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# Analysis of glutathione and glutathione disulfide in human saliva using hydrophilic interaction chromatography with mass spectrometry ☆

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**Abstract**

A sensitive method for the determination of glutathione (GSH) and glutathione disulfide (GSSG) in human saliva was developed and validated. GSH was captured and stabilized by the addition of *N*-ethylmaleimide (NEM). Solid-phase extraction (SPE) using an Oasis MAX® extraction cartridge was employed for sample preparation and analysis was performed on a Shimadzu LCMS-2010 A that was operated in the single ion monitoring mode using positive ion electrospray ionization (ESI) as the interface. The monitored ion for GSH-NEM was *m/z* 433 and that for GSSG was *m/z* 613. Chromatography was carried out on an Atlantis HILIC silica column (150 mm × 2.1 mm, 5 μm) with acetonitrile and formate buffer as the mobile phase at the flow rate of 0.2 ml/min. The calibration curve was linear over the range of 0.1–100 μM for GSH-NEM. The extraction recoveries of GSH-NEM spiked at concentrations of 25 and 50 μM were 97.1 and 104.4%, respectively. Similar results were obtained for GSSG. The newly developed hydrophilic interaction chromatography with mass spectrometry (HILIC/MS) method showed superior sensitivity for the determination of GSH and GSSG in human saliva samples.


*Keywords:* Hydrophilic interaction chromatography; Saliva; Mass spectrometry; Glutathione; Glutathione disulfide

## 1. Introduction

Biological thiol compounds are classified into high molecular weight protein thiols and low molecular weight free thiols. Endogenous low molecular weight thiol compounds, namely, glutathione (GSH) and its corresponding disulfide, glutathione disulfide (GSSG), are very important molecules that participate in a variety of physiological and pathological processes. GSH plays an essential role in protecting cells from oxidative and nitrosative stress, and can be converted into the reduced form by the action of glutathione reductase. It has been proposed that oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage [1]. The decrease in GSH level, the increase in GSSG level, and the lowering of the GSH/GSSG ratio are considered to be important contributors to a number of human diseases. Blood is used as the biological specimen for clinical examination. However, because blood sampling is an invasive procedure and is coupled with various restrictions such as diet and hygiene, it places a burden on the subject. By contrast, human saliva is easily obtainable from patients and might be useful for clinical diagnosis.

Previously reported methods for the measurement of thiol compounds in biological samples include enzymatic assay, HPLC with electrochemical detection (ECD) [2] without sample derivatization, and thiol derivatization followed by HPLC with UV detection [3] or fluorometric (FL) detection [4,5]. However, oxidized thiols could not be detected by HPLC with ECD or FL detection, and it is difficult to determine reduced and oxidized thiol compounds simultaneously. It is necessary to determine the concentrations of disulfides and thiols independently, such as in the assessment of GSH/GSSG ratio in relation to human health. However, such determinations must normally be done sequentially. For example, for the GSH/GSSG ratio, direct determination yields the concentration of only GSH. Reduction of the sample prior to a second analysis yields a signal corresponding to the combined concentrations of GSH and GSSG. GSSG concentration is determined from the difference between total

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GSH and free GSH concentrations [6]. This method could not be determined to GSSG content of a real value. Only a few methods that utilize FL detection for the simultaneous determination of thiols and disulfides are available [7]. However, those methods are very complex. Therefore, an analytical method for the determination of thiol compounds and their corresponding disulfides in biological samples is desirable.

Hydrophilic interaction chromatography (HILIC) is characterized by the presence of a high initial concentration of organic modifier to favor hydrophilic interaction between the solute and the hydrophilic stationary phase [8]. Briefly, HILIC based on silica columns is normal-phase chromatography, and utilizes conventional reversed-phase (RP) mobile phases. Thus, the retention times of highly polar compounds are increased as their hydrophilicity is increased. HILIC is easy to use with a mass spectrometer (MS) because a hydrophilic stationary phase is used in combination with a organic mobile phase, and elution is usually performed by increasing the concentration of aqueous phase. Recently, HILIC/MS was employed to separate and quantify folates in complex food samples [9]. It has also been used to determine polar pharmaceutical compounds that are difficult to retain and separate by RP-HPLC.

