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Direct and simultaneous determination of reduced and oxidized glutathione and homoglutathione by liquid chromatography–electrospray/mass spectrometry in plant tissue extracts

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Abstract

A simple, highly selective, sensitive, and reproducible liquid chromatography–electrospray ionization/mass spectrometry (time of flight) method has been developed for the direct and simultaneous determination of glutathione and related compounds such as homoglutathione in different plant tissues. These compounds are low-molecular mass antioxidants involved in cellular redox homeostasis in plants, and efforts are being made to develop methods to determine the concentrations of oxidized and reduced forms of these compounds and their ratio. Many of the methodologies developed so far, however, are time-consuming and complex; therefore, analytes can decompose and their redox status can change during the analysis process. The method we have developed allows the simultaneous determination of reduced forms (glutathione [GSH] and homoglutathione [hGSH]) and oxidized forms (glutathione disulfide [GSSG]) of these compounds and is also suitable for the determination of ascorbic acid (ASA) and *S*-nitrosoglutathione (GSNO). Quantification was done using isotopically labeled GSH and ASA as internal standards. All compounds were base peak resolved in less than 6 min, and limits of detection were 60 pmol for GSH, 30 pmol for hGSH, 20 pmol for GSSG, 100 pmol for ASA, and 30 pmol for GSNO. The intraday repeatability values were approximately 0.4 and 7% for retention time and peak area, respectively, whereas the interday repeatability values were approximately 0.6 and 9% for retention time and peak area, respectively. Analyte recoveries found were between 92 and 105%. The method was used to determine the concentrations of GSH, GSSG, hGSH, and ASA in extracts from several plant tissues.

Keywords: Ascorbate; Glutathione; Homoglutathione; Liquid chromatography; Mass spectrometry; Oxidized glutathione; *S*-Nitrosoglutathione

Reduced glutathione (GSH,[1] L-Υ-glutamyl-L-cysteine-glycine) and ascorbic acid (ASA) play an important role in maintaining the intracellular redox status in plant cells. Both metabolites act in the so-called ascorbate–glutathione cycle, helping to prevent and/or minimize damages caused by reactive oxygen species (ROS) [1,2]. ROS are produced in cell metabolism under normal conditions, but their production in plants is enhanced when plants are submitted to biotic and abiotic stresses. Both GSH and ASA are key metabolites in the control of the redox-signaling cascades that modulate a variety of metabolic processes [3]. GSH is also implicated in the control of reactive nitrogen species

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[1] *Abbreviations used*: GSH, reduced glutathione; ASA, ascorbic acid; ROS, reactive oxygen species; GSNO, *S*-nitrosoglutathione; NO, nitric oxide; GSSG, glutathione disulfide; DHA, dehydroascorbic acid; hGSH, homoglutathione; HPLC, high-performance liquid chromatography; MS, mass spectrometry; HPLC–ESI/MS(TOF), high-performance liquid chromatography–electrospray ionization/mass spectrometry (time of flight); MPA, metaphosphoric acid; GSH*, isotopically labeled GSH; ASA*, isotopically labeled ASA; EDTA, ethylenediaminetetraacetic acid; PVPP, polyvinyl–polypyrrolidone; *m*/*z*, mass/charge ratio; LOD, limit of detection; S/N ratio; signal/noise ratio; LOQ, limit of quantification; hGSSGh, oxidized homoglutathione; RSD, relative standard deviation; FWHM, full width, half mass; FW, fresh weight.

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[4], and *S*-nitrosoglutathione (GSNO) seems to be a stable transport form of nitric oxide (NO) [5]. When GSH and ASA act as antioxidants in the ascorbate–glutathione cycle, they are oxidized to glutathione disulfide (GSSG) and dehydroascorbic acid (DHA), respectively. On the other hand, GSH is implicated in the detoxification of xenobiotics in conjunction with glutathione *S*-transferases, which react with GSH via the nucleophilic sulfhydryl group of the cysteine moiety [6]. Also, when plants are exposed to heavy metals, GSH is used to synthesize phytochelatins, that is, cysteine-rich peptides able to form complexes with several metal ions [7]. In addition, GSH is the predominant form of reduced sulfur in plants [8]. Homologues of GSH can be found in different plant species, where some of the constitutive amino acids differ from those found in GSH. For instance, homoglutathione (hGSH, L-ϒ-glutamyl-L-cysteine-β-alanine) can be found in several tissues and organs of legumes [9,10], whereas other less studied homologues have been found in a number of plant species [11,12].

From a physiological point of view, the GSH/GSSG ratio often is more relevant than the total concentration of GSH in explaining the effects of different stresses [13,14]. For instance, the GSH/GSSG redox pair ratio could be more influential in the control of gene expression and protein function than is the total concentration of GSH [15]. Under normal conditions, the glutathione pool usually is reduced to a large extent, and the oxidized form (GSSG) often is less than 5% of the total pool. The low concentrations of GSSG make quantifying it accurately very difficult, and consequently highly sensitive methodologies are required.

Methodologies for the determination of both GSH and GSSG have been reviewed recently [16,17]. The selectivity and sensitivity of nonseparative techniques, such as UV–Vis spectroscopy, spectrofluorometry, and amperometry, often are inadequate. Improvements in selectivity and sensitivity were achieved using chromatographic techniques such as thin-layer chromatography and high-performance liquid chromatography (HPLC). Recent methods have used HPLC coupled to a variety of detection techniques, including UV–Vis and diode array detection, fluorometry, electrochemical detection, and mass spectrometry (MS). To further enhance sensitivity, common procedures include derivatization of the free thiol group in the GSH molecule with chromophores, such as the Ellman's reagent, or fluorophores, such as monobromobimane. In general, GSSG is measured by reducing it to GSH with the enzyme glutathione reductase. The difference between the total (after reduction) and initial GSH values is used to estimate the GSSG concentration in the sample. Using this procedure, the GSSG concentration found in a given sample often could be similar to the standard deviation between replicates. An improved approach includes a preliminary step where the thiol group of GSH is blocked with vinylpyridine [18]. Subsequently, the derivatization reaction occurs only with GSH formed during the in vitro GSSG reduction step. The use of such complex procedures implies a very long analysis time, and this may result in both losses in analyte concentration and changes in redox status.

