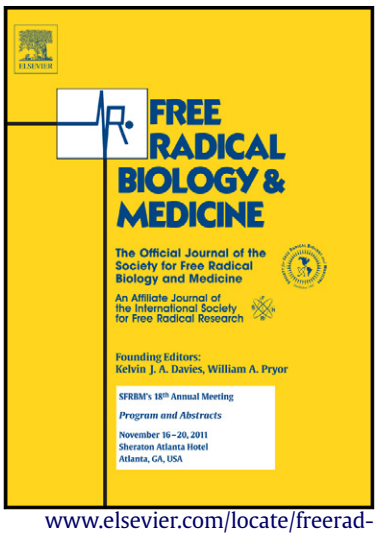

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An Uncommon Redox Behaviour Enlightens the Cellular Antioxidant Properties of Ergothioneine

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Abstract

Ergothioneine (ESH), an aromatic thiol, assumed by diet in humans and accumulated in particular cells, is believed to act as antioxidant. However, its redox mechanism remains unclear and it does not seem to provide any advantage as compared to other antioxidants, such as alkylthiols, which are better reducing agents and generally present in cells at higher levels. Here, we investigated by ESI-MS spectrometry the products of ESH oxidation produced by neutrophils during oxidative burst and, to further elucidate ESH redox behavior, we also analyzed the oxidation products upon reacting ESH with hypochlorite in cell free solutions. Indeed, neutrophils are the main source of hypochlorite in humans. Furthermore, we also tested other biologically relevant oxidants, such as peroxynitrite, and hydrogen peroxide. Results indicate that treatment of human neutrophils with phorbol 12-myristate 13-acetate (PMA) in the presence of ESH leads to a remarkable production of the sulfonated form (ESO_3H), a compound never described before, and hercynine (EH), the desulfurated form of ESH. Similar results were obtained when ESH was subjected to cell free oxidation in the presence of hypochlorite, as well as hydrogen peroxide, or peroxynitrite. Furthermore, when the disulfide of ESH (ESSE) was reacted with those oxidants, we found that it was also oxidized with production of EH and ESO_3H whose amount was dependent on the oxidant strength. These data enlighten a unique ESH redox behavior, entirely different from alkylthiols, and suggest a mechanism, so far overlooked, through which ESH performs its antioxidant action in the cells.

Keywords: ergothioneine; neutrophils; oxidative burst; oxidative stress; hercynine; ergothioneine sulfonic acid.

Abbreviations.

EH, hercynine

EIC, extracted ion chromatogram

$ES(O)_2SE$, ergothioneine disulfide S-dioxide

ES(O)SE, ergothioneine disulfide S-monoxide

ESH, ergothioneine

ESI, electrospray ionization

ESO_2H, ergothioneine sulfinic acid

ESO_3H, ergothioneine sulfonic acid

ESOH, ergothioneine sulfenic acid

ESSE, ergothioneine disulfide

FIA, flow injection analysis

HPLC, high perfomance liquid chromatography

ICC, ion charge control

LC, liquid chromatography

MetMb, ferrimyoglobin

MRM, multiple reaction monitoring

MS/MS, tandem mass

OCTN1, organic cationic transporter 1

PMA, phorbol 12-myristate 13-acetate

ROS, reactive oxygen species

Introduction

Ergothioneine, (ESH; 2-mercaptohistidine betaine), is an ubiquitous substance occurring in fungi, plants and animals, known to be synthesized only by few microorganisms, chiefly actinomycetales [1]. It is supposed that some species of microorganisms occurring in the soil produce and release ESH which is firstly absorbed by plants and, subsequently, by animals. In human body, ESH has been found to accumulate in bone marrow, seminal fluid, eye lenses, kidneys and erythrocytes up to millimolar concentrations [2]. A specific transporter of ESH, the organic cationic transporter 1 (OCTN1), has been discovered in the plasma membrane of various human cell types, with the highest levels of expression in monocytes, macrophages and hematopoietic lineage cells [3,4]. Depleting HeLa cells of ESH through OCTN1 silencing leads to an increased mitochondrial oxidative burden and damage [5]. Moreover, OCTN1 knockout mice, completely deficient in ESH, resulted to be more susceptible to oxidative stress and with higher lethality due to intestinal ischemia and reperfusion injury [6].

Although the prevalent opinion is that ESH acts in the cells mainly as an antioxidant (for an exhaustive review on the ESH function and role see ref. [7]), more than a century after its discovery, it is still obscure the mechanism through which ESH performs its antioxidant action. To date, it has been widely demonstrated that ESH is an effective scavenger of hydrogen peroxide, lipid peroxides, superoxide ion, singlet oxygen [8,9], hydroxyl radical [10,11] and nitric oxide derivatives [12,13]. ESH has been shown to counteract reactive oxygen species (ROS) generated upon interaction of hydrogen peroxide with hemoproteins, such as hemoglobin and myoglobin [14,15]. Indeed, hemoproteins react with hydrogen peroxide turning into the high reactive ferryl form (ferryl hemoproteins), with iron in the oxidation state of Fe^{+4}, taking part to adverse cellular oxidation processes. The prooxidant and proinflammatory effects of ferryl hemoglobin seem to be involved into atherogenesis [16]. Also, it has been proposed that, in the early phases of ischemia, deoxymyoglobin is oxidized by hydrogen peroxide to ferryl myoglobin which, during oxygenated reperfusion, might lead to cellular injury [17]. ESH has been shown to reduce ferryl hemoprotein (containing Fe^{+4}) into methemoprotein (containing Fe^{+3}), thus ameliorating the ischemia-

reperfusion damage [18]. A similar antioxidant mechanism has been proposed for the ESH inhibition of nitrite-induced oxyhemoglobin oxidation [19]. Another relevant ESH antioxidant activity is exerted against hypochlorous acid. ESH, in fact, has been found to be a powerful hypochlorous acid scavenger, being able to protect α1-antiproteinase against the inactivation by this compound [11]. Furthermore, the current hypothesis is that the antioxidant activity of ESH is critical in protecting circulating erythrocytes from the possible damage induced by this highly reactive oxidant produced by activated neutrophils during inflammation or normal function. On the whole, all these studies seem to leave no doubt on the antioxidant properties of ESH.

Chemically, ESH is a particular type of aromatic thiol, resulting from the *N,N,N*-trimethylhistidine (histidine betaine or hercynine) in which a sulfhydryl group is linked to the imidazole ring at C2 position (**Figure 1**). At physiological pH, ESH exists in a tautomeric equilibrium between thiol-thione forms, with the thione form predominating (**Figure 1**).

The free energy of reduction of ergothioneine disulfide (ESSE), lower than those of alkyldisulfides, has been ascribed to the thermodynamic stability of the thione form [20]. Actually, in the totality of the proposed mechanisms of its antioxidant function, ESH is considered to oscillate between the reduced state and the oxidized state represented by the disulfide form, similarly to alkylthiols such as cysteine or glutathione [19, 26]. This is, perhaps, the most compelling reason of concern about the antioxidant role of ESH in cells. Indeed, if ESH behaves like an alkylthiol, it is plausible to wonder what particular advantage is given by its antioxidant function with respect to alkylthiols, generally present in the cells at higher levels and also more easily oxidized than ESH.

In this paper, we aim at elucidating aspects of ESH redox behaviour, gone completely unnoticed so far, which unveil that the chemical antioxidant mechanism of ESH is absolutely different from alkylthiols.

