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Analytical Methods

A rapid HPLC post-column reaction analysis for the quantification of ergothioneine in edible mushrooms and in animals fed a diet supplemented with extracts from the processing waste of cultivated mushrooms

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ABSTRACT

For establishing an efficient and sensitive method for the quantitative determination of 2-thiol-L-histidine-betaine (ergothioneine, ERG) in edible mushrooms and the blood and muscles of animals, a technique using reversed-phase separation and post-column reaction between 2′-dipyridyl disulphide and ERG was developed. A corresponding derivative 2-thiopyridone, detected at 343 nm, was used for estimating ERG concentration. The flow rate, temperature, pH, and composition of the solution were optimised. A low limit of quantification (1.41 ppm) and a simpler sample preparation made this technique more rapid compared to other methods using liquid chromatography–mass spectrometry. The coefficient of variation (CV) values for the reproducibility and recovery of ERG were within the acceptable values of 6% and 97.5–100.0%, respectively. The efficiency of this methodology was compared with that of spectrophotometric and mass-spectrometric quantitative methods, and was assessed in the light of previous studies. The ERG contents in different mushrooms were 12.69–234.85 mg/kg wet weight basis. Dietary supplementation with extracts from mushroom processing waste significantly improved ERG bioavailability in the blood of yellowtail fish and muscle tissue of cattle.

1. Introduction

2-Thiol-L-histidine-betaine (L-ergothioneine, ERG) was first isolated from ergot, a fungal infection of the rye grain (Tanret, 1909). ERG is a naturally occurring amino acid analogue that is synthesised in some bacteria and fungi but not in animals (Melville, Horner, Otken, & Ludwig, 1955). ERG exists as a thione-thiol tautomer, with the thione form predominating in aqueous solutions. Since it scavenges strong oxidants such as hydroxy radicals, hypochlorite, and peroxynitrite *in vitro* (Akanmu, Cecchini, Aruoma, & Halliwell, 1991), and chelates redox-active bivalent cations such as Cu^{2+} (Akanmu et al., 1991; Hartman, 1990), ERG is thought to function primarily as an antioxidant in cells. There are several lines of evidence that cellular antioxidant status, which is reflected in ERG content (Meister, 1992), correlates with resistance to lipid peroxidation in atherosclerosis, smoking, and diabetes (Diaz, Frei, Vita, & Keaney, 1997). The interest in this compound was greatly stimulated by its subsequent discovery in blood as a free form (Kawano et al., 1982). It has also been found to occur in semen and various mammalian tissues, principally the liver and kidneys (Mayumi et al., 1978; Melville, 1959), and in the whole blood of pig (Heath, Rimington, Glover, Mann, & Leone, 1953) and rat (Kawano et al., 1982). Despite the considerable amount of work on this compound, its exact mode of action in various organisms and its origin in the animal body remain to be elucidated. One factor that has hindered the elucidation of the role of ERG is the lack of a convenient and rapid assay for detecting this compound in biological materials. Several methods, including spectrophotometry (Hunter, 1930; Lawson, Morley, & Woolf, 1951; Melville, 1959; Stowell, 1961) and high performance liquid chromatography (HPLC) (Dubost, Beelman, Peterson, & Royse, 2007), have been developed for assaying ERG. Currently, methods that use spectrophotometric analysis to detect ERG in biological samples suffer from several serious drawbacks; for example, certain compounds in biological materials can undergo the assay reaction or inhibit the development of the reaction (Baldridge & Lewis, 1953; Melville & Lubschez, 1953). Therefore, the samples typically require various pretreatments to eliminate the interfering compounds. As pointed out by Jocelyn (1958), these assay techniques are too laborious and time consuming to apply to convenient general use. Although the ERG assay that uses mass spectrometry (MS) is one of the satisfactory quantitative methods, it is not suitable for rapid and routine analysis of ERG in biological materials. Moreover, sodiated and/or potassiated adducts of the ERG molecule distributed in the biological materials or generated during the extraction and/or analysis processes often create difficulties in quantifying the total ERG content by MS. Because of these different issues, the establishment of a suitable chromatographic separation technique without interference from

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co-eluting compounds from the biological materials has remained a challenging task.

The present study reports the development, optimisation, and validation of a rapid HPLC post-column analysis of ERG based on a selective post-column reaction of chromatographically separated ERG with 2′-dipyridyl disulphide (2-Py-S-S-2-Py) under strong acidic conditions. Under optimised conditions, the total ERG contents in several species of edible mushroom and in the blood of several animals and muscles of cattle were evaluated.