HPLC–MS techniques have been a major step toward the determination of GSH and GSSG during recent years [19–25]. These techniques improve selectivity, avoid the GSSG reduction step, and generally have sufficient sensitivity to measure the low concentrations of these compounds without derivatization. In HPLC–MS methods, the autooxidation of the GSH thiol group has been prevented by blocking it with iodoacetic acid [19], Ellman's reagent [20], or *N*-ethylmaleimide [21,22]. So far, only two studies [23,24] have attempted to simultaneously measure GSH and GSSG by MS without blocking the GSH thiol group. Norris and coworkers [23] carried out the extraction of tissue with a methanolic solution, which might not ensure the absence of autooxidation or enzymatic degradation of the thiol groups if they are not blocked [17]. Tissue extraction with acidic solutions can stop ϒ-glutamyl transpeptidase enzyme activity and prevent GSH autooxidation [17,26], although it may induce ionization problems in MS techniques [17]. The work of Gucek and coworkers [24] was the first attempt to measure both forms of glutathione by HPLC–MS in plant extracts, although information on limits of detection and other validation parameters for GSSG was not provided. Klejdus and coworkers [25] recently measured GSH in maize kernels by HPLC–MS, but quantification of GSSG was not carried out.

Because of the reasons indicated above, new reliable methods to accurately determine the reduced and oxidized forms of GSH and its homologues in plant tissues should be developed. The aim of the current work was to develop and validate a new high-performance liquid chromatography–electrospray ionization/mass spectrometry (time of flight) (HPLC–ESI/MS(TOF)) method to simultaneously measure the concentrations of reduced and oxidized forms of glutathione and homoglutathione in extracts of different plant tissues. The method also allows the simultaneous determination of ASA and GSNO. An extraction procedure with 5% metaphosphoric acid (MPA) was used, and the analytes were measured in the same chromatographic run and without any derivatization step. Isotopically labeled GSH (GSH^*) and ASA (ASA^*) where used as internal standards to control factors affecting the process during extraction, separation by HPLC, and ESI–MS.

Materials and methods

Chemicals

All eluents, extraction buffers, and standard solutions were prepared with analytical-grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA, USA). Acetonitrile HPLC–gradient grade was purchased from Panreac Química (Barcelona, Spain); formic acid was purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA); GSH and GSSG were purchased from Calbiochem (San Diego, CA, USA); hGSH was purchased from Bachem (Bubendorf,

Switzerland); ASA, DHA, and GSNO were purchased from Sigma–Aldrich (St. Louis, MO, USA); and labeled GSH ([glycine 1,2-^{13}C,^{15}N]GSH) and ASA ([1-^{13}C]ASA) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Standard solutions

Stock solutions containing 10 mM GSH (M_m 307.3), GSSG (M_m 612.6), hGSH (M_m 321.4), ASA (M_m 176.1), DHA (M_m 174.3), GSNO (M_m 336.3), GSH* (M_m 310.3), and ASA* (M_m 177.1) were prepared in 2.5% (w/v) MPA, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% formic acid. Aliquots of the stock solutions were conserved at −80 °C. Aliquots were thawed only once to prepare the standards and then were discarded. ASA solutions were prepared in a cold chamber at 4 °C and under a green safelight to avoid degradation, as described in Ref. [26].

Plant material

Different plant species and tissues were used. Materials were chosen to cover a range of different species and tissues having different analytes and concentrations. Materials used included leaves of *Beta vulgaris*, *Prunus persica*, *Medicago sativa*, *Hordeum vulgare*, *Lycopersicon esculentum*, *Oryza sativa*, and *Trifolium* sp.; roots of *Beta vulgaris*; and nodules of *Medicago truncatula. B. vulgaris*, *L. esculentum*, and *M. truncatula* were grown in a growth chamber (16 h light/8 h dark photoperiod, 24 °C day/20 °C night). *O. sativa* and *H. vulgare* were grown in a greenhouse during winter at approximately 18 °C day/10 °C night. *P. persica*, *M. sativa*, and *Trifolium* sp. leaves were collected in the field. All material was collected at approximately 12 h solar time.

Tissue extraction

Tissue extraction was performed as described elsewhere [26–31], with some modifications. Plant tissue (100–500 mg) was frozen in liquid N_2, stored at −80 °C until analysis, and ground with mortar and pestle in liquid N_2. GSH* and ASA* were added at the moment of sample grinding. The dry powder was homogenized with 200–1000 µl of cold (4 °C) extraction solution (5% (w/v) MPA and 1 mM EDTA in 0.1% formic acid), supplemented with 1% (m/v) polyvinyl-polypyrrolidone (PVPP) just before use. Homogenates were centrifuged at 15,000*g* for 20 min at 4 °C. Supernatants were collected, and the pellet was resuspended with 150–300 µl of the same extraction solution and centrifuged again under the same conditions. The second supernatant obtained was combined with the first and taken to a final volume of 2 ml with extraction solution. The supernatants were filtered through 0.22-µm polyvinylidene fluoride filters and immediately analyzed or frozen in liquid N_2 and stored at −80 °C until analysis. All steps were done in a cold chamber at 4 °C and under a green safelight to avoid ASA degradation. All instruments (e.g., mortar, pestle) were also precooled. Three replicates of each extract were done.

Using an extraction solution of very low pH stops enzymatic activities, thereby avoiding the degradation of thiols, and also prevents GSH autooxidation [28,30,32]. Extraction with organic solvents, often preferred to acid extraction when using MS for detection, might not prevent thiol group GSH autooxidation [17]. The possible negative influence of MPA on the ionization of analytes was negligible because the signals obtained with standard solutions prepared with 0.1% formic acid or 5% MPA were similar (not shown). This suggests that the effects of the high-ionic strength acidic extraction solvent on ionization possibly are limited to the first minute of the chromatographic run, when the chromatographic front enters the electrospray chamber.

HPLC–ESI/MS(TOF) analysis

Analyses were carried out with a BioTOF II (Bruker Daltonics, Billerica, MA, USA) coaxial multipass time-of-flight mass spectrometer (MS(TOF)) equipped with an Apollo electrospray ionization source (ESI) and coupled to a Waters Alliance 2795 HPLC system (Waters, Milford, MA, USA).