In the present study, we developed the HILIC/MS method to determine reduced and oxidized glutathione in human saliva samples.

## 2. Experimental

### 2.1. Chemicals and reagents

Glutathione (purity >97.0%), glutathione disulfide (purity >95.0%) and *N*-ethylmaleimide (NEM) (purity >98.0%) were obtained from Wako (Tokyo, Japan). γ-Glutamyl-glutamic acid (γ-glu-glu) was obtained from Sigma (Tokyo, Japan). Other chemicals and solvents were obtained from Wako.

### 2.2. Equipment

HPLC analysis was performed with a SHIMADZU (Shimadzu, Tokyo, Japan) system that consisted of an LC-10AD$_{VP}$ pump, an SIL-HT$_{C}$ autosampler, a CTO-10A$_{VP}$ thermostated column compartment, and a DGU-14AM vacuum degasser, and was connected to a SHIMADZU LCMS-2010 A mass spectrometer.

### 2.3. Preparation of N-ethylmaleimide derivatives for use as calibration standards

To the stock solution of 20 mM GSH in 1 mM formic acid was added 1 mM EDTA. To the stock solution of 1 mM GSSG in 1 mM formic acid was added 1 mM EDTA. Working standard solutions of GSH and GSSG were prepared from the stock solutions by mixing NEM (40 mM), EDTA (2 mM) and γ-glu-glu (100 μM) as the internal standard. A calibration curve extending from 0.1 to 100 μM was drawn with the standard solutions at concentrations of 0.1, 1, 2.5, 5, 7.5, 10, 20, 50 and 100 μM. The analysis was conducted using γ-glu-glu as the internal standard. Concentrations of compounds in the samples were calculated from the calibration curves of GSH and GSSG.

### 2.4. Sample preparation

The sample was prepared by adding 500 μl of saliva to a 20 μl solution of 40 mM NEM, 2 mM EDTA and 100 μM γ-glu-glu immediately. The mixed sample was vortexed for 15 s, reacted for 30 min at room temperature and centrifuged (10,000 × *g*, 3 min) after adding 480 μl of ammonium carbonate. Solid-phase extraction (SPE) of GSH and GSSG was carried out with OASIS MAX® 1 cc (30 mg) SPE cartridges (Waters). The cartridges were initially primed with 1 ml of methanol followed by 1 ml of water and 5 mM ammonium carbonate. Derivatized saliva sample was added under reduced vacuum conditions. After aspiration, each cartridge was washed with 1 ml each of 50 mM ammonium carbonate, acetonitrile and 0.5 ml of 10 mM trifluoroacetic acid. The analytical compounds were eluted with 3 ml of 10 mM trifluoroacetic acid under reduced pressure. The eluate was evaporated to dryness and the residue was reconstituted in 100 μl of the mobile phase.

### 2.5. Chromatography and mass spectrometry conditions

Using an autosampler, 10 μl of each sample was injected into an Atlantis HILIC silica column (150 mm × 2.1 mm; Waters, Japan) packed with 5 μm particles. The column oven temperature was maintained at 40 °C and the flow rate was set at 0.2 ml/min. To separate the NEM derivatives of thiols from each other and from the excess NEM, gradient elution was used. The elution profile was as follows: 0–20 min 90–70% B. Mobile phase (A) was 0.5 mM ammonium formate buffer (pH 4.0) and (B), acetonitrile.

Positive ion electrospray ionization (ESI) was employed for MS. Selected ion monitoring (SIM) was conducted to simultaneously monitor ions at *m/z* 433, 613 and 277, which corresponded to the protonated molecular ions of GSH-NEM, GSSG and γ-glu-glu. Both curved desolvation line (CDL) and heat block temperatures for the analysis were set at 300 °C. Nebulizer gas flow was set at 1.5 l/min and detector voltage was set at 1.5 eV.