2. Materials and methods

2.1. Mushroom samples and chemicals

Fresh and edible fruiting bodies of common mushrooms, including *Agaricus augustus*, *Agaricus bisporus* (brown and white varieties), *Auricularia auricular*, *Flammulina velutipes*, *Grifola frondosa* (black and white varieties), *Hypsizygus marmoreus*, *Hypsizygus tessulatus*, *Hyspatys marrcus*, *Lyophyllum decastes*, *Lentinula edodes*, *Pholiota nameko*, and *Pleurotus eryngii* were purchased from local retailers in Tokyo, Japan. All purchased mushrooms were commercially produced and cultivated on an industrial scale in Japan. L-(+)-Ergothioneine was purchased from Biomol International LP (Plymouth Meeting, PA, USA); 2,2′-dipyridyl disulphide, from TCI Chemical Co. (Tokyo, Japan); reduced glutathione, from Sigma–Aldrich (St. Louis, MO, USA); L-cysteine, L-histidine, and L-methionine, from Wako Pure Chemical Industries (Osaka, Japan); and methanol of HPLC grade from Kokusan Chemical Co. Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Preparation of extracts from mushroom fruiting bodies

Crude mushroom extracts for the ERG assay were prepared according to the method of a previous study (Bao, Ochiai, & Ohshima, 2010), with slight modification. The fruiting body of the mushroom was ground in a MK-K75 food processor (Matsushita Electric Industrial Co. Ltd., Osaka, Japan). A 20-g portion of the homogenised material was mixed with 100 ml of water and gently boiled at 95 ± 2 °C for 1 h in a glass beaker. The supernatant was collected by centrifugation (3000*g* for 15 min at 4 °C) and subsequently evaporated at 40 °C *in vacuo*. The resultant residue was suspended in 15 ml of 70% (v/v) aqueous ethanol. The ethanolic solution was vortexed, left to stand at 4 °C for 2 h, and subsequently centrifuged at 3000*g* for 15 min at 4 °C. The supernatant was collected and evaporated at 40 °C *in vacuo* to remove the ethanol. The ethanol-free residue was dissolved in 4 ml of the distilled water. Thus, 1 ml of the extract was obtained from 5 g of wet material. Next, 1 ml of a methimazole solution (0.2 mg/ml in distilled water) was added as an internal standard (IS) to a mixture containing 0.5 ml of the mushroom extract, 4.5 ml of water, and 14 ml of absolute ethanol. The ethanol mixture was vortexed and left to stand at 4 °C for 2 h and subsequently centrifuged at 3000*g* for 15 min at 4 °C. The supernatant was collected and evaporated at 40 °C *in vacuo* to remove the ethanol. The residue was dissolved in 10 ml of deionised water and subjected to analysis.

2.3. Preparation of extracts from the processing waste of mushroom cultivation

The mushroom processing waste was kindly donated by Cosmic Farm Co. Ltd. (Niigata, Japan). The extracts from *F. velutipes* and *Pleurotus cornucopiae* were prepared according to a procedure of Bao, Shinomiya, Ikeda, and Ohshima (2009) using a practical food processing line at Yamaki Foods Co. Ltd. (Matsumae, Ehime, Japan). In brief, 770.8 kg of mushroom processing waste was extracted by 4000 l of hot water at 95 °C for 1 h. Following filtration, centrifugation, and subsequent thin-layer vacuum concentration, the concentrated extracts of *F. velutipes* and *P. cornucopiae*, abbreviated as CE-F and CE-P, respectively, were obtained. The extracts were stored at 5 °C for feeding trials in the present study.

2.4. Rearing conditions and feeding plan

Fish feeding trials were performed offshore of Tojima Island, Uwajima city, Ehime prefecture, Japan, in January, 2011, according to the procedure of Bao et al. (2009). In brief, 300 2-year-old yellowtail (*Seriola quinqueradiata*) (4.1 ± 0.1 kg body weight and 61.6 ± 1.1 cm fork length) were selected at random, divided into 3 groups, and reared in 3 net cages, each 64 m^3 (4 × 4 × 4 m) in size. Fish in all 3 groups were fed a commercial diet of extruded pellets (EP) for yellowtail (New Buri EP, Marubeni-Nisshin Feed Co. Ltd., Tokyo, Japan). During an adaptation period for the first 3 weeks, the fish were fed every 2 days (on average) an amount corresponding to 3% of the body weight. Afterwards, for 2 weeks (on average), each fish in the CE-F and CE-P groups was fed every 2 days an amount corresponding to 3% of the body weight the commercial diet containing 10% concentrated mushroom extract solutions (1.8 mg ERG/kg body weight of fish), while fish in the control group were continuously fed the commercial diet without supplementation. Additionally, in another trial, the dependency of ERG contents in the blood on CE-F concentrations in the diet was evaluated by supplementing the diet with 5% and 10% CE-F according to the above procedure. After 2 weeks of feeding, the fish in all groups were starved for 1 day. The water temperature of the area ranged between 17.3 and 20.8 °C for the 2 weeks of the feeding period.