The BioTOF II was operated with endplate and spray tip potentials of 2.8 and 3.3 kV, respectively, in negative ion mode and of 3.0 and 3.5 kV, respectively, in positive ion mode. Drying gas (N_2) pressure was kept at 30 psi. Nebulizer gas (N_2) pressure was kept at 30 and 60 psi in ESI/MS and LC–ESI/MS experiments, respectively. The mass axis was calibrated using Li-formate adducts in negative ion mode and a mixture of 1 µM leucine–enkephaline and 20 µM methionine in positive ion mode. Spectra were acquired in the mass/charge ratio (*m*/*z*) range of 100–700.

To optimize the MS signal, direct injection of 20-µM solutions of all standards prepared in 0.1% (v/v) formic acid were carried out using a syringe pump (Cole–Parmer Instrument, Vernon Hills, IL, USA) operated at 2 µl min^{-1}. Optimal parameter values after tuning included orifice voltage values of 90 and 120 V in negative and positive ion modes, respectively, and a drying gas temperature of 200 °C in both cases. Orifice voltage values were chosen to maximize the GSSG signal without compromising GSH detection because in plant tissue extracts GSSG always occurs in lower concentrations than does GSH.

HPLC–ESI/MS(TOF) analysis was carried out by injecting 20-µl aliquots of standard solutions and sample extracts in a reverse-phase monolithic column (Chromolith Performance RP-18e, 4.6 × 100 mm, Merck, Darmstadt, Germany) fitted with a 4 × 30-mm precolumn packed with Lichrospher RP C_{18}, end-capped 5-µm spherical particle size (Scharlau, Barcelona, Spain). Autosampler and column temperatures were 6 and 30 °C, respectively. Samples were eluted at a flow rate of 1 ml min^{-1}. The exit flow from the column was split with a T-connector (Upchurch Scientific, Oak Harbor, WA, USA) that led 200 µl min^{-1} (20% of the

total efflux) from the HPLC into the ESI interface of the MS(TOF) apparatus. The mobile phase was built using two solvents: A (0.1% formic acid in Milli-Q water) and B (0.1% (v/v) formic acid in acetonitrile). For separation of the analytes, a linear gradient from 0 to 10% B (0–5 min) was used. Then, to wash the column, the concentration of B was increased linearly from 10 to 50% from 5 to 6 min, and this solvent composition was maintained until 9 min. Finally, to regenerate the column, the solvent was changed linearly to 0% B until 11 min and then was maintained at 0% B until 15 min, when a new sample could be injected.

Validation was carried out by obtaining calibration curves corrected with internal standards (100 μM ASA* was used for the quantification of ASA, and 75 μM GSH* was used for the quantification of GSH, hGSH, GSSG, and GSNO), limits of detection (LODs, signal/noise (S/N) ratio of 3), limits of quantification (LOQs, S/N ratio of 10), and intra- and interday repeatability and recovery, using standard techniques (for a complete description, see Results).

The system was controlled with the software packages BioTOF (version 2.2, Bruker Daltonics) and HyStar (version 2.3, Bruker DaltoniK, Bremen, Germany). Data were processed with Data Analysis software (version 3.2, Bruker DaltoniK).

Results

ESI/MS(TOF) analysis

In the negative ion mode ESI/MS(TOF) spectra, standard analyte solutions (20 μM) showed major $[M-H]^{-1}$ ions at *m/z* values 306.2 for GSH (Fig. 1A), 309.2 for GSH* (Fig. 1B), 320.2 for hGSH (Fig. 1C), and 611.4 for GSSG (Fig. 1D). Minor peaks at *m/z* values 613.3 (Fig. 1A), 619.4 (Fig. 1B), and 641.4 (Fig. 1C) are due to the corresponding dimer $[2M-H]^{-1}$ ions. Neither oxidation of GSH nor reduction of GSSG was observed during the ionization process (Figs. 1A and D). Moreover, neither single-labeled GSSG (GSSG*, formed by one GSH and one GSH* molecule, 614.4 *m/z*) nor double-labeled GSSG (GSSG**, formed by two GSH* molecules, 617.4 *m/z*) was found in 200- and 1000-μM GSH*-spiked plant extracts, indicating that no GSH oxidation occurs (data not shown). ASA, ASA*, and GSNO spectra show major $[M-H]^{-1}$ ions at *m/z* values 175.1, 176.1, and 335.2, respectively (Figs. 2A–C). Under the conditions used, optimized for GSSG analysis, the GSNO spectrum shows peaks at *m/z* values 304.2 and 671.4, corresponding to the $[M-NO-2H]^{-1}$ and $[2M-H]^{-1}$ ions.