### 2.6. Precision and accuracy

The precision of the method was determined with the low, medium and high quality control samples by replicate analysis of GSH-NEM and GSSG at three concentrations (1, 10 and 100 μM). Intraday precision and accuracy were determined by replicate analysis of the group of standards in one day ($n = 6$), and interday precision and accuracy were determined by replicate analysis over six days ($n = 6$). The concentration of each sample was determined using calibration standards prepared on the same day.

### 2.7. Storage stability

The stability of GSH and GSSG was evaluated at −20, 4 and 25 °C every day for one week. The stability was tested by

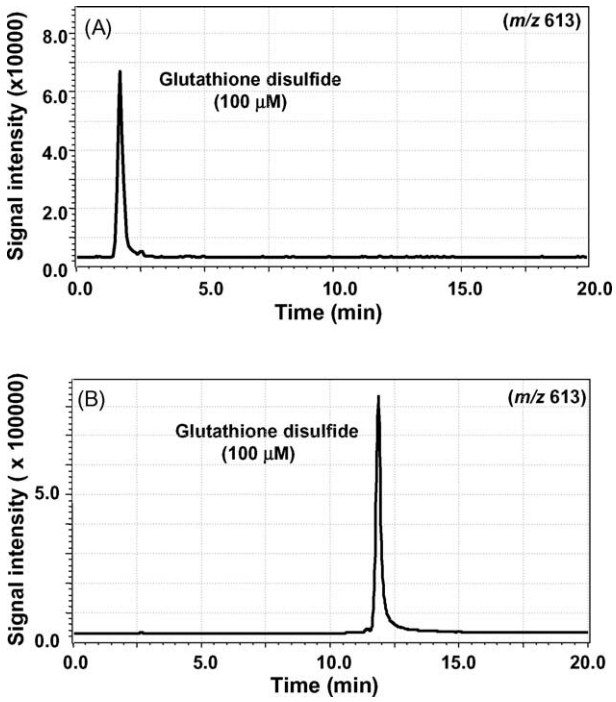


Fig. 1. Chromatograms of glutathione disulfide (100 μM) as obtained by (A) RPLC-MS and (B) HILIC/MS methods. (A) CAPCELL PAK (150 mm × 2.1 mm, 5 μm; SHISEIDO, Tokyo, Japan), (B) Atlantis™ HILIC silica column (150 mm × 2.1 mm, 5 μm; Waters, Tokyo, Japan). The mobile phase of (A) was 0.5 mM formate buffer (pH 4.0)/acetonitrile (95:5, v/v). The elution profile of chromatogram (B) was as follows: 0–20 min 90–70% (B). Mobile phase (A) was 0.5 mM ammonium formate buffer (pH 4.0) and (B) acetonitrile.

analyzing standard samples at two concentrations of GSH and GSSG (50 and 100 μM).

## 3. Results and discussion

### 3.1. Optimization of HILIC/MS conditions

Thiol compounds are difficult to extract without derivatization from biological samples because they are highly polar and water soluble. Moreover, the susceptibility of the thiol group to oxidation often leads to inaccurate analytical results. GSH could be determined by fluorescence and MS after derivatization with *ortho*-phthalaldehyde (OPA) [10], ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) [11] and 5,5′-dithio-(bis-2-nitrobenzoic) acid (DTNB) [12]. Only one study of the simultaneous detection of thiols and disulfides using tandem MS is available [13]. The analytical method is of great value. However, in that study, glutathione ethyl ester was used, which was hydrolyzed under acidic conditions and converted into GSH as internal standard, and it was difficult to determine trace levels of GSH in saliva sample. On the other hand, GSSG, having no thiol group, could not be derivatized. Fig. 1 shows the chromatograms of GSSG that was separated with ODS and HILIC columns. GSH and GSSG could be retained on a HILIC column having a hydrophilic stationary phase. To prevent the autoxidation of GSH during sample pretreatment, GSH was derivatized with