Hybrid F1 (Japanese Black × Holstein) cows were also used as an animal model in this study to evaluate the bioavailability of ERG in feed supplemented with CE-F. Feeding trials were carried out in Miyazaki prefecture, Japan, in February 2011. A total of 12 fattening cattle (11 non-pregnant females and one emasculated male, mean age of 27 months, and mean body weight of ~650 kg) were divided randomly at the start of the feeding trial into a control (n = 6) and treatment group (n = 6). For 23 days, each animal in the control group was fed at 40 kg/(cow day) a basal total mixed ration (TMR) for fattening cattle two times per day. Animals in the treatment group were fed with TMR containing 40 g/(cow - day) of CE-F lyophilised powder (0.08 mg ERG/kg body weight of cattle). All animals in each group were provided with similar feeding and management practices.

2.5. Collection of blood and muscle samples

Five to 10 yellowtail specimens were randomly taken from each group after 2 weeks of feeding and a subsequent 1-day starvation period. Five millilitres of whole blood was collected from the caudal vein by means of a heparinised syringe for ERG analysis. Fresh whole pig blood was obtained from a slaughterhouse in Ehime, Japan. Ethylenediaminetetraacetic acid was used as an anticoagulant. Blood samples were transferred to sodium heparin tubes and kept on ice pending delivery to the laboratory.

After a feeding trial period of 23 days, all the cattle were starved for 1 day and then sacrificed at a commercial slaughterhouse. Carcasses were stored at 5 °C for 2 days. Next, a 200-g sample was collected from the longissimus thoracis muscle of each animal, vacuum packaged, and transported to the laboratory under refrigerated conditions within 24 h.

2.6. Preparation of blood and muscle extracts

A 10-g portion of each sample of cattle meat was cut into small pieces and was finely homogenised in the MK-K75 food processor.

ERG was extracted from the whole blood of yellowtails and pigs and from the cattle muscle samples using the procedure of Bligh and Dyer (1959), with a slight modification by exchanging the water with 0.88% KCl. A 0.4-ml methimazole solution (0.2 mg/ml in distilled water) was added to 0.5 ml of blood or 2 g of muscle sample as an IS prior to homogenisation. The homogenate was centrifuged at 3000*g* for 8 min at 4 °C. The methanol layer was taken and evaporated at 40 °C *in vacuo*. The methanol layer thus obtained was dissolved in 4 ml of deionised water and subjected to analysis.

2.7. HPLC separation and post-column derivatisation

The reaction of 2-Py-S-S-2-Py with chromatographically separated ERG at lower pHs was investigated by monitoring the formation of 2-thiopyridone (Py-2-SH) at 343 nm (Fig. 1A). The system developed in this study is presented schematically in Fig. 1B. The HPLC-separated analytes were reacted with 0.23 mM 2-Py-S-S-2-Py in 0.25 M HCl (pH 1) in a polyetheretherketone tube (1/16″ o.d. × 0.25 mm i.d. × 30 m; GL Science, Tokyo, Japan) used as a post-column reaction coil. The length of the reaction coil was optimised based on the reactivity and baseline noise. The 2-Py-S-S-2-Py solution was pumped with a model L-6020 HPLC inert pump (Hitachi, Tokyo, Japan) at a flow rate of 0.15 ml/min; the formation of Py-2-SH was detected photometrically at 343 nm. The optimisation of the reactant flow rate, pH and composition of the solution, and reaction temperature was conducted by the successive variation of one condition at a time, by using an aqueous authentic ERG solution (20 μg/ml), and by evaluating the peak area of the derivatives. The ERG in the biological extracts was separated in a system comprising a PU-980 Intelligent HPLC pump (JASCO, Tokyo, Japan) coupled with a photodiode array (PDA) detector (SPD-M10A) inlined before a reaction coil for pre-reaction, and a post-reaction PDA detector (SPD-M10AV) (Shimadzu, Kyoto, Japan) inlined after the reaction coil. The samples were loaded on a Symmetry Shield™ C18 column (4.6 mm i.d. × 250 mm, 5-μm particle size; Waters Corp., Milford, CT, USA), eluted with 10% methanol in deionised water at a flow rate of 0.2 ml/min, and monitored at 254 nm. The temperature of the reaction coil was controlled by a CTO-10AC column oven (Shimadzu, Kyoto, Japan).

2.8. Standard solution

A calibration curve was obtained for ERG using a standard series of solutions of authentic ERG dissolved in deionised water containing 20 μg/ml of 1-methyl-3H-imidazole-2-thione (methimazole) as an IS. The peak area ratio of ERG to IS was plotted against the concentration of ERG.