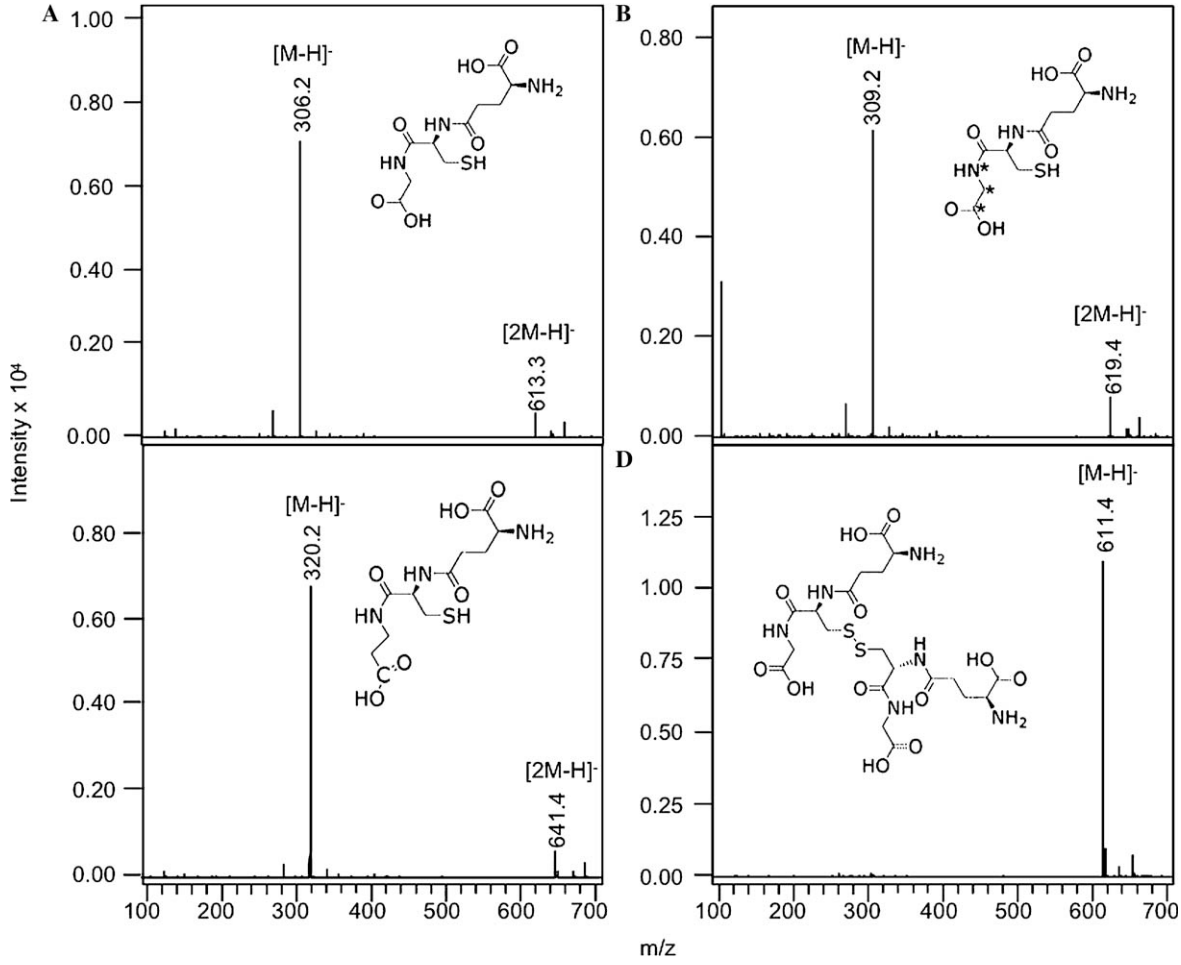

Fig. 1. ESI/TOF mass spectra of GSH (A), GSH* (B), hGSH (C), and GSSG (D) standards in negative ion mode. Data were acquired by injecting 20-μM solutions of each analyte in 0.1% (v/v) formic acid and 50% isopropanol. Labeled atoms are indicated with an asterisk.

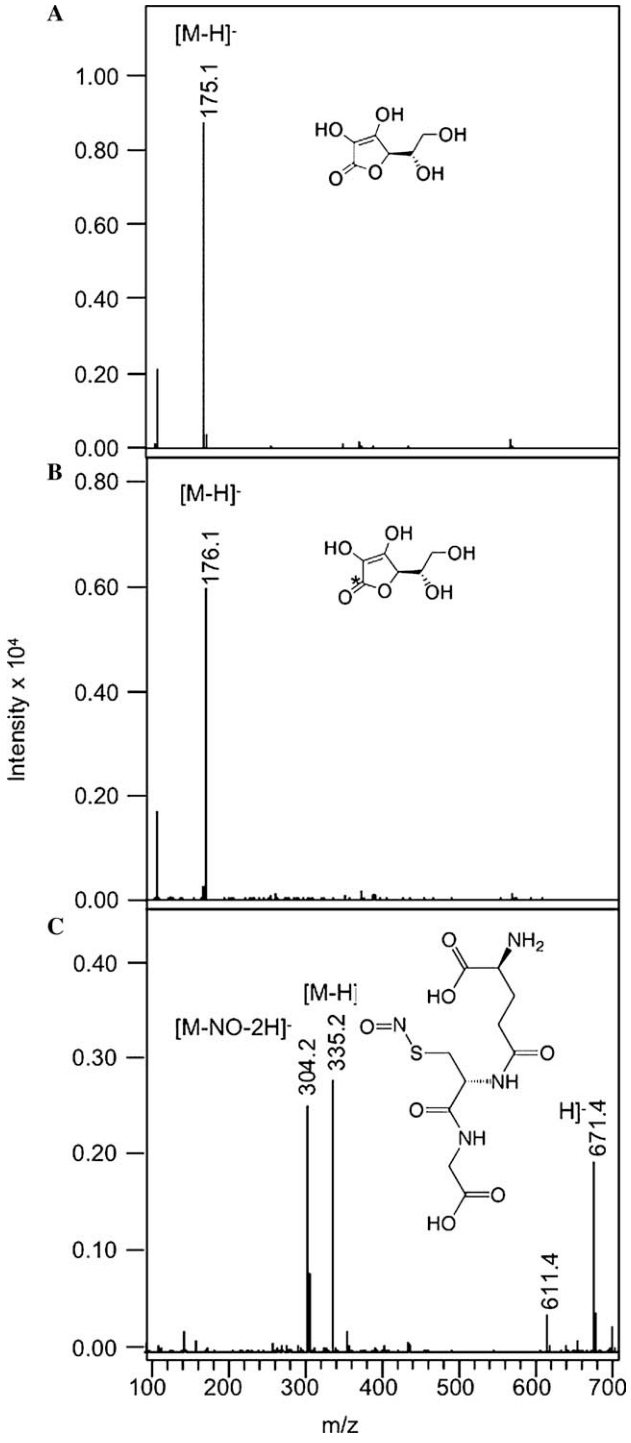

Fig. 2. ESI/TOF mass spectra of ASA (A), ASA* (B), and GSNO (C) standards in negative ion mode. Data were acquired by injecting 20-μM solutions of each analyte in 0.1% (v/v) formic acid and 50% isopropanol. Labeled atoms are indicated with an asterisk.

The signal obtained under the same conditions for the $[M-H]^{-1}$ ion of DHA was too low to permit analysis (see below). In the positive ion mode, major ions found in the MS spectra were at *m/z* values 308.2, 322.2, and 613.4 for GSH, hGSH, and GSSG, respectively, and signals obtained were slightly less intense (with a lower S/N ratio) than those obtained in the negative ion mode (data not shown). In the positive ion mode, signals for the ASA and DHA $[M+H]^{+1}$ ions were too low to permit analysis. Therefore, the negative ion mode was chosen for further experiments.