Materials and Methods

Materials

Ergothioneine, myoglobin from equine heart, histopaque-1077, histopaque-1119, iodomethane, iodomethane-d_3, hydrogen peroxide 30 wt. % in H2O, calcium hypochlorite, sodium nitrite were purchase from Sigma-Aldrich, Milan, Italy. SPE-C18 columns for flash chromatography were obtained from Phenomenex (Anzola Emilia, Italy). All other solvents and reagents used were of analytical grade.

The concentration of the hypochlorite stock solution in 0.1 M NaOH was determined, after appropriate dilution, by the absorbance at 292 nm (ε_{292} =350 M^{-1} cm^{-1}) [21].

Preparations of neutrophils from human peripheral blood.

Neutrophils were isolated from six ml of heparinized peripheral blood from human healthy volunteers after centrifugation (400 x g for 40 min at 4°C) on discontinuous density gradient on Histopaque-1077 and Histopaque-1119, following manufacturer' instructions. After centrifugation, the interface cells between the Histopaque-1119 and the Histopaque-1077 were carefully removed and transferred to a new conical tube. Cells were washed twice with PBS, centrifuged at 300 x g for 10 min at 4°C, seeded on gelatin-coated culture dishes (2 x 10^6 cells/ml) and immediately used for the experiment.

Neutrophil oxidative burst assay.

The purified neutrophils were stimulated in the presence (100 ng/ml) or absence of phorbol 12-myristate 13-acetate (PMA) in a final volume of 3 ml of HBSS containing ESH (0.4 mM). After PMA addition, aliquots of the incubation media (200 µl) were taken from time zero up to 30 min and centrifuged at 16.000 x g for 10 min. The supernatants were then removed and subjected to mass spectrometric analysis.

Preparation of peroxynitrite

Peroxynitrite was synthesized as described [22]. Briefly, 50 ml of ice-cold 1.2 M HCl and 50 ml of 1.4 M H_2O_2 were mixed and maintained under continuous stirring in an ice cold container. Then 100 ml of ice cold 0.6 M sodium nitrite solution was added, followed immediately by addition of

100 ml of 1.5 M NaOH solution. The excess of hydrogen peroxide in the reacting solution was decomposed by passing the solution through a column containing manganese dioxide. The eluate was frozen overnight in a glass cylinder at -20 °C. The top layer which separated out, containing enriched peroxynitrite, was collected in aliquots and used in the successive experiments. The peroxynitrite concentration in the stock solution, after appropriate dilution in 0.4 M NaOH, was determined by its absorbance at 302 nm (ε_{302}= 1670 $M^{-1}cm^{-1}$) [22].

Preparation of ergothioneine disulfide

ESH disulfide was prepared essentially as described [23]. Briefly, 3.9 mg of ESH dissolved in 200 µl of 5 M HCl were reacted with 8.5 µl of a standardized 2.0 M H_2O_2 solution in 1.5 ml conical tube. After standing at room temperature for 10 min, the solution was divided in aliquots of 20 µl in conical tubes, evaporated to dryness under vacuum overnight, and stored in dry nitrogen atmosphere at -20 °C. At the time of use, an aliquot was dissolved again in 100 µl of the due solvent and immediately employed.

Preparation of hercynine and deuterated hercynine.

As hercynine was not commercially available, it was prepared by a modified heterogeneous phase reaction employing iodomethane as the methylation agent in the presence of $KHCO_3$ [24,25]. Also deuterated hercynine (EH-d_9) was prepared in the same way by substituting iodomethane with deuterated iodomethane (methyl-d_3 iodide). In both cases, histidine (50 mg) was dissolved in 5 ml of methanol, then 200 mg of $KHCO_3$ and, subsequently, 2 ml of the methylating agent was added. The mixture was stirred at room temperature overnight. The reaction completeness was monitored by LC-ESI-MS. Then, the mixture was centrifuged and the supernatant collected and evaporated to dryness at 40°C in a rotavapor. The residue was dissolved in 10 ml of Milli Q grade water and purified by flash chromatography on SepPac C18 cartridge (Phenomenex, Anzola Emilia, Italy). The sample-loaded column was washed with 100 ml of Milli Q water and eluted by applying 50 ml of H_2O/acetonitrile solution (80:20 v:v). The eluate containing the purified product was evaporated to dryness under a stream of air and dried overnight in vacuum over P_2O_5.

Mass spectrometric determinations.

The analyses were performed with an HPLC Agilent 1100 series coupled on line with an Agilent LC-MSD SL quadrupole ion trap. All MS acquisitions were performed by using ESI in positive ion mode with nitrogen as the nebulizing and drying gas. Nitrogen was used at a flow rate of 7 L/min and a pressure of 30 psi both as a drying and a nebulising gas. The nebulizer temperature was set at 350°C. The ion charge control (ICC) was applied with a target set at 30000 and maximum accumulation time at 20 ms. The measurements were performed from the peak area of the extracted ion chromatogram (EIC). The quantitation was achieved either by comparison with the calibration curves obtained with standard solutions or by internal standard addition. The optimization of the instrumental parameters for the analyses of ESH, ESSE and EH was performed by continuous infusion (FIA-ESI-MS/MS measurements) of 5 μM standard solution in 0.1% formic acid. The mass cutoff and amplitude were optimized to obtain the most efficient MS/MS transitions from the positively charged precursor ion $[M+H]^+$ to the fragment ions. Multiple reaction monitoring (MRM) was used for the detection of analytes in HPLC-ESI-MS/MS analyses. The most intense transitions utilized for MS/MS detection were 230.1→186 for ESH, 198.1→154 for EH, 207.1→163 for EH-d_9, 229.1→185 for ESSE (as bipositive ion) or 457.2→310 (as unipositive ion), 278.1→154 for ESO_3H. The chromatographic analyses with ESI-MS detection were performed on a 250 mm x 3.0 mm i.d., 5 μm, Discovery-C8 analytical column (Supelco, PA, USA). The mobile phase was composed with 0.1% formic acid in water and pumped at 0.1 mL/min in isocratic conditions at room temperature. Sample volumes of 5-10 μL were injected.

Cell free oxidation of ESH

All the oxidation reactions of ESH were conducted in 1.0 mM sodium phosphate buffer at pH 7.4. The final composition of the reaction mixtures was 100 μM ESH and 100 μM of the oxidant, according to the employed ESH and oxidant concentrations in similar in vitro studies (11). The reactions were initiated by adding into the reaction mixture the due amount of the oxidant stock solution. 5 μl aliquots of the reaction mixture were analyzed over time by flow injection analysis (FIA) with MS detection. When hydrogen peroxide was employed as oxidant, the reactions were conducted both in the presence or absence of ferrimyoglobin (MetMb). MetMb was prepared by

myoglobin oxidation with ferricyanide and purified as reported [14]. In all the experiments, the final concentration of MetMb was 50 μM.

Cell free oxidation of ESSE

All the oxidation reactions of ESSE were conducted in 1.0 mM sodium phosphate buffer at pH 7.4. The final composition of the reaction mixtures was 93 μM ESSE and 93 μM of the oxidant. The reactions were initiated by adding into the reaction mixture the due amount of the oxidant stock solution. 5 μl aliquots of the reaction mixture were analyzed over time by FIA with MS detection.

Hydrolytic decomposition of ESSE

Assay conditions: Solutions of ESSE (93 μM) in 1.0 mM sodium phosphate buffer, pH 7.4. 5 μl aliquots of the solutions were analyzed over time both by FIA with MS detection and by HPLC-ESI-MS/MS.

Oxidative formation of sulfate from ergothioneine.