2.9. Quantification of ERG by LC–MS

The ERG content in the blood samples was quantitatively determined by the method of Dubost et al. (2007), with slight modification. The analysis was carried out using a Shimadzu LCMS-2010EV liquid chromatography mass spectrometer system (Tokyo, Japan) equipped with 3 Develosil TM C30-UG-5 columns (Nomura Chemical Co. Ltd., Aichi, Japan) in series. The isocratic mobile phase was 10% methanol containing 0.1% acetic acid with a flow rate of 0.2 ml/min. The injection volume was 20 μl, and the column oven temperature was set at 40 °C. The ERG eluted from the column was monitored by absorbance measurements at 254 nm with a PDA detector (SPD-M20A) (Shimadzu, Kyoto, Japan). With regard to the conditions for MS, the curved desolvation line temperature was 250 °C, voltage was 1.5 kV, nebuliser nitrogen gas flow was

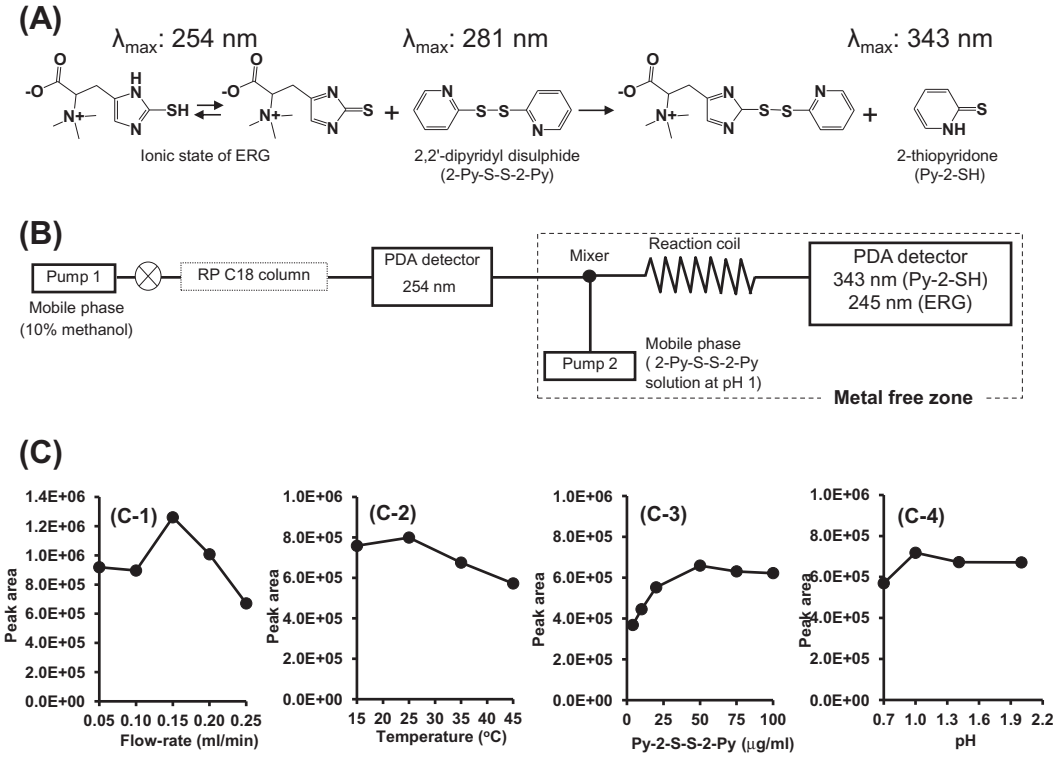

Fig. 1. The reaction scheme for ergothioneine (ERG) with 2,2′-dipyridyl disulphide (Py-2-S-S-2-Py) (A) and a schematic diagram of a new system (B) for the quantitative determination of ERG by HPLC post-column reaction analysis; and the effects of the flow rates of the 2-Py-S-S-2-Py solution as a post-column reaction reagent (C-1), the column oven temperatures (C-2), the concentrations of Py-2-S-S-2-Py (C-3), and the pH of Py-2-S-S-2-Py solution (C-4) on the peak area of the derivative 2-thiopyridone (Py-2-SH).

1.5 l/min, analytical mode was electrospray ionisation (ESI)-positive scan, and the scan range of m/z was 50–1000. For selected ion monitoring, m/z 230 was set for ERG. A calibration curve was obtained from different concentrations of authentic ERG.

2.10. Quantification of ERG by spectrophotometry

ERG content was measured as previously described (Carlsson, Kierstan, & Brocklehurst, 1974b), with slight modification. In brief, the reaction mixture contained 0.35 ml of a 0.23 mM 2-Py-S-S-2-Py solution in 0.25 M HCl (pH 1) and 0.50 ml of blood extract or authentic ERG solution. After incubation at 25 °C for 5 min, the ERG concentration was determined by measuring the absorbance at 343 nm using a Shimadzu UV-1700-PC spectrophotometer.

2.11. Accuracy and precision

For the recovery experiments, different concentrations of ERG were added to the whole blood of yellowtails and pigs. The recovery was determined by comparing the amount of detection and the amount of sample without the added standard.

The intraassay precision was obtained by preparing and analysing a blood sample multiple times ($n = 5$) in a single run. The interassay precision was assessed by calculating the variance of the ERG concentration in a blood control sample analysed periodically throughout 5 days.