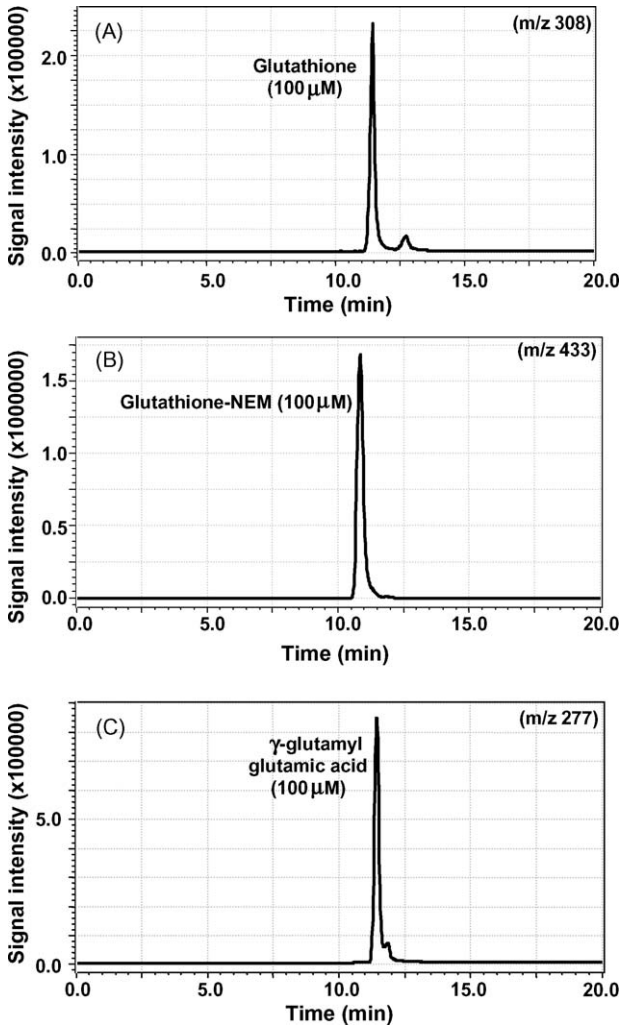


Fig. 2. Chromatograms of (A) glutathione, (B) glutathione-NEM and (C) γ-glutamyl-glutamic acid (100 μM) as obtained by the HILIC/MS method. Analytical separation was performed on Atlantis™ HILIC silica column (150 mm × 2.1 mm, 5 μm; Waters, Tokyo, Japan). The column oven temperature was maintained at 40 °C and the flow rate was set at 0.2 ml/min. The elution profile of chromatogram (B) was as follows: 0–20 min 90–70% (B). Mobile phase (A) was 0.5 mM ammonium formate buffer (pH 4.0) and (B) acetonitrile.

NEM. In the presence of a large excess of NEM (NEM/GSH molar ratio 100/1), the derivatization was completed within 20 min, as confirmed by the detection of the ion at *m/z* 308 [14]. Fig. 2 shows the typical chromatograms for GSH, GSH-NEM and γ-glu-glu. The peak intensity of GSH-NEM was increased with the decrease of the peak intensity of GSH.

We examined whether the presence of trifluoroacetic acid, acetic acid or formic acid in the mobile phase would increase both sensitivity and resolution, to obtain improved peak shapes. Formic acid gave better separation and sensitivity than trifluoroacetic acid or acetic acid (data not shown). Then, to determine whether pH has an effect on the elution and sensitivity of the peaks, the pH of the aqueous phase was varied by adding ammonium formate. HILIC/MS conditions were examined by adjusting the concentration and the pH of the mobile phase (Fig. 3). The mobile phase that gave the most intense peak was formate buffer at 0.5 mM and pH 4.0.