The experiments were conducted using only water as a solvent, in order to avoid interference from phosphate buffer. 1 ml of ESH solution (1.75 mM) in water was reacted with hydrogen peroxide (10 mM) for 18 h. Then, 200 μl of barium chloride solution, 22 mM in water, was added to the reaction mixture. The white precipitate formed was collected by centrifugation in a conical tube and treated with 5 M HCl. The formation of barium sulfate was demonstrated by the insolubility of the white precipitate in 5 M HCl solution. As blanks, two solutions of ESH and H_2O_2 in water, at the same concentrations as in the reaction mixture, were treated, after 18 h, in the same manner with the barium chloride solution.

Statistical analysis

The results of the FIA analyses reported in the figures are the mean±S.E.M of 4 determinations.

Results

Stimulation of human neutrophils in the presence of ergothioneine

ESH has been proven to be a powerful scavenger of hypochlorous acid (11). As the main cellular source of this strong oxidant in human body is represented activated neutrophils, we first analyzed the products of ESH oxidation in whole cell experiments. To this end, human neutrophils were subjected to oxidative burst by stimulation with PMA in the presence of ESH. The time-dependent HPLC-ESI-MS analyses of the incubation medium revealed the increases over time of two unknown peaks at m/z 198.1 and m/z 278.1, with retention times of 12.1 min and 16.2 min, respectively. Both MS/MS fragmentation patterns of the peaks at m/z 198.1 and at m/z 278.1 showed the more intense fragment at m/z 154. Therefore, the transitions 198.1→154 and 278.1→154 were utilized for the analytical determinations (**Figure 2**). In order to ascertain whether the unknown compounds derived from ESH oxidation or were released in the medium by the cells, we performed a set of cell free ESH oxidation experiments in the presence of hypochlorous acid and, as a comparison, also in presence of other important cellular oxidants, such as hydrogen peroxide and peroxynitrite.

Cell free ESH oxidation

Several reaction mixtures were prepared each containing 100 μM ESH and hydrogen peroxide or peroxynitrite or hypochlorite all at the same concentrations (100 μM) in sodium phosphate buffer 1.0 mM, pH 7.4. Furthermore, being reported a more intense oxidative action of H_2O_2 in the presence of hemoproteins (14, 15), ESH oxidation by hydrogen peroxide was also performed in solutions containing 50 μM MetMb.

Each reaction started with the addition of the due amount of the oxidant stock solution. The time courses of ESH consumption during oxidation is reported in **Figure 3**, panel A. As expected, the formation of ESSE was always detected when analyzing over time the reaction mixtures with each oxidant (**Figure 3, panel B**). However, surprisingly, the same unknown peaks at m/z 198.1 and m/z 278.1, found during neutrophil oxidative burst, were always found among the products of the ESH

oxidation by all the oxidants employed (**Figure 3, panels C and D**). The compound at *m/z* 278.1 (**Figure 3, panel D**), formed at different amounts depending on the strength of the oxidant employed, was found to be ESO_3H by successive HPLC-ESI-MS/MS analyses. Also, the identity of the compound at m/z 198.1 was revealed to be hercynine (EH), confirmed by its mass spectrometric and chromatographic behaviour, and substantiated by comparison with authentic standard which was synthesized labelled with deuterium at the methyl groups (*N,N,N*-trimethylhistidine-d_9, EH-d_9). As an example, a typical HPLC-ESI-MS/MS analysis of the reaction mixture in which ESH was reacted with hypochlorite for 10 min, is reported in **Figure 4** (panel A).

The chromatographic analysis allowed, in less than 30 min, the complete chromatographic separation of ESH and its oxidation products, that is, EH, ESSE and ESO_3H. In particular, EH eluted at a retention time (T_R) of 12.1 min, followed by ESO_3H at T_R 16.2 min, ESH at T_R 17.2 min, and ESSE at T_R 25.8 min. The MS/MS fragmentation patterns confirmed the identity of each chromatographic peak (**Figure 4, panel B, C, D, E**).

Indeed, after certain reaction times, HPLC-ESI-MS/MS analyses of the reaction mixtures were performed to identify and characterize the oxidation products. However, to monitor over time these rather fast reactions, the analyses were conducted by FIA, which allowed the determination of the components in the reaction mixtures with higher frequency and in much shorter times. The rate of ESH oxidation follow the order hypochlorite > peroxynitrite > H_2O_2 + myoglobin > H_2O_2 (**Figure 3, panel A**). When ESH oxidation was conducted with hypochlorite, a more relevant formation of ESO_3H was observed (**Figure 3, panel D**). Indeed, some formation of ESO_3H was also observed when ESH was oxidized by peroxynitrite and H_2O_2 but at significantly lower levels than oxidation by hypochlorite. The mass spectrometric characterization of ESO_3H is reported in **Figure 4, panel D and E**.

Oxidation of ESSE

In the light of the above results showing that ESSE was always formed during ESH oxidation, we sought to evaluate oxidative behaviour of ESSE in the presence of the same oxidants.

Each ESSE oxidation reaction started with the addition of the due amount of ESSE to the oxidant solution at physiological pH. At certain reaction times, identification and characterization of the oxidation products were performed by HPLC-ESI-MS/MS analyses of each reaction mixture.

In order to follow over time the oxidation reactions of ESSE, which is oxidized more rapidly than ESH, the analyses of the reaction mixtures were performed by FIA. Results show that the rates of ESSE oxidation follow the order hypochlorite > peroxynitrite > H_2O_2 (**Figure 3, panel E**). The rates of the product formations in the reaction mixtures are reported in **Figure 3** (panels F, G, H). Similarly to ESH, the main product of all ESSE oxidation reactions was EH. Levels of ESO_3H were more consistent only when hypochlorite was employed as an oxidant.

Decomposition of ESSE in the absence of oxidants

ESSE was reported to have an uncommon behaviour compared to alkylthioldisulfides (23). Specifically, ESSE, which is stable in very acidic aqueous solution, slowly decomposes at increasing pH, partially reforming ESH along with an unidentified compound (23). In this respect, we studied over time the decomposition of ESSE dissolved in 1 mM sodium phosphate buffer at pH 7.4 (**Figure 5**). After 36 h, ESSE was no longer detectable in the solution and the concentrations of its decomposition products were identified and quantified by HPLC-ESI-MS/MS. We found that the products of the hydrolytic decomposition of ESSE are ESH and EH, the latter very likely corresponding to the unknown compound reported by Heath and Tonnies (23). The quantitative analyses, reported as mean±STD of four determinations, each in duplicate, on four identical ESSE solutions (93 μM) were the following: [ESH] = 137.7+/-12.5 (μM) and [EH] = 45.2+/-6.3 (μM). The molar ratio [ESH]/[EH] resulted 3.05, thus indicating that 3 moles of ESH and 1 mole of EH are produced from decomposition of 2 moles of ESSE.

Detection of sulfate ion as a reaction product of ESH with hydrogen peroxide

The desulphurization of ESH with the consequent formation of EH was observed in the course of all the oxidation reactions performed. Therefore, we undertook additional experiments, using only hydrogen peroxide as an oxidant, in order to ascertain the fate of sulphur atoms. The experiments, in order to avoid interference from phosphate buffer, were conducted in water as a solvent by

mixing ESH and hydrogen peroxide in the molar ratio of about 1:6 and reacting for 18 h. After the addition to the reaction mixture of a barium chloride solution, a white precipitate soon formed, whose identity as barium sulfate was confirmed by its insolubility in 5 M HCl. Conversely, two blank solutions of ESH and H_2O_2 in water, at the same concentrations as in the reaction mixture, did not give any precipitate when treated in the same manner with the barium chloride solution.