2.12. Influence of metal (ferrous) ion on the stability of 2,2′-dipyridyl disulphide at low pH and the testing of compounds that could potentially interfere with the assay system

The influence of metal (ferrous) ion on the stability of 2-Py-S-S-2-Py at low pH was evaluated by the following procedure: a piece of ferrous metal (10 g) was added to a solution containing 0.23 mM of 2-Py-S-S-2-Py in 0.25 M HCl (pH 1). The mixture was incubated at 25 °C. The change in the absorption spectra of the mixture solution was measured at 0, 0.5, 1, 2, and 3 min and 2 days after incubation using a Shimadzu UV-1700-PC spectrophotometer.

For determining whether L-cysteine, reduced glutathione, L-histidine, and L-methionine could interfere with the determination of ERG levels in biological samples, an authentic ERG solution was spiked with the appropriate amount of the chosen substance to obtain a final concentration of 15 μg/ml for each compound. The effects on ERG detection were then evaluated.

2.13. Statistical analysis

Microsoft Excel 2007 was used to calculate the means and standard deviations for all of the multiple measurements and to generate graphs. SPSS for windows (version 11.5, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Each value is expressed as the mean ± standard deviation (SD). Differences among groups were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Optimisation of HPLC with the post-column reaction

3.1.1. Chromatographic separation

The effects of the mobile phase composition on the chromatographic separation was studied. Different concentrations of methanol in deionised water were chosen for the optimisation. In terms of maximum resolution, the optimum condition for the separation of the analytes was obtained by using 10% methanol as an isocratic mobile phase. Under this condition, ERG and IS were separated with good resolution. Chromatographic peaks were identified by spiking experiments and matching the retention times of the derivatives with those of the authentic compounds.

3.1.2. Flow rate of the post-column reagent

A preliminary experiment showed that the most suitable concentration of 2-Py-S-S-2-Py for acquiring intense peak signals was 0.46 mM. The peak area for the ERG standard solution (20 μg/ml) at an injection volume of 20 μl was monitored at different flow rates for the derivatising reagent between 0.05 and 0.25 ml/min. The post-column flow rate was optimised by maintaining a constant pre-column mobile phase flow rate of 0.2 ml/min. The optimum flow rate of the 2-Py-S-S-2-Py solution for producing the most intense signal was 0.15 ml/min (Fig. 1C-1). The peak area drastically decreased when a flow rate of >0.15 ml/min was used.

3.1.3. Temperature of the derivatisation reaction

For investigating the relationship between the temperature of the post-column reaction module and the peak area of the derivative, a temperature range of 15–40 °C was examined under an optimum flow rate. An increase in the temperature above 25 °C resulted in a decrease in the peak area (Fig. 1C-2). This could be explained by the increase in the reaction temperature increasing side reactions such as aerial oxidation of ERG, which is responsible for the creation of a non-inducing compound from ERG (Carlsson et al., 1974a, 1974b). Shelf degradation of 2-Py-S-S-2-Py to 2-Py-SH can also occur at higher temperatures and result in an increased background. Therefore, the optimal post-column reactor temperature was set at 25 °C.

3.1.4. Concentration of the 2-Py-S-S-2-Py solution

The effect of 2-Py-S-S-2-Py concentrations on the post-column reaction of ERG is shown in Fig. 1C-3. Increasing the concentration of 2-Py-S-S-2-Py to 50 μg/ml was found to result in an increase in the peak area for the 2-Py-SH derivative. The optimum concentration for the 2-Py-S-S-2-Py solution was, therefore, defined as 50 μg/ml (0.23 mM). In addition, the results of this experiment revealed that continually increasing the 2-Py-S-S-2-Py concentrations did not result in a proportional increase in the response. The increase in the concentration of the reactant may lead to an increase in the accumulation of an intermediate that competed for detection. It could also be due to saturation of absorption at higher concentration.

3.1.5. pH of the 2-Py-S-S-2-Py solution

The effect of the pH of the post-column reagent on the formation of Py-2-SH was evaluated over a pH range of 0.7–2.0. As shown in Fig. 1C-4, pH 1 was found to be optimal for the reaction. Correspondingly, the optimal pH of the final reaction mixture was approximately 1–2. A similar effect was reported in a spectrophotometric study (Carlsson et al., 1974a), where the optimum pH for the formation of Py-2-SH was observed to be between 1 and 2. Therefore, the derivatisation reaction input column was carried out at pH 1.

3.2. Calibration curve

Based on the above optimisation process, a combination of a 0.23 mM solution of 2-Py-S-S-2-Py at pH 1, reaction temperature of 25 °C, and flow rate of 0.15 ml/min was used for generating a calibration curve. By performing triplicate injections, a standard calibration curve was obtained for a series of concentrations of authentic ERG containing 20 μg/ml of IS. The calibration curve

Table 1
Recovery and reproducibility on the intra-day and inter-day assay of the ergothioneine contents in the whole bloods of yellowtail and pig.