HPLC–ESI/MS(TOF) analysis

Analytes were separated with a linear solvent gradient in a monolithic C_{18} column, and mass spectra were acquired by ESI/MS(TOF) in the *m/z* range of 100–700 during the whole chromatographic run, to obtain three-dimensional (time, *m/z*, and intensity) chromatograms. Ion chromatograms were extracted for the exact *m/z* values corresponding to the $[M-H]^{-1}$ species of each analyte indicated above. Results show that the HPLC–ESI/MS(TOF) method developed is capable of adequately resolving ASA, GSH, hGSH, GSSG, and GSNO present in a mixed standard solution in a single chromatogram, with retention times of 2.6, 3.2, 4.1, 4.4, and 4.9 min, respectively (Fig. 3). ASA* coeluted with ASA, whereas GSH* eluted at 3.0 min, a slightly shorter retention time than that of GSH (Fig. 3). Although a commercial standard of oxidized homoglutathione (hGSSGh) is not available, legume leaf extract chromatograms show at 5.4 min a peak with *m/z* 639.2, attributable to the $[M-H]^{-1}$ ion of hGSSGh (Fig. 4). Analysis time for all compounds analyzed was approximately 6 min, with column washing and regeneration time accounting for an additional 9 min, leading to a total analysis time of 15 min per sample.

Validation of the HPLC–ESI/MS(TOF) method

The HPLC–ESI/MS(TOF) method was validated preparing solutions of available standards in extraction solu-

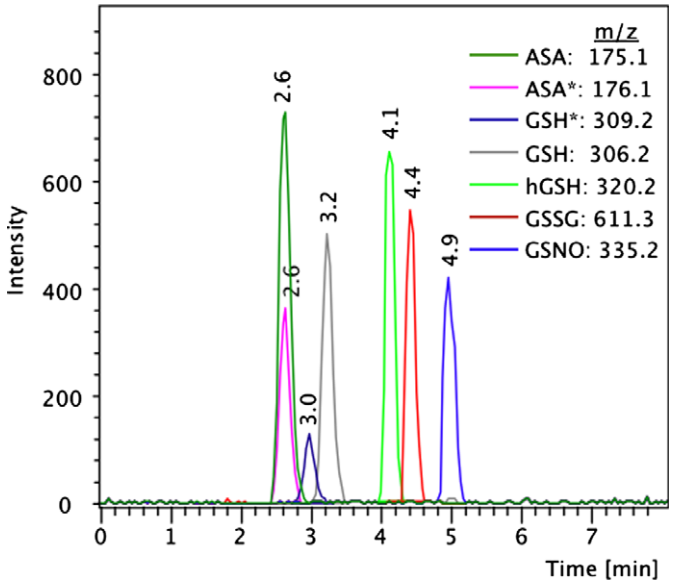

Fig. 3. Chromatogram of a mixture of standards. ASA, ASA*, GSH*, GSH, hGSH, GSSG, and GSNO were at concentrations of 200, 100, 25, 75, 75, 25, and 75 μM, respectively, in 2.5% (w/v) MPA, 1 mM EDTA, and 0.1% (v/v) formic acid.

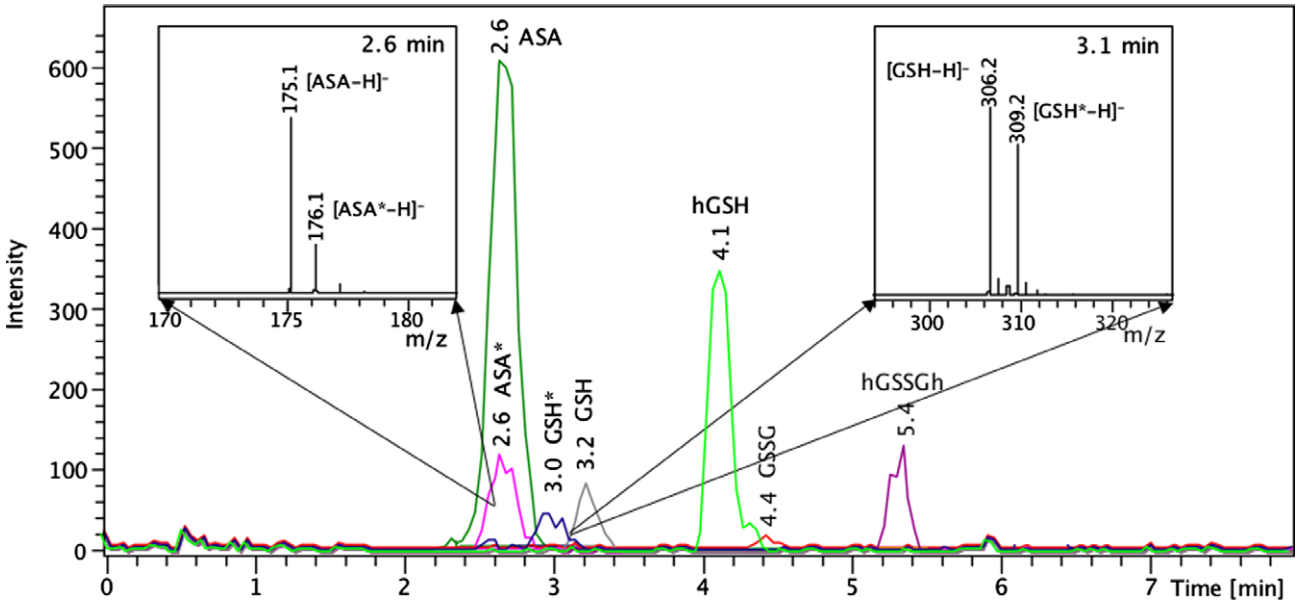

Fig. 4. Chromatogram of a leaf extract from *M. sativa* showing peaks corresponding to ASA (175.1 *m/z*), GSH (306.2 *m/z*), hGSH (320.2 *m/z*), a putative hGSSGh peak (639.2 *m/z*), and peaks corresponding to ASA* (176.1 *m/z*) and GSH* (309.2 *m/z*) used as internal standards. Insets show zooms of the mass spectra at retention times of 2.6 and 3.1 min.

tion (5% (w/v) MPA, 1 mM EDTA, and 0.1% (v/v) formic acid). Calibration curves corrected with internal standardization, LODs, and intra- and interday repeatability and recovery were measured. Real plant extract samples were also analyzed with the method developed.

Calibration curves corrected by internal standardization were obtained by analyzing solutions of standards in the ranges of 5–250 μM (GSH and hGSH), 1–15 μM (GSSG), 25–750 μM (ASA), and 5 to 150 μM (GSNO). Internal standards used were GSH* for GSH, hGSH, GSSG, and GSNO and ASA* for ASA. In all cases, a linear regression was obtained (r^2 of 0.991–0.998) (Fig. 5).