Discussion

In this study we reveal the unique redox behaviour, so far overlooked, that makes ESH different from alkylthiols, shedding a new light on the chemical antioxidant mechanism of ESH in the cell. To date, the antioxidant action of ESH has been described with reaction mechanisms considered, more or less explicitly, to function in cyclic manner: ESH is believed to be first oxidized to disulfide [19], or to a mixed disulfide with another thiol [26], and subsequently regenerated by the disulfide reduction, similarly to what is known for alkylthiols. As demonstrated in this study, in the presence of oxidants, ESH can form the disulfide according to the usual pattern, but this disulfide (ESSE) can behave very differently from alkylthiol disulfides, in that, being unstable at physiological pH, it undergoes a progressive decomposition by disproportion. Our data show that, from two moles of ESSE, three moles of ESH and one mole of EH are formed. Notably, the partial ESH regeneration from ESSE does not require reducing substances, being the process a disproportion. The possible mechanism accounting for that stoichiometry is reported in **Figure 6**.

The pathway starts with the hydrolysis of ESSE (Figure 6, reaction a) into ESH and the highly reactive sulfenic acid (ESOH) which then disproportionates into ESO_2H and EH [27] (Figure 6, reaction b). Then, ESO_2H decomposes into EH + H_2SO_3 (Figure 6, reaction c). The global reaction accounts for the observation that from the decomposition of two moles of ESSE, three moles of ESH and one mole of EH are formed (Figure 6, reaction d). The whole process is driven toward the end products by the irreversible decomposition of the sulfinic acid, ESO_2H (Figure 6, reaction c).

It is well known, in fact, that the oxidative desulfurization of 2-imidazolethiones is to be ascribed to the formation of 2-imidazolesulfinc acids, highly instable intermediates, which rapidly and

irreversibly decompose into the corresponding imidazoles and sulfurous acid [28]. On the contrary, it is important to point out that 2-imidazole sulfonic acids are stable toward hydrolysis [28].

From the overall mechanism, it clearly emerges the great advantage of ESH as compared to alkylthiols from a biological point of view. In fact, ESH can be partially reformed from the disulfide ESSE, without the need of reducing agents. The reaction produces also sulfurous acid, an other effective reducing agent, which could counteract the cell oxidative stress by further conversion into sulfate. This oxidative behaviour also makes ESH a rich source of reducing equivalents as the oxidation of ESH to EH and sulfate involves the irreversible transfer to intracellular oxidants of six electrons *per* each ESH molecule. This is at variance with the classical oxidation of alkylthiols into their respective alkyldisulfides, where only one electron *per* thiol molecule is transferred to the oxidant and cellular reducing equivalents are required to regenerate alkylthiols.

As for the pathway(s) of ESH oxidation in the presence of oxidants, in our experimental conditions, ESSE was always observed to form, more or less rapidly, and then gradually disappear. The main product of the oxidation reactions was EH in all cases (**Figure 3, panel C**). When hypochlorite was used, ESO_3H was observed at the highest levels (**Figure 3, panel D**).

It is likely that ESH oxidation entails the preliminary formation of ESOH, as for other alkyl and aryl thiols [27]. Successively, ESOH could form disulfide by reacting with ESH according to the reversible reaction [27]: ESOH + ESH = ESSE + H_2O. When the oxidant is hypochlorite, the disulfide is likely formed through the preliminary formation of the sulfenyl chloride intermediate ESCl [27, 29], according to the reaction ESCl + ESH = ESSE + H^+ + Cl^-. In the presence of excess of oxidants, ESSE could not only disproportionate but be further transformed into more oxidized species, such as disulfide S-monoxide (ES(O)SE) and disulfide S-dioxide ($ES(O)_2SE$) (**Figure 7**, **left side**), which can be seen as the thiosulfinate and the thiosulfonate derivatives of ESH.

Our inability to observe these derivatives directly could be due to their instability inasmuch as the higher electrophylicity of the sulphur atom bound to oxygen makes their decomposition in water much faster than that of ESSE. It is well known, in fact, that thiosulfinates and thiosulfonates are easily hydrolyzed to sulfenic and sulfonic acid [27]. These reactions closely resembles the proposed

hydrolytic decomposition of ESSE. Obviously, only one molecule of ESH and one of EH and sulfurous acid are formed from the hydrolysis of one molecule of ES(O)SE. While one molecule of ESH and one of ESO_3H are formed when one molecule of $ES(O)_2SE$ is hydrolyzed (**Figure 7, left side**).

However, we cannot rule out that, depending on the strength and concentration of the oxidants, ESH could be, at least in part, also sequentially oxidized to ESOH, ESO_2H and ESO_3H (**Figure 7**, right side), without passing through the formation of disulfide and its oxidation products. Anyway, no matter how ESO_2H, the key intermediate of both pathways, is produced, its irreversible decomposition leads to the formation of EH and sulfurous acid in both cases (**Figure 7**).

Our findings that ESH can be oxidized through the proposed oxidative route give reason of the observation, so far unexplained, that, while being not metabolized to any notable extent in mammalian tissues [30, 31], ESH is excreted by humans as EH and free sulfate [32,33], which are the ultimate products of the oxidative pathway described for the first time in this paper. While these studies [30-33] give no explanation of how EH and sulfate are formed, their conclusions strongly reinforce our hypothesis that excreted sulphate and EH derive from the proposed oxidative degradation of ESH.

In this regard, the remarkable lower affinity of the ESH transporter for EH [34] could suggest an evolutionary purpose according to which cells possessing the transporter can avidly accumulate ESH, while its oxidative by-product EH can be easily excreted.

Conclusions

Our studies show peculiar antioxidant properties of ESH which make this compound a cell adjuvant of noticeable importance, mainly because of its capacity to be irreversibly oxidized by relevant oxidants occurring in cells. In particular, we report for the first time that the oxidation of ESH by those oxidants produces mainly EH and variable amounts, depending on the oxidant strength, of ESO_3H, a compound never described before. It is worth to point out that, being available in the diet, ESH represents a free and rich source of reducing equivalents that, through the herein described

redox mechanism, could be slowly and continuously utilized by cells in basal conditions and ensure additional protection during extreme oxidative stress.

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Competing financial interests

The authors declare no competing financial interests.

Legends to figures

Figure 1. Chemical structures. Ergothioneine (ESH), hercynine (EH), ergothioneine sulfenic acid (ESOH), ergothioneine sulfinic acid (ESO_2H), ergothioneine sulfonic acid (ESO_3H), ergothioneine disulfide (ESSE), ergothioneine disulfide S-monoxide (ES(O)SE), ergothioneine disulfide S-dioxide $ES(O)_2SE$. The equilibrium between the thiol-thione forms of ergothioneine is indicated.

Figure 2. HPLC-ESI-MS/MS analyses of ESH oxidation by stimulated neutrophils. The chromatographic conditions and the stimulation of human neutrophil by phorbol-myristate-acetate (PMA) in the presence of ergothioneine are described in the experimental section. 10 µl of incubation medium was analyzed immediately after PMA addition (t = 0 min), and then at 15 min and 30 min. The extracted ion chromatograms (EIC) at the indicated MS/MS transitions are reported. The fragmentation patterns of the peaks corresponding to *m/z* 198.1 and *m/z* 278.1 are the same as those reported in panel B and panel D of Figure 4.