Recovery assay Blood origins	Spiked concentration (μg/ml)	In sample (before addition) (μg/ml)[a]	Expected (μg/ml)	Actual (μg/ml)	Recovery (%)
Yellowtail	80	154.20 ± 8.94	234.20	240.20 ± 10.14	102.56
	40	154.20 ± 8.94	194.20	198.38 ± 5.81	102.15
	20	154.20 ± 8.94	174.20	177.50 ± 9.87	101.89
Pig	5	29.00 ± 0.60	34.00	34.66 ± 0.95	101.94
	14	29.00 ± 0.60	43.00	41.91 ± 4.12	97.47
	25	29.00 ± 0.60	54.00	54.30 ± 4.56	100.55
Reproducibility assay	Blood origins	Mean (μg/ml)[b]	SD[c]	CV (%)	
Within-assay	Yellowtail	124.34	6.74	5.42	
	Pig	28.13	1.32	4.70	
Between-assay	Yellowtail	124.64	6.71	5.39	
	Pig	25.54	1.52	5.95	

[a] $n = 3$.
[b] $n = 5$ for all samples in all cases.
[c] SD of mean for all samples.

showed linearity with the concentrations to at least 90 μg/ml (data not shown). The derived linear equation was $f(X) = 0.0275X - 0.0302$ ($r^2 = 0.9997$), where $f(X)$ is the peak area ratio (ERG/IS) and X is the concentration of ERG. The limit of quantification, defined as the lowest concentration that could be determined with the linear calibration curve, was 1.41 μg/ml for the 20 μl volume of sample solution injected.

3.3. Recovery

A standard addition recovery experiment was carried out using the samples obtained from yellowtail and pig blood spiked with ERG at different concentrations before the extraction. As shown in Table 1, the recoveries of authentic ERG from the blood samples after spiking ranged from 97.5% to 102.5%, indicating the high accuracy and reliability of this method.

3.4. Precision

The precision of the method was evaluated by measuring by both the intraday and interday reproducibility of the analyses for the yellowtail and pig blood samples (Table 1). The intraday and interday precisions were determined to be within 6%, suggesting that the HPLC post-column reaction analysis provided satisfactory reproducibility for quantitative determination of ERG.

3.5. Selectivity

It has been reported that 2-Py-S-S-2-Py reacts not only with ERG but also with other thiones at pH 1 (Carlsson et al., 1974a). In particular, L-cysteine, reduced glutathione, L-histidine, and L-methionine have been observed to undergo this reaction and produce 2-Py-SH. Therefore, these compounds were examined for their ability to interfere with the assay procedure. However, no interference from these compounds was found, with all of the compounds having a reasonably separate elution time from ERG and/or showing no evident reaction with 2-Py-S-S-2-Py (data not shown).

3.6. Comparison and validation of the method

The optimised post-column assay for ERG was also compared with the traditional spectrophotometric (Fig. 2A) and the advanced MS (Fig. 2B) methods. Although a highly linear correlation for the authentic standard was observed over a wide range (1–100 μg/ml) for both the spectrophotometric assay and the present method, the spectrophotometric analysis resulted in an overestimation of the ERG levels in the biological samples. Several biologically abundant compounds (e.g., L-cysteine, glutathione, purines, tyrosine, and histidine) can undergo and/or inhibit the development of the spectrophotometric assay reaction (Baldridge & Lewis, 1953; Melville & Lubschez, 1953). Therefore, for the spectrophotometric method to achieve precise values, several sample pretreatments are needed in order to eliminate the interfering compounds. On the other hand, MS using ESI revealed a highly linear correlation for both the ERG standard and the ERG in the biological samples by monitoring the molecular ion $[M+H]^+$ at m/z 230 (Fig. 2B). Next, the sodiated cluster ions $[M+Na]^+$ at m/z 252 and $[2M+Na]^+$ at m/z 481 and the retention time of authentic ERG and biological ERG were determined and quantified (Supplementary Fig. S1). The results clearly indicated that the present post-column assay for

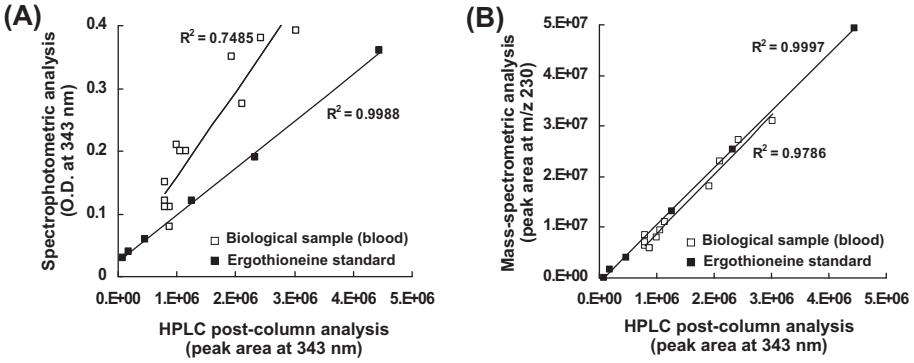

Fig. 2. Relationship between the ergothioneine contents determined by the proposed HPLC post-column reaction analysis and those obtained by spectrophotometric (A) and mass-spectrometric (B) methods.

