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# Simultaneous Determination of Oxidized and Reduced Glutathione in Eel's (*Monopterus albus*) Plasma by Transient Pseudoisotachophoresis Coupled with Capillary Zone Electrophoresis

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Both the reduced form of glutathione (GSH) and the oxidized form of glutathione (GSSG) in eel's (*Monopterus albus*) plasma were for the first time determined by transient pseudoisotachophoresis coupled with capillary zone electrophoresis. The method of transient pseudoisotachophoresis coupled with capillary zone electrophoresis has been thoroughly optimized and adequately evaluated for the simultaneous determination of GSH and GSSG in eel's plasma. The detection limits (S/N = 3) of the method developed were 0.2 and 0.05 $\mu$mol/L for GSH and GSSG, respectively. The linearity of the calibration curves was valid in the range of 0–10 $\mu$mol/L GSH and 0–0.70 $\mu$mol/L GSSG. The method was simple, fast, and reproducible. It was found that the respective concentrations of GSH and GSSG were in the range of 9.1−14.5 and 0.31−0.58 $\mu$mol/L in the adult eel's plasma, and 10.8−17.9 and 0.49 – 0.68 $\mu$mol/L in the juvenile eel's plasma of the three populations determined. Each blood sample was a composite of five eels. For each of the three populations, the concentrations of GSH and GSSG in the adult eel's plasma were lower than those in the juvenile eel's plasma, and the concentrations of GSH and GSSG in the plasma of population 1 (deep yellow finless eels) were higher than those in populations 2 (light yellow finless eels) and 3 (green finless eels) for either the adult or the juvenile eels.

**KEYWORDS: Glutathione; eel; plasma; transient pseudoisotachophoresis; capillary zone electrophoresis**

## INTRODUCTION

*Monopterus albus* can be found in the tropics (34° N to 6° S) from India to southern China, Malaysia, and Indonesia. It lives in muddy ponds, swamps, canals, and rice fields, where it burrows in moist earth in dry season, surviving for long periods without water during summer (*1*). *M. albus* is not only an economically important species in Asia for fish production but also a good model vertebrate for studies in comparative genomics, evolution, and developmental biology studies. Recent evidence have shown that anthropogenic xenobiotics have the potential to disrupt the endocrine systems of both humans and aquatic organisms (*2*).

Glutathione (GSH) is a tripeptide of glycine, glutamate, and cysteine, and oxidized glutathione (GSSG) is a dimer of GSH, where two GSH molecules are linked through a disulfide bond. The crucial role of glutathione is in antioxidant defense, nutrient metabolism, and regulation of essential pathways for whole body homeostasis. Glutathione is a ubiquitous molecule that is produced in all organs, especially in the liver (*3*). Nevertheless, since blood glutathione concentrations may reflect glutathione status in other less accessible tissues, measurement of both reduced (GSH) and oxidized glutathione (GSSG) in blood has been considered essential as an index of the whole subject oxidative status and as a useful indicator of disease risk (*4, 5*). In fact, the ratio of reduced to oxidized glutathione within cells is often used scientifically as a measure of cellular toxicity.

Over the years, a wide variety of methods have been introduced for the determination of glutathione in biological samples. The early techniques for the measurement of GSH and GSSG were based on an enzymatic recycling method by Tietze et al. (*6*). Recently, applications of electroanalytical methods to the determination of glutathione have been reported by several research groups (*7–11*). These methods are generally simple and sensitive but vulnerable to electrode contamination and less tolerant to matrix interference. Spectrofluorometry provided sensitive determination of glutathione (*12–14*). The shortcoming of spectrofluorometry includes the requirement of a derivation step and special reagents. In addition, the determination of both reduced and oxidized glutathione usually requires more than one measurement. For simultaneous determination of both reduced and oxidized glutathione in biological samples, analytical separative methods, such as HPLC and CE, are advantageous. There have been many reports on HPLC in the simultaneous determination of both reduced and oxidized glutathione in biological samples (*15–22*). CE is a modern