Figure 3. Time course oxidations of ESH and ESSE. Upper panel. FIA analyses of ESH oxidation with the oxidants hypochlorite (●); peroxynitrite (○); hydrogen peroxide in the presence of myoglobin (▲); hydrogen peroxide (Δ). All the reaction mixtures contained 100 µM ergothioneine and 100 µM of the oxidant, in phosphate buffer 1 mM pH 7.4. The concentration of myoglobin, when present, was 50 µM. The reactions started with the addition of the oxidant solution. At set time intervals, 5 µl of the reaction mixture was injected in a stream of 0.1% formic acid in water at flow rate of 100 µl/min. FIA analyses were performed in full scan in the range 190-290 amu. The data are reported as the peak area by extracting at *m/z* 198.1, for EH; at *m/z* 229.1 for ESSE; at *m/z* 230.1 for ESH; at *m/z* 278.1 for ESO_3H. **Panel A**. Time courses of ESH consumption. **Panel B**. Time courses of ESSE production. **Panel C.** Time courses of EH production. **Panel D**. Time courses of ESO_3H production. **Lower panel.** FIA analyses of ESSE oxidation with the oxidants hypochlorite (●); peroxynitrite (○); hydrogen peroxide (Δ). All the reaction mixtures

contained 93 μM ESSE and 93 μM of the oxidant, in phosphate buffer 1 mM pH 7.4. The reactions started with the addition of the oxidant solution. At set time intervals, 5 μl of the reaction mixture was injected in a stream of 0.1% formic acid in water at flow rate of 100 μl/min. FIA analyses were performed in full scan in the range 190-290 amu. The data are reported as the peak area by extracting at *m/z* 198.1, for EH; at *m/z* 229.1 for ESSE; at *m/z* 230.1 for ESH; at *m/z* 278.1 for ESO_3H. **Panel E**. Time courses of ESSE decrease**.** **Panel F**. Time courses of ESH production. **Panel G**. Time courses of EH. **Panel H**. Time courses of ESO_3H production.

Figure 4. LC-ESI-MS/MS chromatogram of a reacting mixture containing ESH and hypochlorite. Panel A. Chromatography of an aliquot of the reaction mixture containing initially ESH and hypochlorite at the same concentrations, after 10 min of reaction. The initial composition of the reaction mixture and the chromatographic conditions are reported in the experimental section. Synthesized EH-d9 was added to the reaction mixture as an internal standard. The extracted ion chromatograms (EIC) at the indicated MS/MS transitions are reported. **Panel B.** The upper part shows the MS/MS fragmentation pattern of EH, produced by the oxidation reaction, by isolating at *m/z* 198.1. The lower part shows the MS/MS fragmentation pattern of EH-d_9, added as an internal standard to the reaction mixture, by isolating at *m/z* 207.1. **Panel C**. The upper part shows the MS/MS fragmentation pattern of ergothioneine disulfide (ESSE) produced by the oxidation reaction, isolated as mono-charged positive ion at *m/z* 457.2. The lower part shows the MS/MS fragmentation pattern of ESSE isolated as doubly charged positive ion at *m/z* 229.1. **Panel D.** MS/MS fragmentation pattern of the oxidation product of ESH isolated at m/z 278.1. The MS/MS fragments clearly indicate the identity of this compounds as the sulfonate derivative of ESH. **Panel E.** MS/MS fragmentation pattern of the oxidation product of ESH isolated at m/z 280.1, corresponding to the sulfonate containing ^{34}S. It can be observed that, excepting the fragments containing the sulphur atom, all other fragments are the same as those of the compound isolated at *m/z* 278.1.

Figure 5. Time course of ESSE decomposition at neutral pH. The HPLC-ESI-MS/MS analyses of a solution of ESSE in 1 mM buffer phosphate at pH 7.4. The initial concentration of ESSE was 93 μM. At the times of 0, 5, 10, and 15 h are reported, 10 μL of the solution were injected. The extracted ion chromatograms (EIC) at the indicated MS/MS transitions are reported.

Figure 6. Pathway of ESSE decomposition at neutral pH. The pathway starts with the hydrolysis of ESSE (reaction a) into ESH and the highly reactive sulfenic acid (ESOH) which then disproportionates into ESO_2H and EH (reaction b). Then, ESO_2H decomposes into EH + H_2SO_3 (reaction c). The global reaction accounts for the observation that from the decomposition of two moles of ESSE, three moles of ESH and one mole of EH are formed (reaction d).

Figure 7. Oxidative patterns of ESH. Left side. ESH is first oxidized into the disulfide (ESSE); ESSE is oxidized into S-monoxide (ES(O)SE) and, then, ES(O)SE into S-dioxide ($ES(O)_2SE$). The hydrolytic decompositions of ESSE, ES(O)SE and $ES(O)_2SE$ have in common the partial formation of ESH which can restart a new oxidative cycle. Moreover, the hydrolytic decomposition of $ES(O)_2SE$ generates the stable ESO_3H, whereas the decomposition of ES(O)SE generates the highly instable thiosulfinate, ESO_2H, which rapidly and irreversibly decomposes into hercynine (EH) and sulfurous acid. Instead, the hydrolytic decomposition of ESSE produces ESOH which disproportionate into an additional ESH molecule and ESO_2H which, as seen above, decomposes into hercynine (EH) and sulfurous acid. **Right side.** ESH is first oxidized into the sulfinic acid, ESOH, which is successively oxidized into the sulfenic acid, ESO_2H and, then, this into the sulfonic acid ESO_3H. The fate of these three compounds is the same as described above.

Highlights

1) Ergothioneine (ESH) is oxidized by cell oxidants differently from alkylthiols

2) Upon oxidation, ESH mainly produces hercynine and sulfurous acid.

3) Upon oxidation with hypochlorite, ESH forms its sulfonated derivative

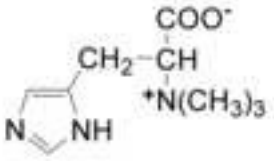
4) The sulfonated derivative of ESH is produced by stimulated human neutrophils

5) Its peculiar oxidative behavior makes ESH a rich source of reducing equivalents

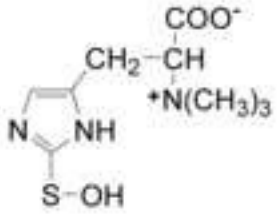
Figure 1

Thiol -Thione Tautomerism of Ergothioneine **(ESH)**

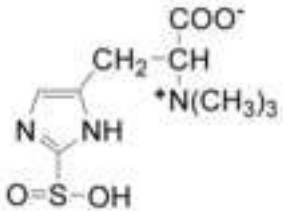
Hercynine (N,N,N-trimethylhistidine) **(EH)**



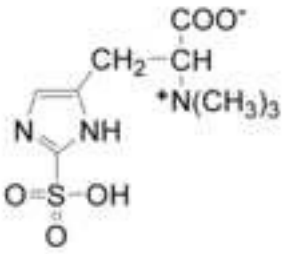
Ergothioneine sulfenic acid **(ESOH)**



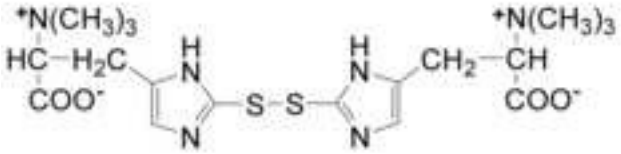
Ergothioneine sulfinic acid **(ESO_2H)**



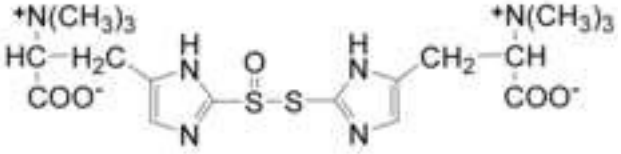
Ergothioneine sulfonic acid **(ESO_3H)**