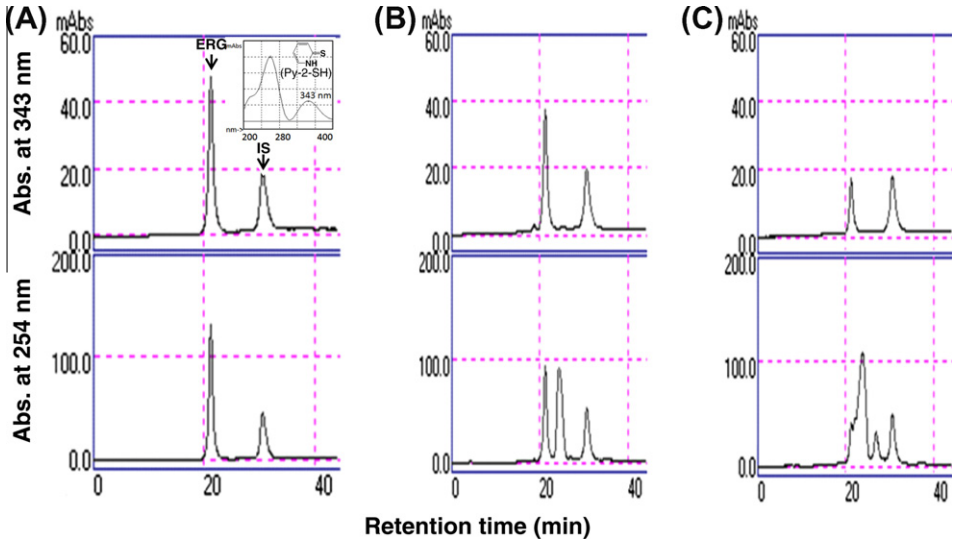

Fig. 3. Typical HPLC chromatograms of 60 μg/ml authentic ergothioneine (A) and yellowtail blood (B), and pig blood (C) extracts after post-column derivatisation. ERG, ergothioneine; IS, methimazole used as an internal standard. (UV-spectrum of the derivative, Py-2-SH, is presented inset).

ERG is as precise as advanced MS analysis. Under the optimised conditions of the post-column assay, the authentic ERG, biological ERG from the blood samples, and IS were evaluated as shown in Fig. 3. These results clearly indicated that the interference of unresolved coeluting compounds monitored at 254 nm can be successfully eliminated by the post-column reaction as clearly observed in Fig. 3C. In addition, spiking of the biological samples with the authentic ERG confirmed the elution time and reactivity towards 2-Py-S-S-2-Py (data not shown). The absorption spectra of Py-2-SH obtained as a derivative of 2-Py-S-S-2-Py reacting with ERG and IS, as shown in Fig. 3, showed similarities to those reported in previous studies (Na, Woo, & Lee, 1999; Ševčíková, Glatz, & Tomandl, 2003). It is noteworthy that the stability of 2-Py-S-S-2-Py was highly vulnerable to ferrous ions, with the rapid formation of Py-2-SH occurring in the presence of metal ions at pH 1 (data not shown). For avoiding this problem, a metal free/Teflon®-coated instrumental system is recommended for use in the post-column reaction analysis (Fig. 1B).

3.7. Application to edible mushrooms and animal tissues

The HPLC post-column reaction analysis system was applied to the quantitative determination of ERG in the mushroom fruiting body, processing waste of several edible mushrooms, and blood and muscles of several animals. The ERG contents of the extracts prepared from the fruiting bodies of several mushroom species ranged between 12.69 and 234.85 mg/kg wet weight basis, while those for the CE-F and CE-P extracts were 0.65 and 0.06 mg/ml, respectively (Table 2). The ERG contents in the whole blood of yellowtail (*S. quinqueradiata*) fed the diet supplemented with CE-F were significantly ($p < 0.05$) higher than those in the controls (Fig. 4A). However, no significant difference ($p > 0.05$) in the ERG content was observed between the yellowtails fed the diet supplemented with CE-P extract and the controls (Fig. 4A). This result suggested that supplementation with CE-F significantly improved the bioavailability of ERG in the yellowtail. On the basis of these results, the CE-F extracts were used for further investigations. Yellowtails and cattle were fed diets containing different concentrations of CE-F extracts. These results indicated that the mean values of the ERG content in the yellowtails fed on diets containing 5% and 10% CE-F extracts were 1.7 and 1.5 times higher, respectively, than the mean value of the ERG content of the yellowtails fed on the control diet (Fig. 4B). However, there was no significant difference ($p > 0.05$) in the ERG contents between the 2 treated yellowtail groups. A similar tendency was observed in the feeding trials for the cattle. Dietary supplementation with the mushroom waste extract powder (1 g/kg diet) containing ERG (1.4 mg/g extract powder) significantly ($p < 0.05$) increased the ERG contents in the muscles (Fig. 4C). The average absorption efficiency of ERG in the cattle was approximately 90%. A similar result was found in a previous study (Encarnacion, Fagutao, Hirono, Ushio, & Ohshima, 2009), where the ERG content in the muscle of kuruma shrimp (*Marsupenaeus japonicus*) fed on a supplemented diet containing mushroom extract was 4.5 times higher than that of the control group. Bao et al. (2009) demonstrated that dietary supplementation with ERG-containing extracts prepared from the processing waste for *F. velutipes* could prevent discoloration and lipid oxidation in the dark muscle of yellowtail on an industrial scale. The results from the present study emphasised that supplementation of ERG using the mushroom extracts could increase the