LODs, defined as the analyte amounts giving an S/N ratio of 3, were 100, 60, 30, 20, and 30 pmol for ASA, GSH, hGSH, GSSG, and GSNO, respectively. Using a 20-μl injection volume, these values are equivalent to analyte concentrations in the injected sample solution of 5.0, 3.0, 1.5, 1.0, and 1.5 μM for ASA, GSH, hGSH, GSSG, and GSNO, respectively. LOQs, defined as the amounts giving an S/N ratio of 10, were 300, 120, 80, 50, and 80 pmol for

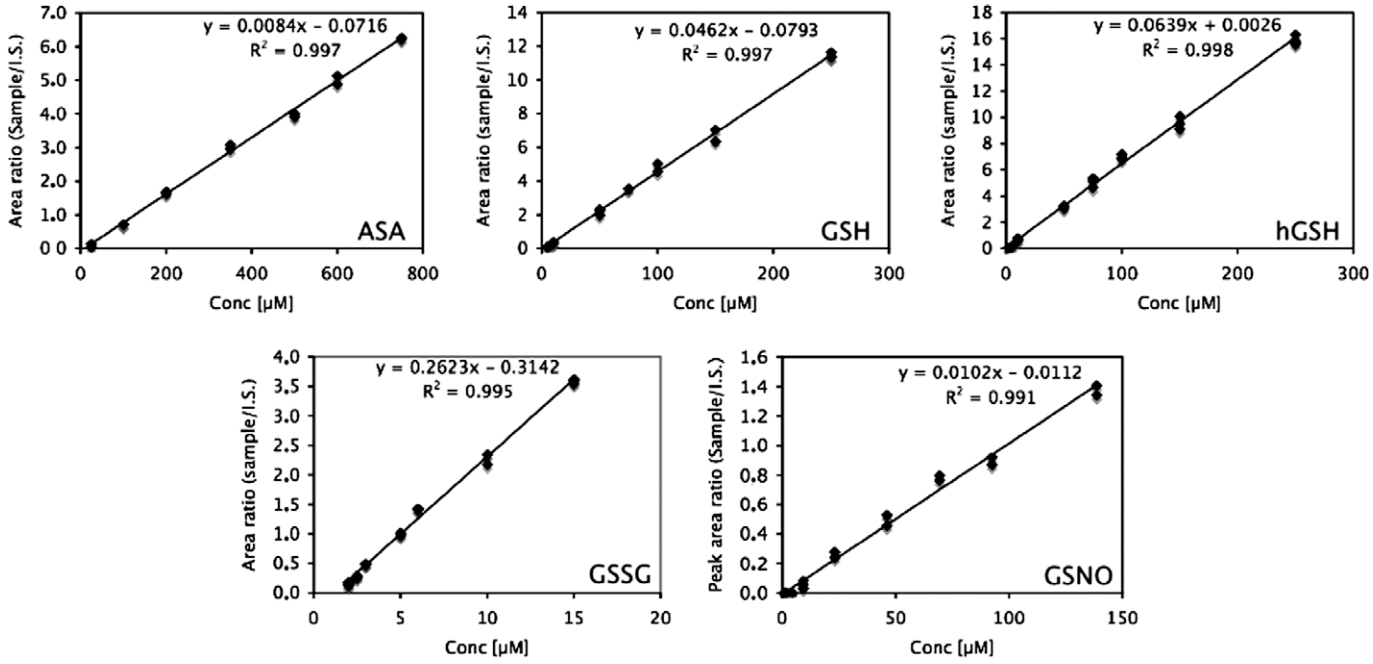

Fig. 5. Calibration curves of ASA, GSH, hGSH, GSSG, and GSNO obtained by analyzing a mixture of standards and internal standards by the ESI/MS(TOF) method. I.S., internal standard.

ASA, GSH, hGSH, GSSG, and GSNO, respectively. For DHA, and possibly due to strong analyte fragmentation, the LOD was higher than 500 μM, a value much larger than the concentrations expected in plant tissue extracts.

The intraday repeatability of the HPLC–ESI/MS(TOF) method was assessed from 10 consecutive chromatographic runs using a standard solution with 75 μM GSH, hGSH, and GSNO; 25 μM GSSG and GSH*; 200 μM ASA; and 100 μM ASA* in 2.5% (w/v) MPA, 1 mM EDTA, and 0.1% (v/v) formic acid. The variation in retention time and peak area was tested for each analyte (Table 1). The interday repeatability of the method was assessed by analyzing the same standard solution for 5 consecutive days (Table 1). The relative standard deviation (RSD) for peak retention time always was lower than 0.41% in the intraday test and 0.95% in the interday test. The RSD for peak area was in the range of 4.4–7.0% in the intraday test and 7.4–13.1% in the case of the interday test.

Recovery assays were carried out for GSH, GSSG, hGSH, ASA, and GSNO by spiking *B. vulgaris* leaf extracts with known amounts of both analyte standards and labeled analytes (GSH* and ASA*). Recovery was calculated by dividing the amount of analyte found in the spiked sample by the sum of the amount found in the sample plus the amount added. Analyte recoveries found were between 92 and 105% (Table 2).

Table 2
Recoveries obtained for GSH, hGSH, GSSG, ASA, and GSNO using *Beta vulgaris* leaf extracts

	Amount added (pmol)	Amount found (pmol)	Recovery (%)
GSH	0	2328 ± 64	
	1704 ± 56	3692 ± 178	91.5 ± 3.7
hGSH	0	0	
	1722 ± 78	1724 ± 114	100.1 ± 4.5
GSSG	0	20 ± 2	
	60 ± 4	78 ± 4	94.4 ± 7.5
ASA	0	3264 ± 90	
	9514 ± 210	12,412 ± 288	98.9 ± 4.4
GSNO	0	0	
	1888 ± 214	1872 ± 52	104.9 ± 10.6

Note. Results are means ± SE ($n = 5$).

Analysis of plant tissue extracts

A chromatogram example of *M. sativa* leaf extracts is shown in Fig. 4. Five peaks, corresponding to ASA, GSH, hGSH, GSSG, and hGSSGh, were detected at retention times of 2.6, 3.2, 4.1, 4.4, and 5.4 min, respectively, as shown in Fig. 4 (in this figure, the internal standards ASA* and GSH* show peaks at 2.6 and 3.0 min, respectively). No trace of double-labeled (617.4 *m*/*z*) or single-labeled (614.4 *m*/*z*) GSSG was found.