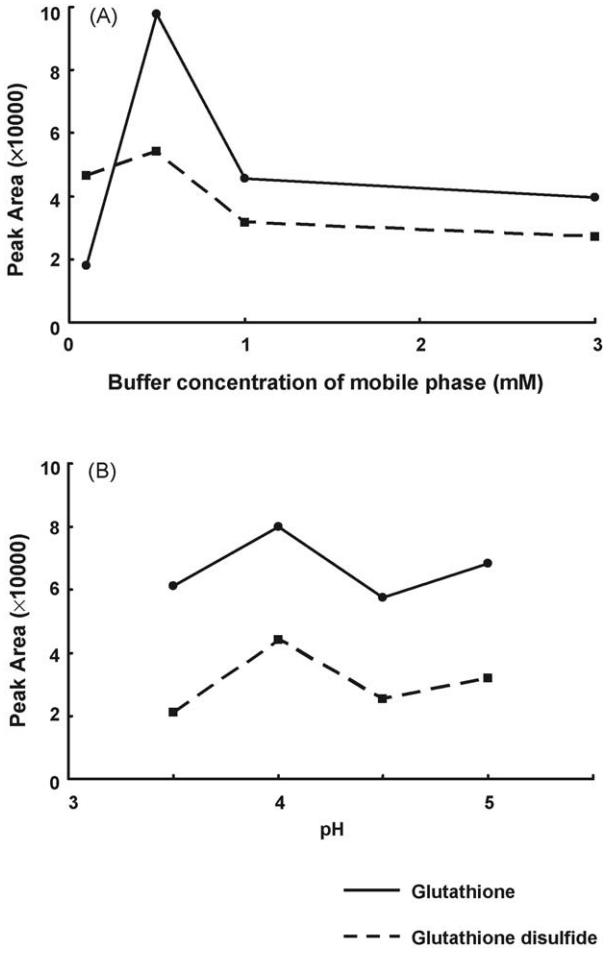


Fig. 3. Examination of the pH and the concentration of the mobile phase.

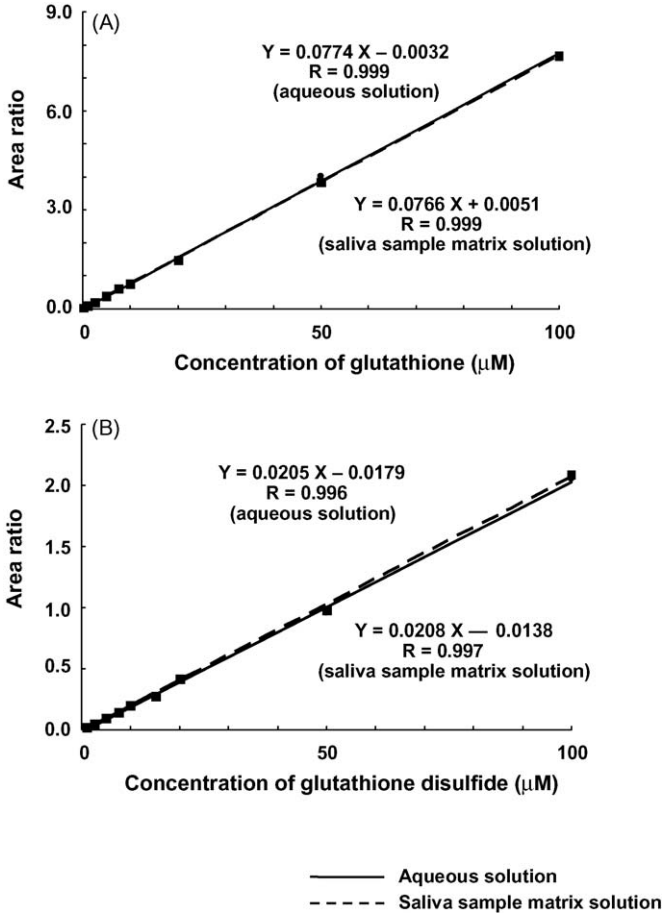


Fig. 4. Calibration curves of glutathione-NEM and glutathione disulfide in (A) aqueous solution and (B) saliva sample matrix solution.

### *3.2. Pretreatment of GSH-NEM and GSSG in human saliva samples using SPE cartridge*

The difficulty lies in not only the very low concentrations of GSH and GSSG in the sample matrix but also their hydrophilicity, which makes isolation and separation from other polar endogenous compounds an extremely challenging task. Conventional method was determined to GSH-NEM and GSSG in blood sample [15]. By contrast, it is necessary to treat and concentrate saliva sample prior to GSH and GSSG determination. We compared the Oasis HLB® SPE cartridge with the Oasis MAX® SPE cartridge in terms of sample extraction. GSSG could not be recovered with the Oasis HLB® SPE cartridge because GSSG, a highly polar compound, was not well retained by the cartridge. In contrast, GSSG was well extracted with the Oasis MAX® SPE cartridge that has a mixed phase mode (RP and anion exchange). Saliva samples were spiked with GSH and GSSG at concentrations of 25 and 50 μM ($n = 6$ at each concentration). The average extraction recoveries and relative standard deviation (R.S.D.) were 87.3 and 10.4% for GSH, and 91.7 and 10.3% for GSSG at the spiked concentration of 50 μM (Table 1). Therefore, the SPE method was suitable for the pretreatment of GSH and GSSG.