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technology of high separation efficiency. It well meets the requirements of the analysis of biomolecules in biological samples in many aspects. The low concentration sensitivity of the technique with UV detection is the main limitation when trace-level compounds are to be determined in complex matrices such as biological samples (*23*). Recently, several research groups have shown their interest in the determination of glutathione in biological samples by CE. Either a fluorometric (*24–26*) or electroanalytical detection (*27–29*) scheme was employed in most of the published research papers. Although detection sensitivity of fluorometric and electrochemical detection was high for glutathione, only the reduced form of glutathione was determined except for one published work (*26*). Because glutathione lacks a strong chromophore and the concentration of glutathione in plasma is at about the micromole per liter level, few reports have been published on the simultaneous determination of both the reduced and oxidized glutathione in biological samples by CE with UV detection (*30–37*). To obtain adequate sensitivity for detection of glutathione in biological samples, sample concentration strategies were employed. There were two general strategies for concentration of glutathione in the reports. One was the transient pseudoisotachophoresis stacking (*33, 34*), and the other was the pH-mediated base stacking (*35*). The transient pseudoisotachophoresis stacking strategy employing high a concentration of acetonitrile and sodium chloride seemed to be quite successful for glutathione in biological samples. Acetonitrile played mainly the role of deprotein, and sodium chloride played the role of the leading electrolyte in transient pseudoisotachophoresis.

Until now, no report has been made on the concentration of glutathione in *M. albus* plasma. In this work, we adopted the tp-ITP strategy coupled with CZE and modified the method reported by Kong et al. (*33*) for the sensitive and rapid determination of GSH and GSSG in eel's plasma. It was accomplished with the aim at providing an analytical technique and information of GSH and GSSG in eel's plasma for fish chemistry and related studies.

## EXPERIMENTAL PROCEDURES

**Apparatus.** The experiments were performed on a CE-L1 instrument (CE Resources, Singapore) equipped with a linear UVIS 200 detector (Alltech, Deerfield, IL). Electropherograms were recorded with the CSW (Chromatography Station for Windows) (DataApex, Prague, Czech Republic). Fused-silica capillaries of 60 cm total length and 50 cm effective length and of 375 $\mu$m o.d. and 50 $\mu$m i.d. were purchased from Yongnian Optical Fiber Factory (Hebei, China). Nylon filters with a pore size of 0.45 $\mu$m were obtained from Quandao Technical Co. (Shanghai, China). The pH values of the running buffers were measured with a pHS-3C meter from Mettler Toledo Instruments (Shanghai, China). Centrifugation was performed on a TGL-16G high-speed tabletop centrifuge from Shanghai Anting Scientific Instrument Co. (Shanghai, China).

**Chemicals.** All reagents used were of analytical grade unless otherwise stated. Glutathione (both reduced and oxidized forms), boric acid, sodium chloride, benzoic acid, and acetonitrile (HPLC grade) were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). All of the solutions and samples were prepared in redistilled water daily and filtered through a 0.45 $\mu$m filter before use. Water purified using a Honest DZG-303A system (Shanghai Bai-Te Scientific and Technical Co., China) was used throughout the experiments.

**Preparation of Eel Blood Samples and Standard Solutions.** There are three different geographical populations of *M. albus* (deep yellow finless eels, light yellow finless eels, and green finless eels) in China (*38*). Thirty eels (*M. albus*), ten eels (five adult and five juvenile) for each of the three geographical population, were purchased from a local market and kept in fresh water at a temperature range of 25–30 °C and starved for 2 days before experiments. Blood was collected from the caudal vein, filtered through a 0.45 $\mu$m filter, and centrifuged at 12000 rpm for 15 min. The supernatant (200 $\mu$L) was transferred into a 2 mL centrifuge tube. Sodium chloride solution (100 $\mu$L) of 350 mmol/L and 700 $\mu$L of acetonitrile were added to the centrifuge tube, and the resultant solution was then vortex-mixed and centrifuged again at 12000 rpm for 10 min. The mixture was ready for sample introduction into the electrophoresis system for separation.

