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Analysis of Ergothioneine Using Surface-Enhanced Raman Scattering: Detection in Mushrooms

Federico Puliga [1,*,†], Veronica Zuffi [1,†], Alessandra Zambonelli [1], Pavol Miškovský [2], Ornella Francioso [1] and Santiago Sanchez-Cortes [3]

[1] Department of Agricultural and Food Sciences, University of Bologna, Viale G. Fanin 40-50, 40127 Bologna, Italy; veronica.zuffi3@unibo.it (V.Z.); alessandr.zambonelli@unibo.it (A.Z.); ornella.francioso@unibo.it (O.F.)
[2] SAFTRA Photonics s.r.o., Moldavská cesta 51, 040 11 Košice, Slovakia; pavol.miskovsky@saftra-photonics.org
[3] Instituto de Estructura de la Materia, Consejo Superior de Investigaciones Cientificas (IEM-CSIC), Serrano, 121, E-28006 Madrid, Spain; s.sanchez.cortes@csic.es
* Correspondence: federico.puliga2@unibo.it
† These authors contributed equally to this work.

Abstract: Surface-enhanced Raman scattering (SERS) spectroscopy is a straightforward analytical technique capable of providing detailed information about metabolites in biological samples. The objective of this study was to perform a SERS analysis of ergothioneine (EGT), an amino acid synthesized by microbes and fungi, across a range of pH values (acidic to alkaline) and concentrations (2×10^{-5} M to 2×10^{-7} M), to understand the dynamic interactions between EGT and silver (Ag) nanoparticles. Furthermore, SERS was applied in situ on mushroom fruiting bodies to detect the presence of EGT. The SERS spectra revealed that the interaction of EGT with Ag nanoparticles underwent significant alterations at varying pH levels, primarily due to isomerization. These changes were associated with modifications in the aromaticity and ionization of the imidazole ring, driven by both metal adsorption and alkaline conditions. Our results indicated the formation of distinct tautomeric forms of the imidazole group, namely the thione and thiol forms, in aqueous solution and on the Ag surface, respectively. Furthermore, the EGT spectra at different concentrations suggested that ionization occurred at lower concentrations. Notably, the SERS spectra of the mushroom fruiting bodies were dominated by prominent bands attributable to EGT, as corroborated by the comparison with the EGT fungal extract and EGT standard. These findings underscore the utility of SERS spectroscopy as a rapid and effective tool for obtaining comprehensive molecular fingerprints, even directly from complex biological matrices such as mushroom fruiting bodies.

Keywords: imidazole ring; tautomerization; nanoparticles; Raman; pH effect; complexation; mushroom tissue

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1. Introduction

Ergothioneine (EGT) is a naturally occurring non-proteinogenic hydrophilic amino acid, first isolated from *Claviceps purpurea* (Fr.) Tul. (ergot) in 1909 [1]. Chemically, EGT is defined by its IUPAC name, (2S)-3-(2-thioxo-2,3-dihydro-1H-imidazol-4-yl)-2-(trimethylammonio)propanoate [2]. EGT is a derivative of histidine, characterized by the presence of a sulfur atom attached to the imidazole ring, which exists in a tautomeric equilibrium between its thiol and thione forms. Unlike common thiols, such as glutathione, EGT predominantly adopts the thione form at physiological pH, which contributes to its remarkable stability and resistance to autooxidation [3]. Furthermore, it does not facilitate

the generation of hydroxyl radicals from H_2O_2 and Fe^{2+} ions [4]. These properties render EGT a highly effective antioxidant and cytoprotectant, with notable metal-chelating capabilities [2,5,6]. For these reasons EGT has garnered significant attention for medicinal, supplemental and cosmetic applications, leading to market expansion from an estimated value of USD 15 million in 2020 to projected growth of USD 125 million by 2027 [7].

EGT is a secondary metabolite that is naturally synthesized by certain bacterial species, fungi and actinobacteria. In animals and humans, EGT can be acquired through the diet, mainly through mushrooms and fermented food, via the pH-dependent activity of a membrane transporter [8]. Paul et al. (2010) [6] demonstrated that EGT is transferred via specific carriers to organelles that are particularly susceptible to oxidative stress, such as mitochondria. In plants, EGT can be acquired through the roots from soil fungi or bacteria as part of a mycorrhizal symbiosis [9,10]. Therefore, fungi represent a notable source of EGT, although its precise location within the organism remains under investigation. In some species, EGT is found in high concentrations in both asexual and sexual reproductive structures, such as conidia and basidiocarps, where it plays a crucial role due to its antioxidant properties [11]. Furthermore, some studies have shown that EGT concentrations in mushroom significantly vary among species [12]. For example, *Pleurotus* spp., *Boletus* spp. and *Lentinula* spp. appear to have the highest EGT contents [13,14].