Table 1
Recovery test of glutathione-NEM and glutathione disulfide in human saliva samples

| Analytical compound | Spiked concentration | | | | | |
|---|---|---|---|---|---|---|
| | HLB | | MAX | | | |
| | 50 μM | | 25 μM | | 50 μM | |
| | Average | R.S.D. | Average | R.S.D. | Average | R.S.D. |
| Glutathione-NEM | 79.7 | 5.6 | 97.1 | 10.2 | 87.3 | 10.4 |
| Glutathione disulfide | 2.7 | 1.1 | 104.4 | 13.5 | 91.7 | 10.3 |

$n = 6$; R.S.D.: relative standard deviation.

Table 2
Analytical parameters for HILIC/MS of glutathione-NEM and glutathione disulfide

| Analytical compound | LOD (μM) | LOQ (μM) | Correlation coefficient |
|---|---|---|---|
| Glutathione-NEM | 0.03 | 0.1 | 0.999 (0.1–100) |
| Glutathione disulfide | 0.3 | 1 | 0.996 (1–100) |

LOD: S/N = 3; LOQ: S/N = 10.

Table 3
Intraday and interday precision and accuracy of quantifying glutathione-NEM (μM) and glutathione disulfide (μM) using HILIC/MS

| Analytical compound | Actual concentration | Detected concentration (mean ± S.D., $n = 6$) | Precision (R.S.D.) (%) | Percent recovery (%) |
|---|---|---|---|---|
| Intraday | | | | |
| Glutathione-NEM | 1 | 0.99 ± 0.12 | 12.0 | 98.8 |
| | 10 | 10.01 ± 0.56 | 5.6 | 100.1 |
| | 100 | 99.6 ± 6.78 | 6.8 | 99.6 |
| Glutathione disulfide | 1 | 0.98 ± 0.06 | 6.3 | 98.0 |
| | 10 | 10.1 ± 0.33 | 3.2 | 101.0 |
| | 100 | 99.1 ± 4.4 | 4.4 | 99.1 |
| Interday | | | | |
| Glutathione-NEM | 1 | 1.01 ± 0.17 | 16.4 | 101.3 |
| | 10 | 10.00 ± 0.24 | 2.4 | 99.9 |
| | 100 | 99.8 ± 1.1 | 1.1 | 99.8 |
| Glutathione disulfide | 1 | 1.15 ± 0.13 | 11.4 | 114.7 |
| | 10 | 9.93 ± 0.35 | 3.6 | 99.4 |
| | 100 | 101.8 ± 0.9 | 0.9 | 101.8 |

S.D.: standard deviation; R.S.D.: relative standard deviation.

### 3.3. Validation of the HILIC/MS method

To validate of the HILIC/MS method, several experimental parameters, such as limit of detection (LOD), limit of quantitation (LOQ) and linearity, were examined. LOD was found to be 0.03 μM for GSH-NEM and 0.3 μM for GSSG with a calculated signal-to-noise ratio (S/N) of 3. LOQ was 0.1 and 1 μM for GSH-NEM and GSSG, respectively (S/N = 15) (Table 2). The calibration curves were obtained by analyzing mixtures containing the two compounds and the internal standard (γ-glu-glu) in the range of 0.1–100 μM and 1–100 μM for GSH-NEM and GSSG, respectively. Fig. 4 shows the calibration curves of GSH and GSSG in aqueous solutions and in pool saliva sample matrix solutions. The slopes and intercepts were identical between the aqueous solutions and the pool saliva sample matrix solutions. Intraday precision was defined as R.S.D., which was calculated from the values measured from six samples at concentrations of 1, 10 and 100 μM, in one day ($n = 6$ each). Intraday accuracy was defined as the relative value on the same measurements of intraday precision. Interday precision and accuracy were calculated using the values measured from six different samples (one sample each day) at concentrations of 1, 10 and 100 μM, respectively. The acceptable intra- and interday precisions and accuracy were set at <15% (Table 3). Those results show that all of low, middle and high concentration ranges are precise and accurate.