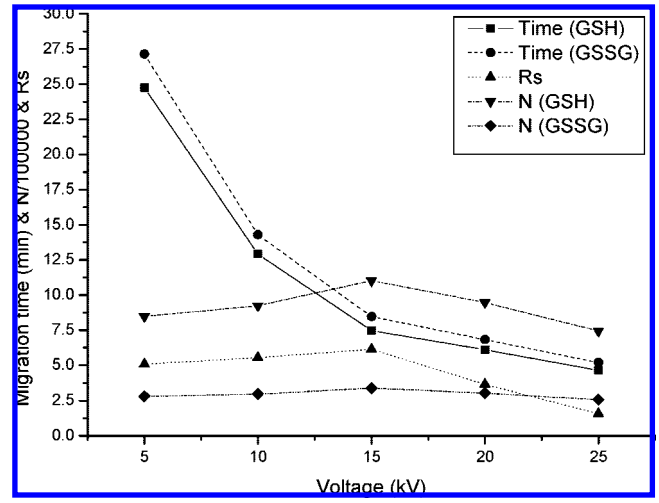


**Figure 1.** Effect of voltage. Capillary: 50 $\mu$m i.d. × 375 $\mu$m o.d. $L_{tot}$ = 60 cm, $L_{eff}$ = 50 cm. Electrophoretic buffer: 300 mM borate (pH = 8.0). Detection wavelength: 200 nm. Sample injection: 5.5 kPa × 30 s. Temperature: 25 °C.

Standard stock solutions of GSH (1.0 mM) and GSSG (1.0 mM) were prepared quantitatively in water and stored at 4 °C when not in use. The working standard solutions were obtained by appropriately diluting the stock solutions and finally made to contain 50 mM sodium chloride and 70% (v/v) acetonitrile (or as specified). Benzoic acid was employed as the internal standard, and its stock solution was prepared in water to have a concentration of 0.82 mM.

**Electrophoresis Procedure.** Prior to running a sample for separation, a new capillary was rinsed with 0.1 mol/L sodium hydroxide for 40 min, water for 10 min, and the separation buffer for 3 min in order. Between runs, the capillary was flushed with the separation buffer for 3 min. Borate buffer of 300 mM (pH 8.0) was used as the separation buffer for both the tp-ITP and the CZE. A voltage of 15 kV was applied for the separation. Sample introduction was facilitated by applying a pressure of 5.5 kPa for 30 s. The detection wavelength was set to 200 nm. The tp-ITP and the CZE experiments were performed at an ambient temperature of 25 °C.

## RESULTS AND DISCUSSION

**Selection of Separation Voltage and Detection Wavelength.** In theory, increasing the run voltage in CE will improve the efficiency of the separation while also shortening migration times. The gains achieved, however, need to be weighed against the resultant peak broadening and irreproducible migration times due to Joule heating effects (*37*). With the increase of voltage from 5 to 15 kV, the migration time of GSH and GSSG was reduced considerably and continuously (**Figure 1**). But when the voltage was higher than 15 kV, the separation efficiency and the resolution started to decrease. The migration time still became shorter, but not remarkably. Therefore, 15 kV was adopted as the separation voltage.