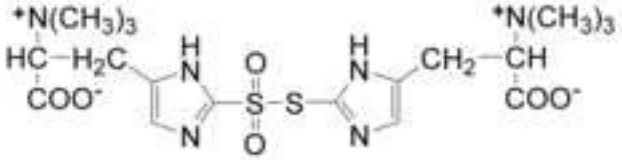
Ergothioneine disulfide **(ESSE)**



Ergothioneine disulfide S-monoxide **(ES(O)SE)**



Ergothioneine disulfide S-dioxide **($ES(O)_2SE$)**



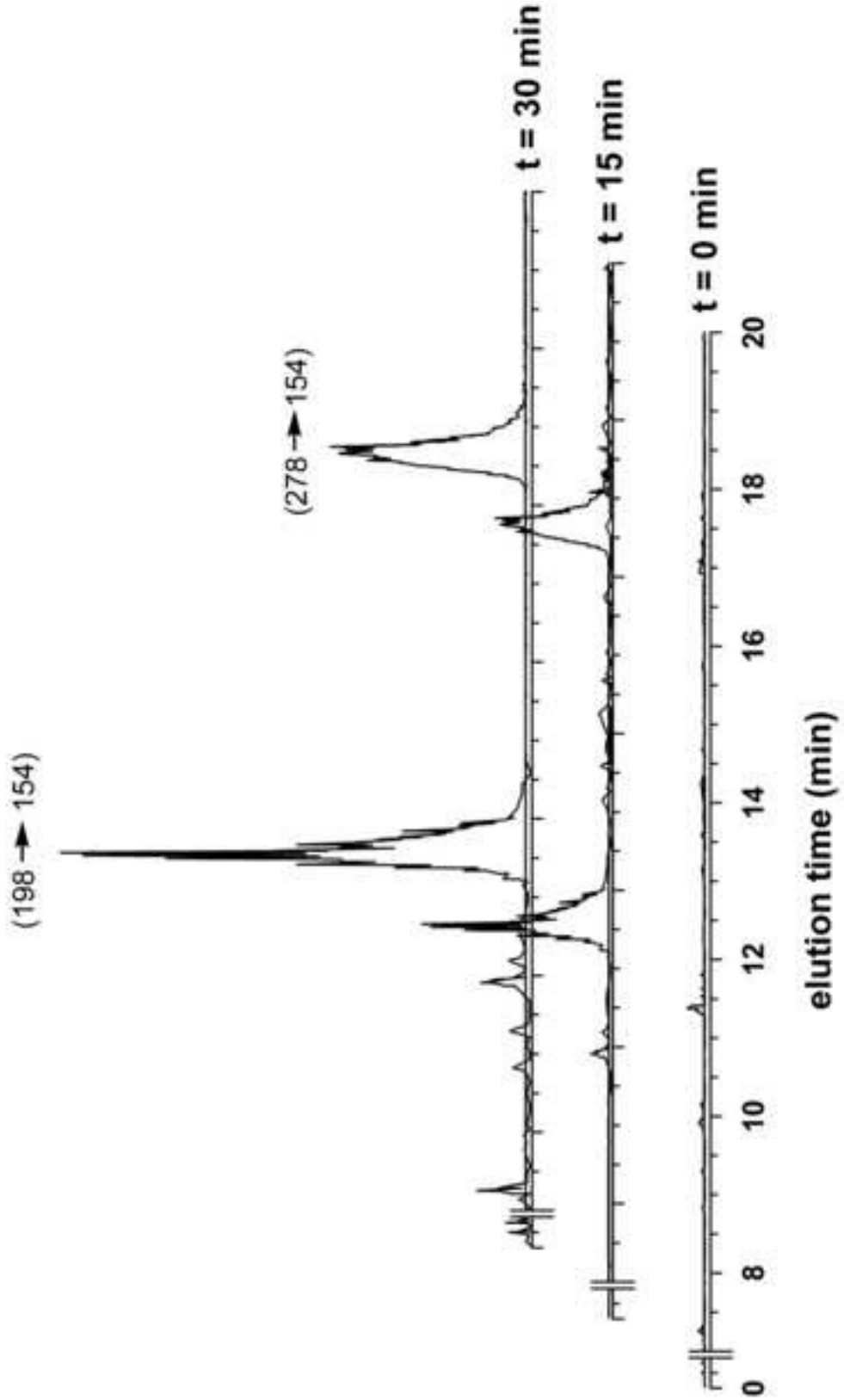

(198 → 154)
(278 → 154)
t = 30 min
t = 15 min
t = 0 min
0
8
10
12
14
16
18
20
elution time (min)

