cys, γ-GC and GSH.

The recovery of GSH, Cys and γ-GC from *Brassica rapa* L. tissues was tested by adding an internal standard at concentrations similar to the endogenous ones in the 0.1 M HCl solution used to extract the thiols from the samples. The analyses were performed in triplicate by comparing samples with and without internal standard. Estimated recoveries were 97.4 ± 1.4% for GSH, 96.7 ± 1.6% for γ-GC and 97.6 ± 1.2% for cysteine.

The lower detection limit was determined for GSH by carrying out serial dilutions of GSH. It was evidenced that a signal-to-noise ratio higher than 3 could still be measured when 100 femtomol of GSH in 30 µL were sampled for the derivatization (corresponding to 3 nM).

3.8. Determination of Thiols in Brassica rapa L. Subsp. Sylvestris Var. Esculenta

The method was tested to determine the oxidized and reduced form of Cys, γ-GC and GSH content in florets, stems and leaf blades of *Brassica rapa* L. subsp. Sylvestris var. esculenta grown in hydroponics in Hoagland medium supplemented with sufficient sulphate (2 mM) (Figure 7; Table 2).

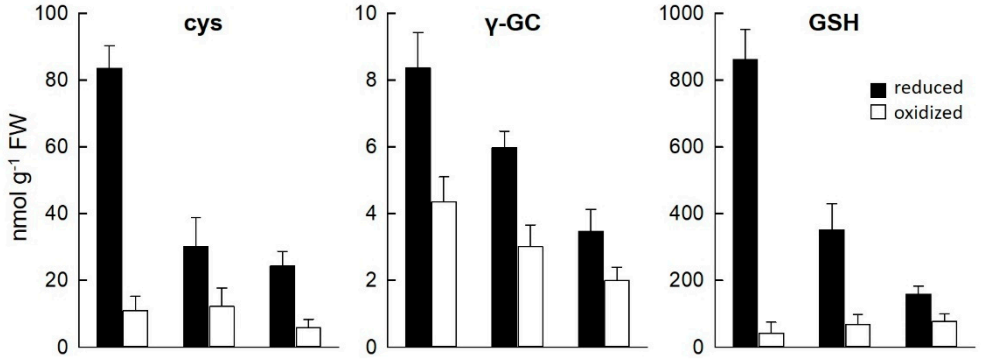

Figure 7. Oxidized and reduced content of Cys, γ-GC and GSH in florets, leaf blades and stems of *Brassica rapa* L. subsp. Sylvestris var. esculenta.

Table 2. Recovery percentage of GSH, Cys and γ-GC in plant part of *B. rapa*. The amount of analyte that was added in the range of concentrations nmol of added analyte varied dependent upon endogenous concentration in each vegetable. Ranges were as follows: GSH, 78–253; GSSG, 41–142; CYS, 41–104; CSSC, 53–253.

Plant Part	Analyte	Control (pmol·g^{-1})	Recovery (%)	Area (Lu/sec)
Inflorescences	GSH	895	98 ± 5	3500 ± 50
	Cys	92	103 ± 7	3020 ± 100
	γ-GC	12	98 ± 9	3100 ± 100
Laminae	GSH	410	101 ± 3	3600 ± 50
	Cys	41	95 ± 4	3100 ± 100
	γ-GC	9	96 ± 10	3050 ± 100
Petiole	GSH	220	102 ± 7	3700 ± 40
	Cys	30	97 ± 8	3200 ± 100
	γ-GC	5	103 ± 8	3200 ± 100

The average contents of GSH/2GSSG were around 840/45, 345/70 and 150/70 neq g^{-1} FW for the inflorescences, laminae and petiole, respectively; those of Cys/2Cis were 80/12, 29/12 and 24/6 neq g^{-1} FW; and those of γ-GC/2γ-GCCG-γ were 8.0/4.0, 6.0/3.0 and 3.0/2.0 nmol g^{-1} FW, respectively. Similar values have been found for Brassicaceae and other plants [33–37]. Glutathione showed the highest concentration in the florets, being two- and three-fold higher than that found in the leaf blades and in the stems, respectively. Even though the Cys and γ-GC showed a similar pattern of distribution among the plant parts, this occurred at concentrations much lower than that of glutathione for Cys and γ-GC.