It is evident that research into the EGT molecular structure in mushroom fruiting bodies is lacking, despite its nutraceutical properties, including antioxidant, anti-inflammatory and antiaging effects [3,15,16], and its application in food being well documented [17]. In order to address this issue, it is crucial to develop robust, fast and reliable analytical techniques for determining EGT in situ. It is also of great importance to ensure that such techniques are able to prevent any possible interference from other co-existing fungal constituents. To date, studies have been published in which various techniques, including spectrophotometry [18], high-performance liquid chromatography [19,20], liquid chromatography-mass spectrometry [12,13] and other chromatographic methods have been used to detect EGT [20]. However, none of these studies have evaluated the possibility of detecting this molecule directly in situ. Moreover, the current techniques necessitate laborious and time-consuming extraction and purification processes.

Surface-enhanced Raman scattering (SERS) is a powerful tool for identifying molecular species by collecting molecular spectral signals at the single molecule level. This technology has achieved substantial development in a wide range of scientific disciplines, including environmental science, medical and biological analyses and food safety [21–23]. SERS is a technique that exploits the significant enhancement of the electromagnetic field (10^4–10^8) provided by localized surface plasmon resonances (LSPRs) in metal nanoparticles (NPs) [24–28]. Consequently, molecular sensitivity can be adapted to the attomolar range, and single-molecule sensitivity can be achieved. In the domain of biomolecules, SERS has garnered significant attention with regard to their detection [29,30]. This has facilitated the acquisition of structural information in a variety of biological matrices and environments. Among the influencing factors, pH, temperature and the concentration of analytes are considered to influence the surface adsorption of target molecules and, consequently, their spectral fingerprint [27]. In the context of the identification of EGT, SERS has been employed by Fornasaro et al. [31,32] in biofluids, including lysates or red blood cells. More recently, Zuffi et al. (2023) [33] took advantage of SERS analysis in *Pleurotus* mushrooms to identify EGT. The SERS analysis provided a unique spectral fingerprint that allowed EGT to be distinguished from other compounds present in the mushroom. The rapid, non-destructive nature of SERS, combined with its potential for portable applications, makes it a promising tool for precise and efficient EGT detection [34].

The aim of the present study was to achieve further insight into the interaction mechanism of EGT with the surface of metal nanoparticles and to investigate the effects of pH and EGT concentration on this interaction. Both parameters are crucial for elucidating the dynamic behavior of EGT in SERS analysis. In addition, this study investigated the in situ presence of EGT in fruiting bodies of different mushroom species, including *Pleurotus cornucopiae* (Paulet) Quél. (PCO), *Pleutorus eryngii* (DC.) Quél. (PER) and *Pleurotus ostreatus* (Jacq.) P. Kumm. (POS) and finally corroborated the presence of EGT after extraction from fruiting bodies.

2. Materials and Methods

2.1. Chemicals

Ergothioneine, silver nitrate, hydroxylamine hydrochloride, hydrochloric acid and sodium hydroxide, all of analytical grade, were obtained from Sigma-Aldrich (Darmstadt, Germany). All solutions were freshly prepared using Milli-Q® ultrapure distilled water (Merck KGaA, Darmstadt, Germany) prior to the experiments and were used immediately.

2.2. Extraction of EGT from POS Fruiting Bodies

First, 5 g of dried fruiting bodies were ground and mixed with 40 mL of a 70% acetone solution [7,35]. The sample was stirred at 10,000 rpm for 4 min at 40 °C and subsequently, centrifuged at 5000× *g* for 15 min to collect the supernatant. The remaining precipitate was further stirred with 70% acetone solution and centrifuged under the same conditions. The supernatants from both centrifugation steps were combined and lyophilized for subsequent SERS analysis.

2.3. Nanoparticles Preparation and SERS Analyses

Prior to the preparation of silver nanoparticles (NPs), all glassware was thoroughly cleaned with aqua regia and rinsed with Milli-Q® ultrapure distilled water to eliminate any surface impurities. For the preparation of the silver colloid, solutions of silver nitrate (10^{-2} M) and hydroxylamine hydrochloride (1.67×10^{-3} M) containing 3.33×10^{-3} M sodium hydroxide were used, following the method described by Leopold and Lendl (2003) [36]. In brief, 10 mL of the silver nitrate solution was added dropwise to 90 mL of the hydroxylamine hydrochloride/sodium hydroxide solution under continuous stirring (300 rpm) at room temperature. The resulting colloidal solution was then stored at 4 °C in the dark and exhibited an extinction maximum at 406 nm and a mean diameter of about 50 nm (Figure S1).