### 3.4. Storage stability

The stability data of GSH and GSSG are presented in Table 4. GSH and GSSG were stable in solution at all the temperatures investigated for at least one week.

Table 4
The stability of glutathione and glutathione disulfide under variety of conditions

| Analytical compound | Temperature (°C) | Actual concentration | Detected concentration (mean ± S.D., $n = 6$) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Days of storage | | | | | |
| | | | 1 | 2 | 3 | 4 | 5 | 6 |
| Glutathione | 25 | 50 | 48.4 ± 0.7 | 52.8 ± 0.9 | 48.1 ± 0.8 | 48.0 ± 1.8 | 47.0 ± 1.8 | 47.3 ± 1.3 |
| | | 100 | 96.8 ± 2.2 | 98.2 ± 1.1 | 99.3 ± 0.4 | 95.0 ± 0.6 | 95.0 ± 1.9 | 93.4 ± 1.1 |
| | 4 | 50 | 51.4 ± 1.5 | 52.0 ± 1.2 | 49.6 ± 0.6 | 50.3 ± 1.9 | 48.1 ± 0.8 | 47.9 ± 1.1 |
| | | 100 | 97.4 ± 2.6 | 103.2 ± 2.4 | 98.1 ± 0.4 | 97.1 ± 3.4 | 97.9 ± 2.1 | 97.6 ± 1.1 |
| | −20 | 50 | 51.8 ± 1.1 | 53.0 ± 2.2 | 49.1 ± 0.3 | 50.5 ± 1.2 | 50.1 ± 1.9 | 49.8 ± 1.0 |
| | | 100 | 98.5 ± 1.9 | 103.1 ± 1.8 | 98.7 ± 0.6 | 97.5 ± 1.3 | 101.9 ± 1.2 | 97.9 ± 1.6 |
| Glutathione disulfide | 25 | 50 | 49.4 ± 1.3 | 49.2 ± 1.3 | 49.2 ± 1.3 | 49.7 ± 1.2 | 48.3 ± 1.3 | 49.3 ± 1.2 |
| | | 100 | 99.5 ± 2.0 | 98.2 ± 1.4 | 98.4 ± 1.7 | 98.8 ± 3.4 | 98.2 ± 0.4 | 98.5 ± 1.9 |
| | 4 | 50 | 49.4 ± 1.6 | 50.6 ± 1.7 | 50.3 ± 1.5 | 49.5 ± 1.2 | 51.0 ± 1.2 | 50.4 ± 2.3 |
| | | 100 | 97.8 ± 1.3 | 102.3 ± 2.4 | 100.5 ± 1.5 | 97.8 ± 1.3 | 101.3 ± 2.3 | 100.6 ± 2.5 |
| | −20 | 50 | 50.5 ± 1.7 | 49.9 ± 1.3 | 49.9 ± 1.4 | 49.4 ± 1.2 | 50.3 ± 1.7 | 51.4 ± 1.4 |
| | | 100 | 103.7 ± 1.8 | 98.8 ± 1.7 | 99.7 ± 1.7 | 98.0 ± 1.3 | 103.2 ± 2.5 | 102.4 ± 2.3 |