Since the compounds of interest lack strong chromophoric properties, their detection is limited to the low-UV region. The effect of wavelength on peak height for GSH and GSSG was examined. It was found that the peak height for GSH and GSSG decreased with increasing the wavelength of detection. There-

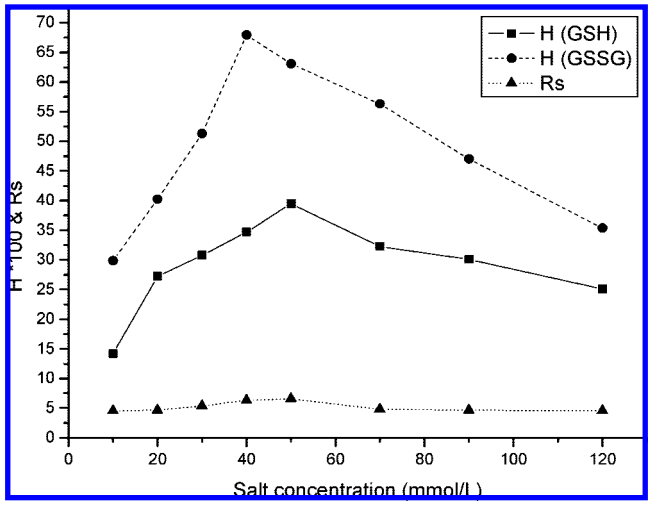


**Figure 2.** Influence of salt concentration in samples on peak heights (*H*) and Rs. Voltage: 15 kV. For other experimental conditions, see the legend to **Figure 1**.

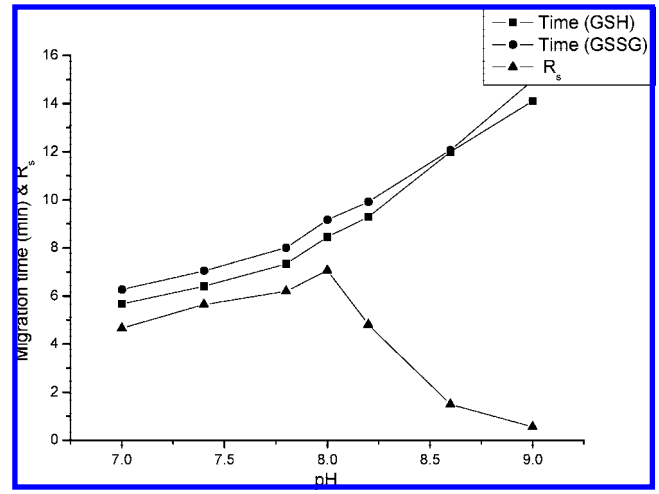


**Figure 3.** Effect of buffer pH on migration time and resolution. Voltage = 15 kV. For other experimental conditions, see the legend to **Figure 1**.

fore, the detection wavelength was set at 200 nm of the lowest end of the detector to obtain the detector response as high as possible in this work.

**Effect of Acetonitrile and Sodium Chloride.** Mixing acetonitrile with the sample is mainly for removing proteins. However, the presence of acetonitrile in the sample has several additional important advantages. Because acetonitrile has low conductivity by itself, it can enhance stacking effect as a result of high field strength (*39*). The optimal concentration of acetonitrile for the peak height and resolution of GSH and GSSG was found to be 70% in volume. This was in agreement with the results in a previous report (*33*).

**Figure 2** shows the influence of the concentration of sodium chloride in the sample on the peak height (*H*) and resolution (Rs) between GSH and GSSG. A salt concentration range of 40–50 mM was considered to be favorable for stacking both GSH and GSSG. It was reported that the total salt concentration of eel plasma was about 58 mM (*40*). So when the eel plasma was treated as described in Preparation of Eel Blood Samples and Standard Solutions, the salt concentration in the treated plasma was about 50 mM, which was just in the favorite range of the optimized salt concentration. The chloride ion, which had a high mobility and was in high concentration in the sample, moved quickly ahead of the analytes as a wide band and acted as the leading ions. Borate ion of the lowest mobility in the separation buffer might act as the terminating ion. GSH and GSSG migrated behind the leading ion ($Cl^-$) as sharp bands in a short period of time after applying voltage for separation by the mechanism of the transient pseudoisotachophoresis (tp-ITP) (*39*).