Table 2
Total ergothioneine contents of hydrophilic extracts prepared from fruiting body and processing waste of edible mushrooms.

Mushroom samples	Ergothioneine contents[a]
Fruiting body[b]	
Agaricus augustus	19.85 ± 1.58
Agaricus bisporus (brown variety)	24.17 ± 4.01
Agaricus bisporus (white variety)	78.98 ± 3.72
Auricularia auricular	32.29 ± 0.91
Flammulina velutipes	151.17 ± 14.95
Grifola frondosa (black variety)	20.19 ± 1.37
Grifola frondosa (white variety)	103.92 ± 8.15
Lentinula edodes	123.01 ± 2.18
Hypsizygus marmoreus	84.06 ± 3.87
Hypsizygus tessulatus	55.52 ± 3.10
Hyspatys marrcus	14.97 ± 0.51
Lyophyllum decastes	77.07 ± 6.31
Pholiota nameko	12.69 ± 0.93
Pleurotus eryngii	234.85 ± 9.75
Processing waste[c]	
Flammulina velutipes	0.65 ± 0.01
Pleurotus cornucopiae	0.06 ± 0.01

[a] Values are expressed as mean ± SD ($n = 3$).
[b] Values are calculated based on the wet weigh basis of fruiting body (mg/kg wet weigh basis).
[c] Values are calculated as mg/ml of mushroom processing waste extract.

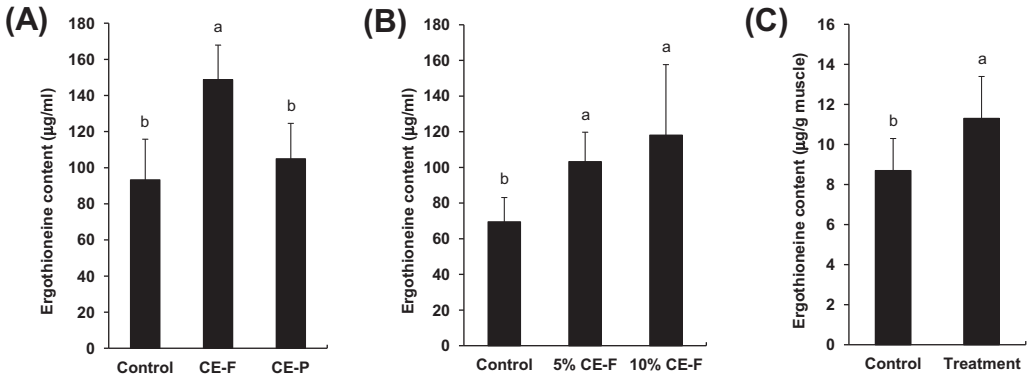

Fig. 4. Ergothioneine contents in the whole blood of yellowtails and in cattle muscle quantitatively determined under the present optimised conditions. A, yellowtail fed with the extracts from the processing waste of different mushroom species (CE-F, *F. velutipes*; CE-P, *P. cornucopiae*); B, yellowtail fed diets supplemented with 5% or 10% CE-F; C, cattles fed with 40 g/(cattle day) of CE-F powder. Results are presented as mean ± SD. Values with different superscript letters represent significant difference ($p < 0.05$).

contents of ERG in the blood and muscle of the animals and thus might be a novel strategy for preventing the oxidation and discoloration of certain animal muscles in post-harvest. The present results suggested that the HPLC-flow injection system developed in this study could be used as a sensitive, specific, and rapid methodology for the quantitative determination of ERG in biological materials.

4. Conclusions

The post-column assay developed and validated in this study for the analysis of ERG was found to be reliable, simple, fast, precise, accurate, and sensitive. For the quantification of ERG, the results of the MS method showed no significant differences from those obtained with the method developed in this study. The purpose of the new assay method is not only to replace the available HPLC methods currently used for assaying ERG but also to serve as a better alternative method where advanced instruments (e.g., HPLC–MS) are not available for routine analysis. The ERG content in different biological samples was satisfactorily measured by the present method. Several feeding trials indicated an improvement in the bioavailability of ERG in different animals by supplementing the feed with the mushroom extracts. In summary, the proposed method can be used for the routine analysis of ERG in biological materials.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2012.01.061.

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