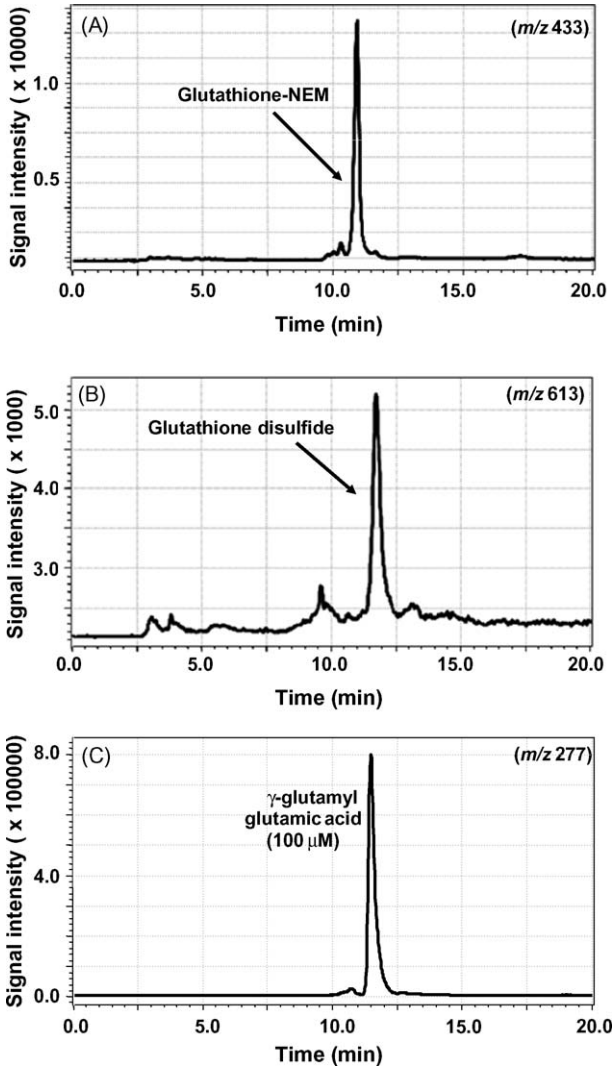


Fig. 5. Chromatograms of (A) glutathione-NEM, (B) glutathione disulfide and (C) γ-glutamyl-glutamic acid (100 μM) in human saliva samples, as obtained by the HILIC/MS method. The elution profile was as follows: 0–20 min 90–70% (B). Mobile phase (A) was 0.5 mM ammonium formate buffer (pH 4.0) and (B) acetonitrile. Positive ion ESI was employed for MS. SIM was conducted to simultaneously monitor ions with *m/z* of 433, 613 and 277, which corresponded to the protonated molecular ions of glutathione-NEM, glutathione disulfide and γ-glutamyl-glutamic acid.

### *3.5. Determination of GSH and GSSG in human saliva samples*

The proposed method was applied to the analysis of saliva samples from six healthy volunteers. The representative chromatograms of the saliva samples are shown in Fig. 5. The GSH/GSSG ratios from the saliva samples ranged from 0.58 to 0.87, with the average being $0.72 \pm 0.54$. The results are shown in Fig. 6.

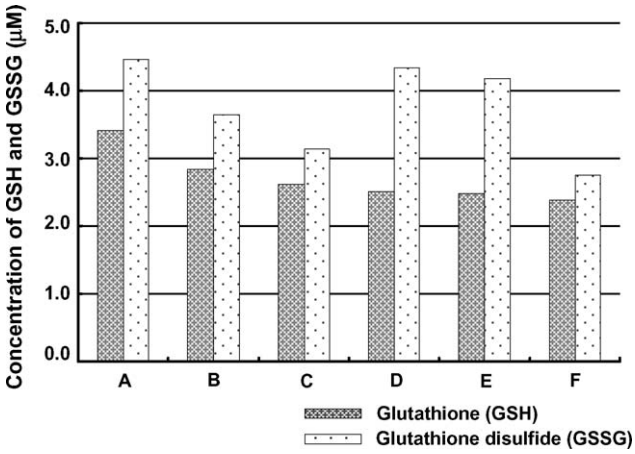


Fig. 6. Concentration of glutathione and glutathione disulfide in human saliva samples.

## 4. Conclusion

The proposed HILIC/MS method enabled the simultaneous determination of GSH and GSSG in human saliva samples. In addition, interferences from endogenous compounds was removed by using the SPE technique. Our method is expected to be useful for the assessment of the roles of GSH and GSSG in the oxidative state.

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