**Effect of pH of the Buffer.** The dependences of the migration time and resolution of GSH and GSSG on pH of the buffer are shown in **Figure 3**. The migration time increased with increasing buffer pH. The explanation for it might be that the peptides became similarly more negatively charged as the buffer pH went to high. It was worthy to note that the migration order of GSH and GSSG was reversed for pH varying from 8.0 to 9.0, and the resolution of the two peptides was significantly reduced due to the little difference in the mobilities in this pH range. The best resolution (Rs = 7.1) for GSH and GSSG was achieved in 300 mmol/L borate solution of pH 8.0 with the migration time of less than 10 min.

**Optimization of Sample Size.** To ensure high separation efficiency in CZE, the sample size has to be very small,

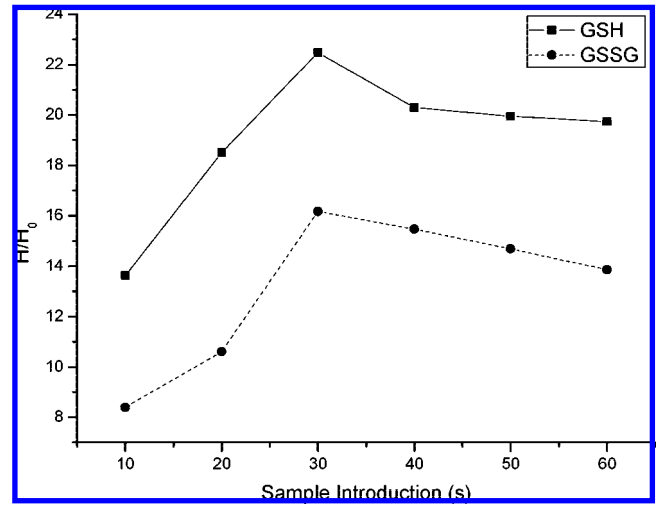


**Figure 4.** Effect of sample injection time on $H/H_0$ of GSH and GSSG. *H* represents the peak height of glutathione obtained using the tp-ITP-CZE and $H_0$ that obtained using the CZE without the tp-ITP. Voltage = 15 kV. Electrophoretic buffer: 300 mM borate (pH = 8.0). For other experimental conditions, see the legend to **Figure 1**.

preferably less than 1% of the effective capillary length. Unfortunately, the sensitivity of common commercial UV–vis detectors is low, and analysis of analytes of low concentration in samples by CZE is difficult. So the sample injection time, i.e., the sample size, needs to be optimized to obtain the best results in tp-ITP (*39*). Time duration in the range of 10−60 s was examined. Both the peak height and peak area for GSH and GSSG increased with increasing injection time up to 30 s. **Figure 4** shows the dependence of peak height on sample injection time. However, the peak of GSH became deviated from Gaussian shape when the sample injection time was longer than 40 s. Consequently, 30 s (equal to 7.8 cm in length of the capillary) was set for sample injection in subsequent measurements. The amount and the zone length of the sample introduced into the capillaries by applying a pressure of 5.5 kPa for 30 s were about 1.5 and 3.4 times those in an earlier report (*33*), respectively.

**Analytical Performance of the Method Developed.** Analytical performance of the tp-ITP-CZE method developed was evaluated. The results are summarized in **Table 1**. To improve the precision of quantitative results, benzoic acid was selected

**Table 1.** Analytical Characteristics of the tp-ITP-CZE Method Developed

| | GSH | GSSG |
|---|---|---|
| time (RSD, %) ($n = 6$) | 0.71 | 0.45 |
| height (RSD, %) ($n = 6$) | 1.08 | 0.87 |
| area (RSD, %) ($n = 6$) | 2.25 | 1.96 |
| linearity range ($\mu$mol/L) | 0–10 | 0–0.7 |
| $R$ | 0.9989 | 0.9991 |
| LOD (S/N = 3 $\mu$mol/L) | 0.2 | 0.05 |