The method was also applied to evaluate total and reduced contents of glutathione in inflorescences, laminae, petioles and roots of *Brassica rapa* L. cv Sylvestris plants grown in nutrient solutions containing 0.01, 0.05, 0.5 and 2 mM sulphate. Higher glutathione amounts were recorded in the inflorescences with a content two- and three-fold higher than that found in leaves and petioles, respectively. Total cysteine concentration (cys+cis) in broccoli plants (Table 3) was about 20-fold lower than the average glutathione content; however, like glutathione, the total cysteine content was also affected by sulphate availability. Cysteine concentration in the inflorescences grown under high sulphur nutrition was tripled compared to that recorded in the inflorescences grown with low sulphur medium and increased ten-fold compared to that recorded in the inflorescences grown under sulphate deficiency. In leaves, petioles and roots, the total cysteine content also increased in response to sulphate, but only up to the 0.5 mM sulphate concentration (Table 3).

The fraction of reduced glutathione was about 90% in the inflorescences, and it was not influenced by sulphate availability (Table 3). Conversely, in the other analysed tissues, the glutathione-reduced fraction was dependent on sulphate; in fact, it was reduced to 75% of that recorded in leaves of plants grown with sulphate deficiency. The cysteine reduced fraction (Table 3) followed the same trend as that observed for glutathione, with lower values recorded in roots and leaves grown under sulphur deficiency (50% and 60% of reduced cysteine, respectively). As for glutathione, reduced cysteine content was not influenced by sulphate availability. The maximum concentration of reduced cysteine (90% of total cystine content) was recorded in the plants grown under high sulphur availability.

Table 3. Total glutathione (GSH+2GSSG), total cysteine and total y-glutamilcisteine in nanoequivalents per gram of fresh weight (neq/g FW), as well as the percentage of the reduced forms in inflorescences, laminae, petioles and roots of *Brassica rapa* L. cv Sylvestris plants grown in nutrient solutions containing 0.01, 0.05, 0.5 and 2 mM sulphate.

Plant Material	Sulphate Nutrition (mM)	GSH+2GSSG (neq/g FW)	GSH (%)	Cys+2Cis (neq/g) FW	Cys (%)	ãGC+2yGCCGy (neq/g) FW	γGC (%)
Inflorescences	0.01	20 ± 5	90 ± 5	5 ± 1	88 ± 5	2.5 ± 1	80 ± 5
laminae	0.01	50 ± 5	87 ± 5	3 ± 1	70 ± 5	1.5 ± 1	65 ± 5
petioles	0.01	15 ± 5	88 ± 5	3 ± 1	93 ± 5	1 ± 1	82 ± 5
roots	0.01	120 ± 10	92 ± 5	4 ± 1	70 ± 5	2.2 ± 1	66 ± 5
Inflorescences	0.05	544 ± 20	90 ± 5	18 ± 2	92 ± 5	5 ± 1	85 ± 20
laminae	0.05	59 ± 5	87 ± 5	16 ± 2	67 ± 5	3 ± 1	60 ± 20
petioles	0.05	29 ± 5	87.80869	5 ± 1	93 ± 5	3 ± 1	70 ± 20
roots	0.05	220 ± 10	91.72207	22 ± 2	81 ± 5	5 ± 1	80 ± 20
Inflorescences	0.5	950 ± 50	90 ± 5	20 ± 5	92 ± 8	10 ± 2	90 ± 10
laminae	0.5	400 ± 30	94 ± 5	12 ± 3	95 ± 8	7 ± 2	85 ± 10
petioles	0.5	105 ± 20	93 ± 5	5 ± 3	88 ± 8	5 ± 1	80 ± 10
roots	0.5	220 ± 20	96 ± 5	15 ± 3	90 ± 8	11 ± 2	95 ± 10
Inflorescences	2.0	1144 ± 50	90 ± 5	53 ± 5	86 ± 8	20 ± 2	95 ± 5
laminae	2.0	374 ± 30	94 ± 5	14 ± 3	94 ± 8	10 ± 2	85 ± 5
petioles	2.0	118 ± 30	93 ± 5	10 ± 3	88 ± 8	7 ± 2	80 ± 5
roots	2.0	231 ± 20	96 ± 5	21 ± 3	90 ± 8	11 ± 3	95 ± 5