The resolution of the MS(TOF) detector used is higher than 10,000 FWHM (full width, half mass). Therefore, MS(TOF) spectra provide information on the elemental isotopic distribution of any compound detected in the chromatographic run. An example can be seen in the insets of Fig. 4, which show the MS resolution of the major ions of ASA and ASA*, coeluting at a retention time of 2.6 min, as well as that of GSH and GSH*, which have slightly different retention times of 3.0 and 3.2 min, respectively, and coelute at an intermediate retention time of 3.1 min.

Analyte concentrations found in these and other plant tissues are shown in Table 3. Concentration ranges found were 43–707 nmol g^{-1} FW (GSH), 47–2300 nmol g^{-1} FW (hGSH), 6–47 nmol g^{-1} FW (GSSG), and 1470–8700 nmol g^{-1} FW (ASA), where FW is fresh weight. These values are within the values reported in the literature for plant tissues (Table 4).

Discussion

The relevance of the GSH/GSSG redox pair in different plant metabolic processes makes necessary having reliable methods to analyze these compounds with high sensitivity while minimizing analyte decomposition and changes in redox status from that originally occurring in the plant. In this work, we have developed and validated an HPLC–ESI/MS(TOF) method capable of measuring GSH, GSSG, hGSH, ASA, and GSNO in plant tissue extracts. The method is based in a separation with reverse-phase HPLC, ionization by electrospray, and highly selective detection of the analytes, using exact mass measurements with a TOF

Table 1
Intraday ($n = 10$) and interday ($n = 5$) repeatability of the LC–ESI/MS(TOF) method

	ASA		GSH		hGSH		GSSG		GSNO	
	RT	A_s/A_{IS}	RT	A_s/A_{IS}	RT	A_s/A_{IS}	RT	A_s/A_{IS}	RT	A_s/A_{IS}
Intraday										
Mean	2.57	2.10	3.17	4.66	4.08	4.99	4.39	3.02	4.93	3.56
SD	0.01	0.09	0.01	0.22	0.01	0.24	0.02	0.21	0.01	0.40
RSD (%)	0.41	4.35	0.33	4.67	0.28	4.91	0.37	6.99	0.29	11.20
Interday										
Mean	2.57	2.13	3.17	4.60	4.08	5.63	4.39	4.03	4.93	3.99
SD	0.01	0.16	0.01	0.42	0.01	0.66	0.02	0.61	0.01	0.45
RSD (%)	0.61	7.36	0.81	9.10	0.62	11.66	0.95	13.11	0.47	11.35

Note. Retention times (RTs) and peak area ratios (sample area/area of internal standard [A_s/A_{IS}]) were obtained using 200 μM ASA, 75 μM GSH, 75 μM hGSH, 25 μM GSSG, 75 μM GSNO (using 100 μM ASA*, and 25 μM GSH* as internal standards) in 2.5% (w/v) MPA, 1 mM EDTA, and 0.1% (v/v) formic acid.

Table 3
Contents of GSH, hGSH, GSSG, and ASA found in plant tissues using the LC–ESI/MS(TOF) method

Plant tissue	GSH (nmol g^{-1} FW)	hGSH (nmol g^{-1} FW)	GSSG (nmol g^{-1} FW)	ASA (nmol g^{-1} FW)
Beta vulgaris (leaf)	152.2 ± 8.8	ND	23.7 ± 0.2	2240 ± 78
Prunus persica (leaf)	155.4 ± 9.5	ND	5.8 ± 0.1	8730 ± 225
Medicago truncatula (nodule)	202.8 ± 21.7	47.4 ± 14.7	7.0 ± 1.7	1471 ± 54
Medicago sativa (leaf)	ND	1333.3 ± 152.8	ND	3523 ± 68
Hordeum vulgare (leaf)	187.5 ± 25.1	ND	22.9 ± 0.9	2344 ± 51
Lycopersicon esculentum (leaf)	707.3 ± 54.4	ND	47.3 ± 5.5	4370 ± 306
Beta vulgaris (root)	92.1 ± 14.8	ND	46.1 ± 10.1	ND
Trifolium sp. (leaf)	42.6 ± 24.8	2332.2 ± 7.6	ND	7620 ± 106
Oryza sativa (leaf)	252.1 ± 11.2	ND	12.9 ± 0.4	7928 ± 1288

Note. Values are means ± SE ($n = 3$). ND, not detected (below LOD). FW, fresh weight.

Table 4
Plant tissue contents of GSH, GSSG, and hGSH found in the literature and the analytical techniques used for their determination

Tissue	Analytical technique	Content	Ref.
Sugar beet roots	GR–DTNB	30 and 10 nmol g^{-1} FW for GSH and GSSG	[33]
Vigna radiata leaves	GR–DTNB	2000 and 200 nmol g^{-1} FW for GSH and GSSG	[34]
Tomato, potato, and broccoli leaves	Amperometric inhibitor biosensor	20–321 μg GSH g^{-1} FW	[35]
Sunflower leaves	GR–DTNB	700 nmol GSH g^{-1} FW	[36]
Arabidopsis thaliana (protoplasts)	mCB/mBB–CLSM/ TPLSC	8–9 nmol GSH g^{-1} FW; 3–4 mM in cells; 3 mM in cytoplasm	[37]
Arabidopsis thaliana trichome cells	mCB/mBB–CLSM	0.24 mM GSH in trichome cytoplasm; 0.08 mM GSH in base cell cytoplasm; 0.14 mM in GSH epidermic cell cytoplasm.	[38]
Arabidopsis thaliana root epidermic cells	mCB–CLSM/TPLSM	2.7 mM GSH in cytoplasmic trichoblasts; 5.5 mM GSH in cytoplasmic atrichoblasts	[39]
Arabidopsis thaliana root epidermic cells	mCB–CLSM	6 mM GSH in external cell cytoplasm; 2–3 mM GSH in other cells	[40]
Populus tremula x Populus alba leaf epidermis	mCB–CLSM and mCB/ mBB–HPLC	0.2–0.3 mM GSH in cytoplasm	[41]
Arabidopsis thaliana root apex	mCB–CLSM	2–4 mM GSH in cytoplasm	[42]
Arabidopsis thaliana leaves	HPLC–DTNB	200–800 nmol GSH g^{-1} FW	[43]
Tomato and azuki bean cells	HPLC–DTNB	500–12,500 nmol GSH g^{-1} cells	[44]
Nodules, roots, and leaves from different legumes	mBB–HPLC	150–820, 1–147, and 1–445 nmol GSH g^{-1} FW in nodules, roots, and leaves, respectively; 0–427, 0–235, and 0–887 nmol hGSH g^{-1} FW in nodules, roots, and leaves, respectively	[10]
Poplar leaves	GR–DTNB mBB–HPLC OPA–HPLC	699–1239 nmol GSH g^{-1} FW; 640–1169 nmol GSH g^{-1} FW; 668–1109 nmol GSH g^{-1} FW	[45]