**Table 2.** Recoveries of GSH and GSSG in Eel's Plasma

| | concn added ($\mu$M) | concn determined ($\mu$M ± SD) | recovery (%) |
|---|---|---|---|
| GSH | 0 | 9.30 ± 0.32 | |
| | 5 | 14.14 ± 0.56 | 96.5 |
| | 10 | 19.53 ± 0.38 | 101.8 |
| | 15 | 24.12 ± 0.49 | 98.7 |
| GSSG | 0 | 0.32 ± 0.02 | |
| | 0.3 | 0.65 ± 0.04 | 112.7 |
| | 0.6 | 0.91 ± 0.06 | 98.5 |
| | 1.0 | 1.34 ± 0.03 | 102.2 |

as the internal standard. The detection limits for GSH and GSSG were determined on the basis of the signal-to-noise ratio of 3. Compared with the results reported by Kong et al. (*33*), the detection limits in the present work were a little lower. It might be due to the enlarged sample size and differences in sample matrix. The precisions of migration time, peak height, and peak area were examined and found to be good (see **Table 1**). The reproducibilities of both the peak height and the peak area were dependent on reproducibilities of sample introduction, but the peak area was more dependent on electroosmotic flow and the parameter settings of the detector and integrator. Regression equations of the calibration curves were obtained by relating the peak height ratio of GSH and GSSG to the internal standard with the concentration of GSH and GSSG. For GSH, $y = 0.3125x - 0.1799$ (correlation coefficient, 0.9989), where $x$ stands for the concentration of GSH (in micromoles per liter) and $y$ for the ratio of the area of GSH to the area of the internal standard of benzoic acid. For GSSG, $y = 0.887x + 0.0841$ (correlation coefficient, 0.9991), similarly, where $x$ stands for the concentration of GSSG (in micromoles per liter) and $y$ for the ratio of the area of GSSG to the area of the internal standard of benzoic acid. The linearity of the calibration curves was valid in the range of 0–10 $\mu$mol/L GSH and 0–0.70 $\mu$mol/L GSSG, respectively, with the concentration of 8.0 $\mu$mol/L benzoic acid as the internal standard. The recovery of GSH and GSSG was determined by spiking eel plasma samples with standards of GSH and GSSG at three different concentrations as shown in **Table 2**. The recoveries of GSH and GSSG were in the range of 96.5−101.8 and 98.5−112.7, respectively.

The proposed tp-ITP-CZE method was applied to the determination of GSH and GSSG in eel's plasma, and a typical electropherogram is shown as trace b in **Figure 5**. For comparison, a typical electropherogram obtained in the CZE without the tp-ITP is shown as trace c in the same figure. Trace a is an electropherogram of the standards of GSH and GSSG and the internal standard of benzoic acid obtained by the tp-ITP-CZE. On the basis of **Figure 5**, it can be concluded that GSH and GSSG in eel's plasma could be determined by the tp-ITP-CZE method developed with satisfaction while it was difficult to determine them by the CZE method without the tp-ITP.

**Table 3** shows the concentrations of GSH and GSSG in three different geographical populations of eel's plasma determined by the proposed tp-ITP-CZE method. The $F$-test (*41*) was first