Figure 2

Figure 3

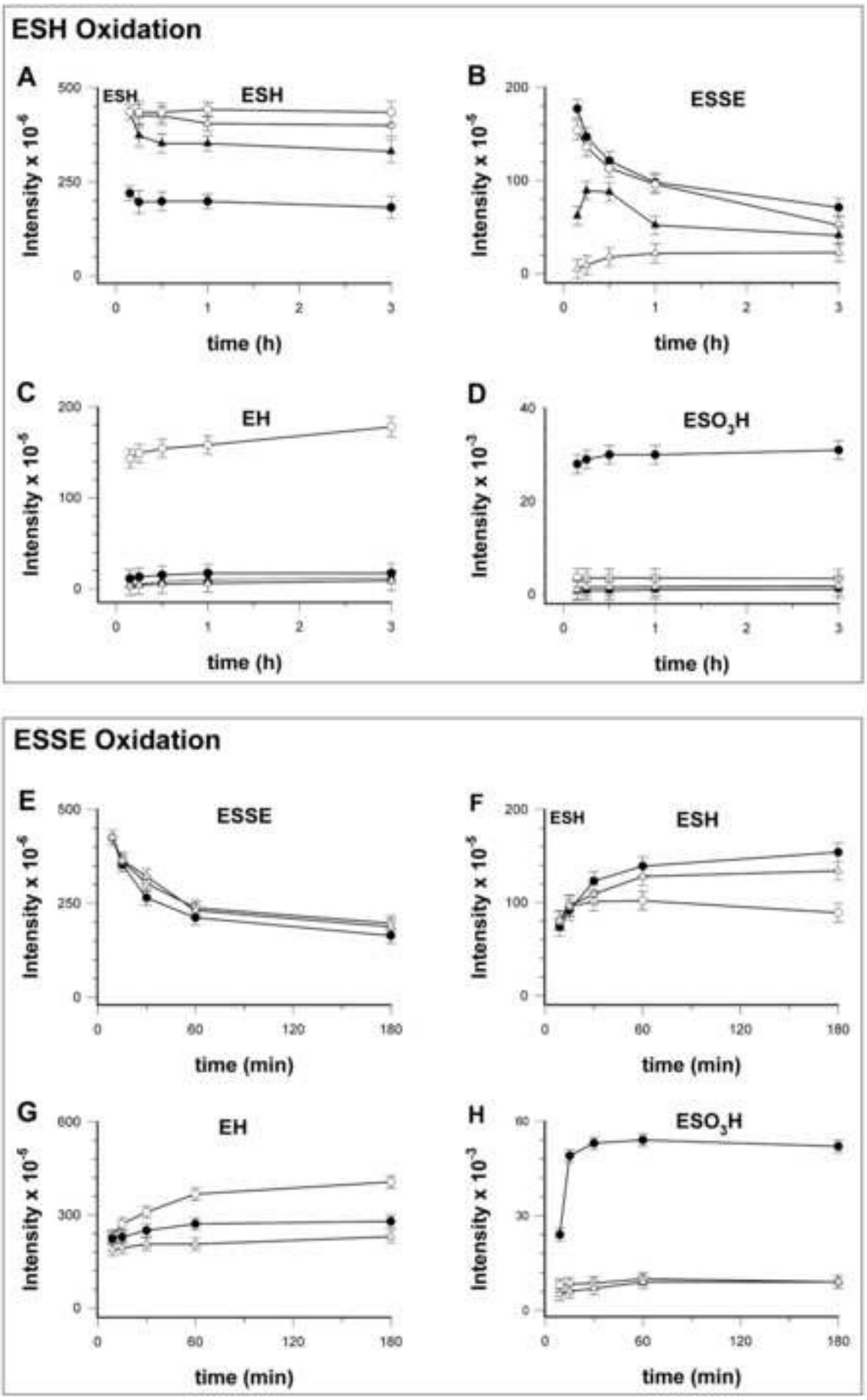
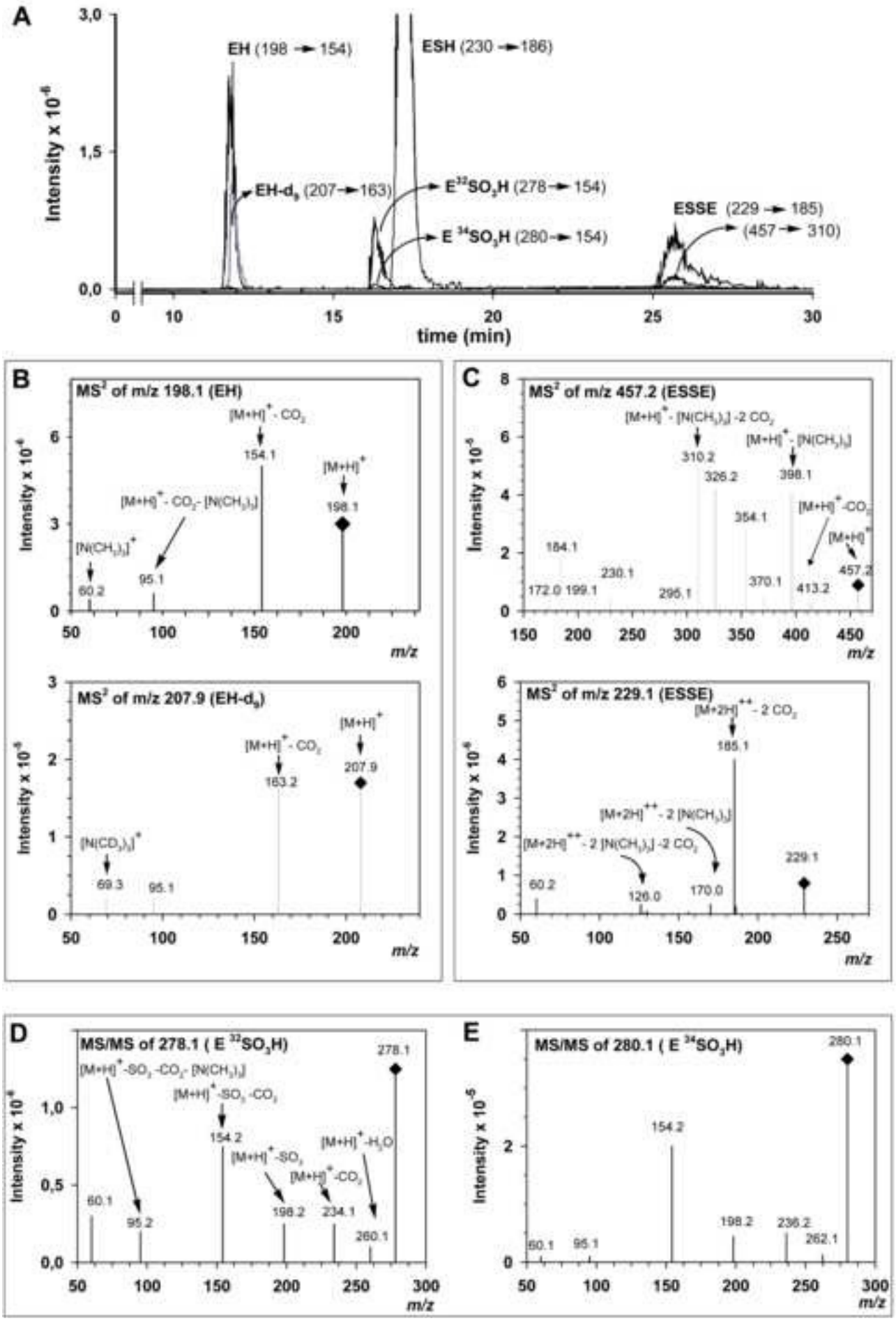