4. Discussion

The determination and quantification of glutathione and other thiols in biological samples require a very sensitive, fast and selective method. Their concentrations, in fact, in particular those of glutathione precursors, Cys and γ-GC, are generally low in plant samples. Furthermore, due to their extreme sensitivity to oxidation, the concentration ratio between the reduced and oxidized form (GSH/GSSG), which provides important information on the cellular redox state, can easily be altered during sample handling.

When MBB is used for thiols derivatization, being a highly reactive compound, it is important to avoid the formation of different products that could complicate the chromatographic analysis and lead to an incorrect determination of the tested compounds. The method for the quantification of thiols described in this paper allows obtaining an efficient chromatographic separation in HPLC, accompanied by a high sensitivity guaranteed by the high fluorescent thiol-bimane adducts. Furthermore, having demonstrated the stability of the MBB solution in acetonitrile even at room temperature (Figure 3), a fully automated sample handling method could be designed, allowing many samples to be run without changing the reagent solution. Another optimization consisted in the grinding of the tissues in liquid nitrogen and the extraction carried out in 0.1 N HCl, which strongly limited the oxidation of the samples. After 2 days of storage at room temperature (data not shown), the samples extracted in 0.1 N HCl still retained 90% of reduced glutathione. Furthermore, the method provided for the immediate injection of the reaction mixture, avoiding the need to add acid to stop the reaction [31]. On the other hand, the stability of thiol-bimane adducts has been previously demonstrated [33]. The optimization of the elution gradient in HPLC made it possible to obtain an excellent peaks resolution maintaining a good speed of analysis. The gradient applied (Table 1) allowed us to elute cysteine, γ-GC and GSH far from the main interfering peaks due to the presence of the reagent, itself fluorescent. The latter were removed from the column with 100% methanol.

The derivatization reaction carried out at pH 9 (Figure 4), a value higher than that described in the literature [16,31], and the reaction time reduced to 7.5 min (Figure 5), which allowed the reaction of over 97% of the glutathione, rendered clean the chromatogram with a signal-to-noise ratio of 3.3 when 100 fmol of glutathione were injected. In conclusion, the method developed showed an effective separation of the peaks of interest, a high sensitivity (up to 100 fmol), a very good repeatability (maximum 1.6% error), a linear correlation coefficient of the straight calibration ($R^2 > 0.99$) (Figure 6) and a high internal standard recovery (>96%), allowing the analysis of about 50 samples per day in total automation without any intervention by the operator.

We tested our optimized method to determine the endogenous content of Cys, γ-GC and GSH in plants under enriched sulphur nutrition. The method was suitable for this evaluation, and our results showed that the S deficiency during growth led to an increase in sulphate in the inflorescences compared to leaf blades. In plants grown under sulphur deficiency and low sulphur availability, in fact, sulphate accumulated mainly in the inflorescences, conversely to that observed in plants grown in medium under high sulphur nutrition (Table 3). These results suggest that plants are able to redistribute the available sulphate to tissues that have the major need in order to guarantee plant survival and reproduction.

Notwithstanding this representing an optimization of the well-known MBB method [16] and the fact that other attempts have been done, also recently, to offer improvements of this method by fluorescence detection [34,35], this is the first time it has been optimized for a complex tissue like that of *Brassica rapa*, in which quenching caused by interference with components present in the plant extract makes it difficult to perform a fluorescent detection.

5. Conclusions

The determination of glutathione and other sulfur derivatives in plant tissues represents an important analytical method that until now has given some problems due to these metabolites' susceptibility to oxidation. The chromatographic methods using HPLC are widely applicable because of the instrument's availability and the less complicated sample preparation procedures.

The optimization of MBB method developed in this work to investigate the glutathione and γ-Glu-Cys-Gly tripeptide present in different tissues of Brassicaceae performs well.

The method allows a very sensitive glutathione and thiols determination, enabling one to analyse about 50 samples/die in a totally automated manner with no operator intervention.

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