Note. GR, glutathione reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); mCB, monochlorobimane; mBB, monobromobimane; CLSM, confocal laser scanning microscopy; TPLSC, two-photon laser scanning microscopy; OPA, *o*-phthalaldehyde.

mass spectrometer. This method permits the direct and simultaneous determination of all analytes in a very short time, approximately 6 min, with high sensitivity.

This is the first time, to our knowledge, that GSH, GSSG, ASA, hGSH, and GSNO are determined simultaneously and directly. The method represents significant advantages over traditional methods for the determination of GSH and GSSG. First, the identification of analytes is unequivocal based on both its retention time and its exact *m*/*z* ratio. Also, all compounds are measured directly because no reduction step is needed to measure GSSG (thereby reducing by half the number of analyses) and no derivatization step is used. All of these features, along with the short analysis time required for each sample and the fact that the extraction process is carried out under safe conditions (i.e., sample extracted with liquid nitrogen, dim green light, acidic pH), allow a more precise and reliable analysis of the GSH/GSSH redox status in plant tissues.

The method has been validated with respect to LODs, LOQs, calibration curves, reproducibility and analyte recoveries, always using isotopically labeled standards. Sensitivity was good, with LODs for analyte concentrations between 1 and 5 μM. The LOD for GSSG, 1 μM, is similar to the best values obtained in plant analysis using capillary zone electrophoresis (1 μM [30]) but not as good as those found using electrochemical detection (0.02 μM [46]). The LOD found for GSH, 3 μM, compares well with the LODs of other methods based on MS [19–22] and other techniques [17], which are in the ranges of 10 nM to 0.82 μM and 5 nM to 10 μM, respectively. LODs are in any case adequate, considering that the content ranges for the analytes in plant tissues are 6–47 nmol g^{-1} FW for GSSG, 43–707 nmol g^{-1} FW for GSH, and 1470–8730 nmol g^{-1} FW for ASA (Table 3). These contents would result in analyte concentrations in the extracts in the range of 1.5 μM to 2.3 mM, with the lower values being for GSSG. The method

run-to-run interday reproducibility for chromatographic time, with RSD values of 0.8–1.0% for GSH and GSSG, respectively, is better than the reproducibility levels reported previously for other HPLC–MS methods that are in the range of 1.7–6.0% [16–19]. RSD values for peak area, 9 and 13% for GSH and GSSG, respectively, are within the range of values found with other HPLC–MS methods that are between 0.06 and 28.3% [19–23]. If needed in specific tissues, improvements in peak area reproducibility, as well as in LODs, could also be achieved using narrower columns, where lower fluxes are used and no flow split is needed. The recovery for analytes spiked in plant tissue extracts is between 92 and 105%, compared with recoveries found with other methods that are in the range of 70–120% [17].

The method has wide possibilities of application. So far, it has been tested with different plant tissues (leaves, roots, and nodules) from different plant species (*B. vulgaris*, *P. persica*, *Z. mays*, *M. sativa*, *M. truncatula*, *H. vulgare*, *L. esculentum*, *Trifolium* sp., and *O. sativa*), showing its suitability to perform analyses in a variety of studies. In addition to GSH and GSSG, the redox pair hGSH and hGSSGh (with the latter putatively assigned to the peak at 5.4 min with a 639.2 *m*/*z*) could also be analyzed in leaf and nodule legume extracts [10], thereby providing a tool for the study of their redox status [47]. Furthermore, the method also seems to be suitable to analyze other plant thiols such as hydroxymethylglutathione. Unfortunately, with the conditions optimized for GSSG and GSH analysis, it was not possible to determine oxidized ASA, DHA, supporting previous findings that fragmentation occurs with this compound even in mild ionization procedures such as ESI [48].

Recent evidence has shown that micromolar concentrations of GSSG could arise from oxidation of GSH during blood denaturation in acidic conditions [22,49], although it should be kept in mind that these authors did not use low temperature for the extraction procedure. Under the extraction conditions used in this work, which involved the use of liquid nitrogen (−196 °C), low safelight, and low pH, no GSH oxidation occurred during the ionization process and no significant oxidation of GSH* spiked to the samples during extraction was found. In fact, no signal at all was found for single- or double-labeled GSSG (*m*/*z* values 617.4 and 614.4) in the samples spiked with 1 mM GSH* at the time of extraction (data not shown). Because our LOD for GSSG was 1 μM, this would imply that if any oxidation exists, it would account for less than 0.2% of the GSH amount, a value much lower than the 2–3% found in blood by Steghens and coworkers [22]. Therefore, from the GSH and GSSG values found in plant samples (Table 3), one can estimate that less than 0.8–11.6% of the GSSG found may come from GSH oxidation during extraction, depending on the sample considered. This is lower than the interday RSD for GSSG (Table 1). However, the possibility that GSH oxidation may occur should be taken into consideration when analyzing plant materials that cannot be frozen rapidly. For instance, plant fluids such as xylem of phloem require some time for collection and therefore are prone to GSH oxidation by molecular oxygen and/or oxidizing substances in a similar way to the GSH oxidation mediated by oxyhemoglobin in blood [22]. In this context, it should be investigated whether changing the extraction conditions may affect the GSSG/GSH ratios found in plant fluids.

In summary, the method developed permits the direct and simultaneous analysis of GSH, GSSG and homologues, ASA, and GSNO with extreme selectivity, high sensitivity, and sufficient reproducibility. The rapidity of the analysis allows for a high analysis throughput. Furthermore, the high resolution of the MS(TOF) spectrometer used can give information on isotopic distribution (see insets in Fig. 4), allowing its use as a tool in metabolic studies with stable isotopes. For example, plants can be fed with stable isotopically labeled compounds to follow the analyte metabolic pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.05.032.

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