Figure 4



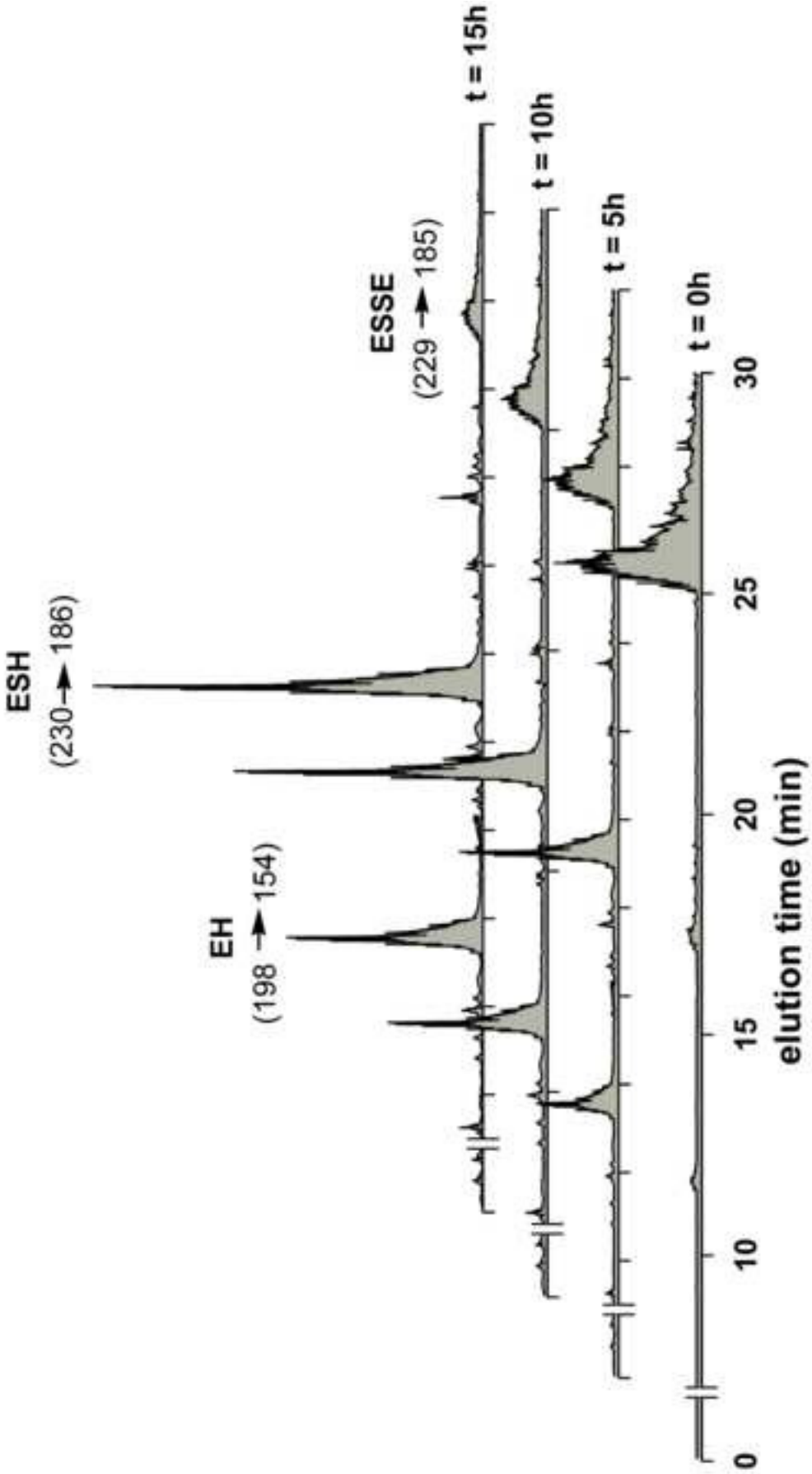
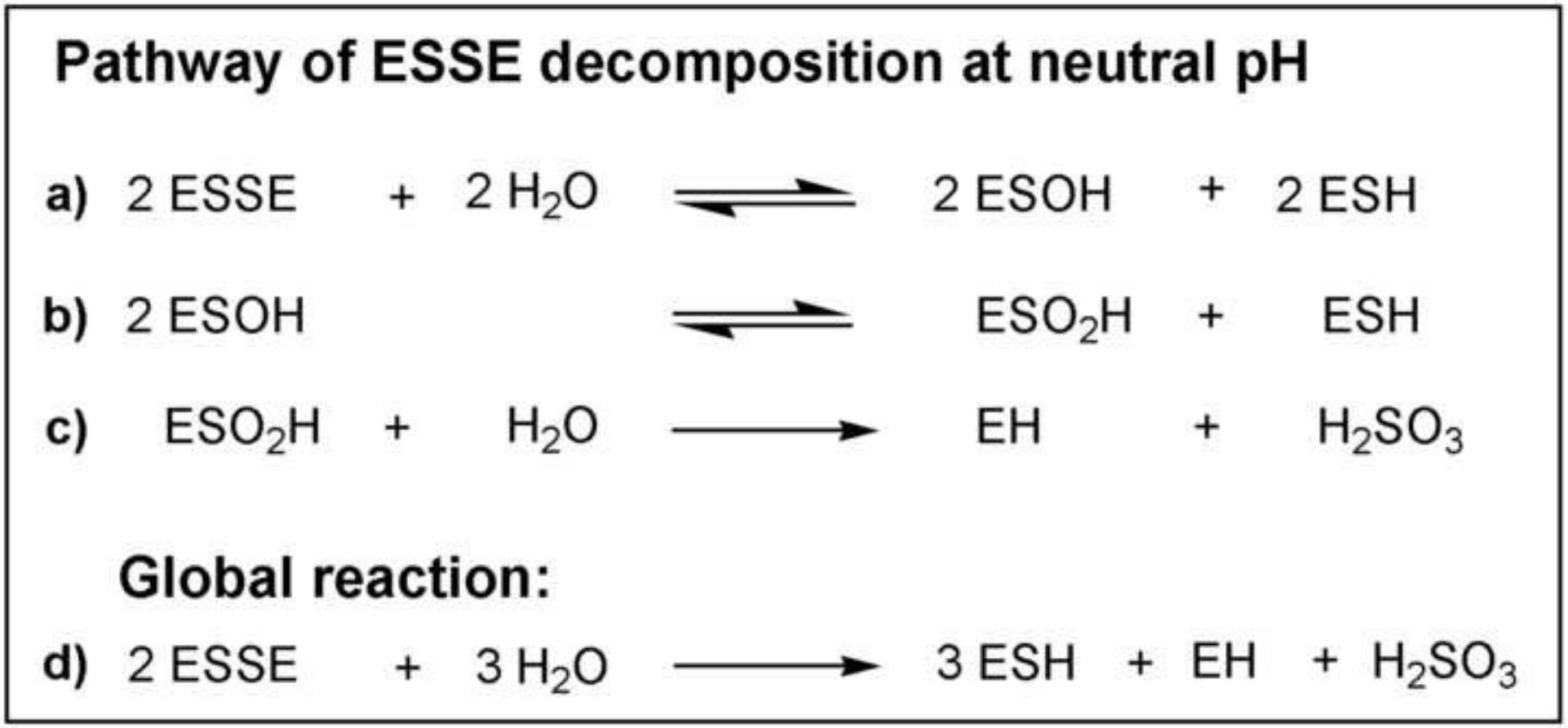

ESH
(230→186)
EH
(198→154)
ESSE
(229→185)
t = 15h
t = 10h
t = 5h
t = 0h
0
10
15
20
25
30
elution time (min)

Figure 5

Figure 6



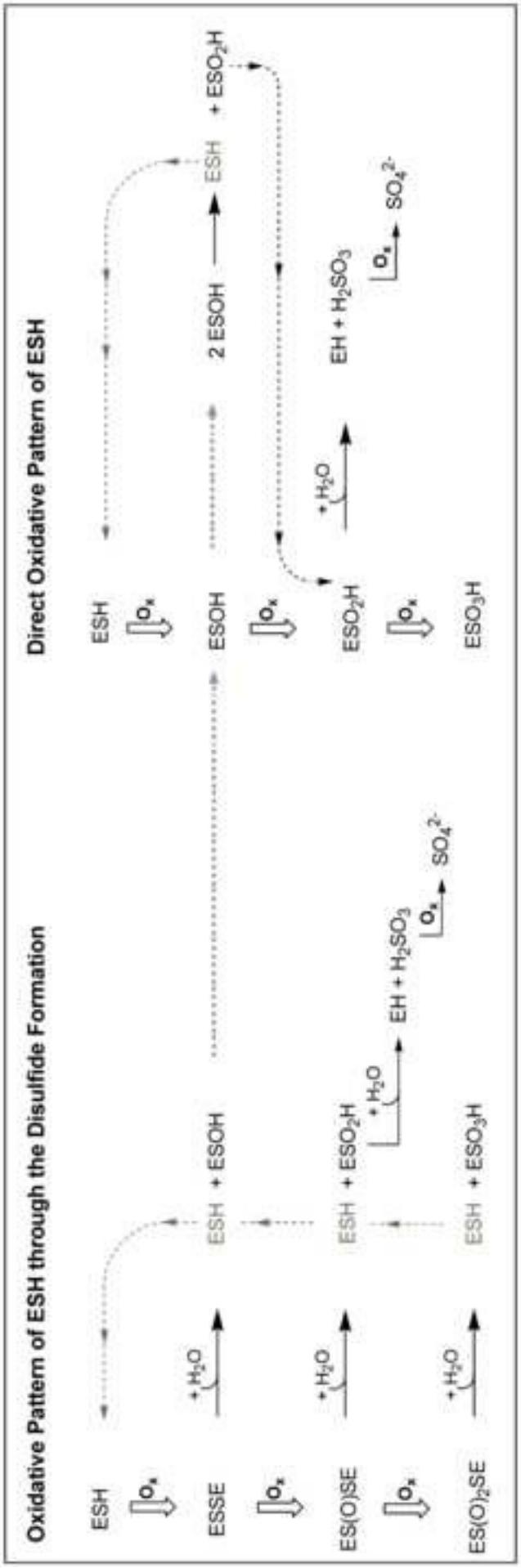

Oxidative Pattern of ESH through the Disulfide Formation
Direct Oxidative Pattern of ESH
ESH
ESSE
ES(O)SE
ES(O)2SE
ESOH
ESO2H
ESO3H
2 ESOH
EH + H2SO3
SO4 2-
+ H2O
Ox

Figure 7