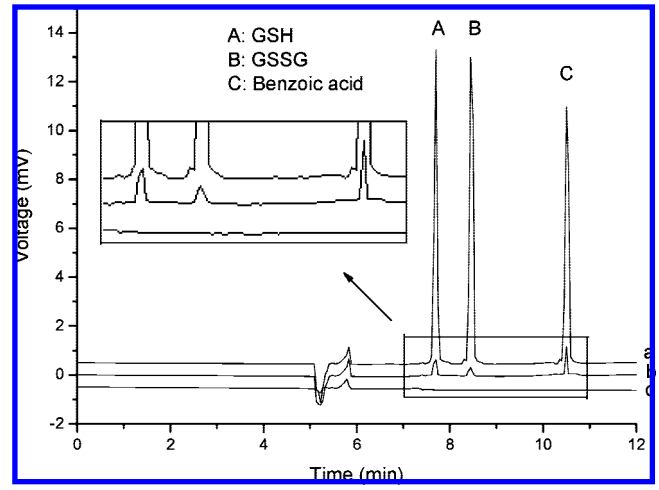


**Figure 5.** Typical electropherograms of the standards **(a)** and eel's plasma sample obtained by using the tp-ITP-CZE **(b)** and the CZE **(c)**. **(a)** A typical electropherogram of the standards obtained by using the tp-ITP-CZE. Sample concentration: GSH, 100 $\mu$mol/L GSH; GSSG, 50 $\mu$mol/L. **(b)** A typical electropherogram of the eel's plasma obtained by using the tp-ITP-CZE. The eel's plasma sample was treated as described in Experimental Procedures. **(c)** A typical electropherogram of the eel's plasma sample obtained by using the CZE. The eel's plasma sample was treated with acetonitrile as described in Experimental Procedures but without the addition of sodium chloride. Sample injection: 2.1 kPa for 3 s. The other experimental conditions were the same as those for **Figure 1**.

**Table 3.** Results of GSH and GSSG in Adult and Juvenile Eel's Plasma of the Three Populations

| | GSH ($\mu$M ± SD) | GSSG ($\mu$M ± SD) |
|---|---|---|
| adult eel | | |
| 1 (deep yellow finless eels) | 14.51 ± 0.39 | 0.58 ± 0.03 |
| 2 (light yellow finless eels) | 9.30 ± 0.32 | 0.32 ± 0.02 |
| 3 (green finless eels) | 9.13 ± 0.42 | 0.31 ± 0.05 |
| juvenile eel | | |
| 1 (deep yellow finless eels) | 17.92 ± 0.45 | 0.68 ± 0.06 |
| 2 (light yellow finless eels) | 11.43 ± 0.52 | 0.45 ± 0.03 |
| 3 (green finless eels) | 10.81 ± 0.48 | 0.49 ± 0.04 |

applied to all of the two sets of results (adult and juvenile eels) of each population and all of the two sets of results of either adult or juvenile eels; it was found that there was no significant difference between all of the two sets of standard deviations based on data in **Table 3**. Then, the $t$-test (*41*) was performed to compare all of the two means. It was found that there was a significant difference between all the two means except the two means of population 2 and population 3 of GSH or GSSG for either adult or juvenile eels. Both the $F$-test and $t$-test were conducted with $P = 0.05$, that is, to be significant at the 0.05 level. For each of the three populations, the concentrations of GSH and GSSG in the adult eel's plasma were lower than those in the juvenile eel's plasma, and the concentrations of GSH and GSSG in the plasma of population 1 were higher than those in the other two populations for either the adult or the juvenile eels. The concentrations of GSH and GSSG in the plasma of population 2 and population 3 were not significantly different for either the adult or the juvenile eels.

Simultaneous determination of both the reduced form of glutathione (GSH) and the oxidized form of glutathione (GSSG) in eel's plasma could be accomplished by the tp-ITP-CZE method with UV detection with satisfaction. The transient pseudoisotachophoresis with use of acetonitrile and sodium

chloride could provide effective concentration of GSH and GSSG in eel's plasma. Benzoic acid was well served as the internal standard to ensure good precision of the quantitative results. The method developed was simple, fast, and reproducible. It has the potential of an analytical method for routine simultaneous determination of both GSH and GSSG in eel's plasma. Concentrations of both GSH and GSSG in eel's (*M. albus*) plasma were reported for the first time. For each of the three populations, the concentrations of GSH and GSSG in the adult eel's plasma were lower than those in the corresponding juvenile eel's plasma, and the concentrations of GSH and GSSG in the plasma of population 1 were higher than those in the other two populations for either the adult or the juvenile eels. The concentrations of GSH and GSSG in the plasma of population 2 and population 3 were not significantly different for either the adult or the juvenile eels.

## ACKNOWLEDGMENT

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