To explore the effect of concentration, SERS analysis was performed using EGT solutions at concentrations of 2×10^{-5}, 2×10^{-6}, and 2×10^{-7} M. Furthermore, 1 mL of nanoparticles (NPs) adjusted to different pH values (3, 5, 6.5, 9, and 11) using 1N HCl or 1N NaOH was placed in a quartz cuvette, along with 5 µL of EGT (10^{-3} M).

In order to investigate the presence of this molecule in different fungal tissue, SERS analyses of PCO, PER and POS fruiting bodies as well as EGT extracted from POS basidiomata, and the corresponding POS residue after extraction (negative control, i.e., mushroom without EGT) were performed using the method described by Puliga et al. (2022) [37]. The measurements were carried out using a PickMol™ Ramascope spectrophotometer (SAFTRA Photonics, Košice, Slovakia) with a 785 nm laser line (laser power was set to 10 mW with an integration time of 2400 ms, resolution 2 cm^{-1}, and sensor temperature of 10 °C). In addition, the Raman Renishaw InVia equipment (Renishaw plc, Gloucestershire, UK) with laser excitations of 785 and 532 nm, a laser power of 2 mW, resolution of 0.3 cm^{-1} and detector operating temperature of −70 °C was used under macro conditions.

Three replicates were conducted for each pH value and biological sample. The spectra were collected using the PickMol™ and Renishaw WiRE 5.5 software and processed with OriginPro 2019 (OriginLab, Northampton, MA, USA).

2.4. Fourier Transform Infrared Spectroscopy (FTIR)

FT-IR spectra of the EGT standard and EGT mushroom extract, were recorded with an ALPHA II FTIR spectrometer (Bruker Optics, Ettlingen, Germany) equipped with an attenuated total reflectance (ATR) sampling device containing a diamond crystal. Spectra were collected from 4000 cm^{-1} to 400 cm^{-1}, with a spectral resolution of 4 cm^{-1}, with 128 scans averaged and summed. A background spectrum of the air under the same instrumental conditions was scanned before each series of measurements. All the attributions are reported in Figure S2.

3. Results and Discussion

The SERS spectra of EGT (Figure 1a, Table S1) exhibit prominent bands at 485 (ν_1), 1221 (ν_2), 1448 (ν_3) and 1590 (ν_4) cm^{-1} when excited at 785 nm. The ν_1 band can be attributed to ring structural vibrations [38], mainly δNCS and δNCN coupled with νC-S vibrations [39,40]. In addition, the ν_2 band is associated with ring stretching motions, with major contributions from νC-C and νC-N vibrations, while the ν_3 and ν_4 vibrations are associated with νC=C and νC=N vibrations in the imidazole (Imz) ring. Additional bands appearing at 1347 and 1297 cm^{-1} are attributed to νCN stretching vibrations in the imidazole (Imz) ring with a high double bond character [41]. In comparison to the Raman spectrum of the solid EGT (Figure 1b) the SERS spectrum exhibits strong changes indicating a deep structural change in EGT upon interaction with the metal. The normal Raman spectrum of the solid presents three main bands at 1213 (ν_2), 1503 (ν_3) and 1644 (ν_4) cm^{-1} which can be assigned to the νC-N, the imidazole ring stretching (ν(Imz)) and νC=C vibrations, respectively [42,43]. The FTIR spectrum of EGT (Figure S2) also shows a band at 1640 cm^{-1}, attributed to the νC=C vibration, but, in addition, a broad band at 1609 cm^{-1} can be attributed to the νCOO^- of the carboxylate and N-H bending of amino groups. Other prominent bands observed in the FT-IR spectrum appear in the 1300–1400 cm^{-1} interval corresponding to νC-N vibrations coupled to ring stretching and C-H bending [42].

When compared to the Raman spectrum of the solid, the ν_4-ν_2 and ν_3-ν_2 differences are significantly reduced from 431 to 369 cm^{-1}, in the case of ν_4-ν_2, and from 290 to 227 cm^{-1} for the ν_3-ν_2. The observed changes suggest an increase in the aromaticity of the Imz group due to its interaction with the metal. Therefore, we suggest that the interaction of EGT with the silver surface promotes a tautomerization of the Imz group from the thione form in the solid (Figure 2a) to the thiol form on the Ag surface (Figure 2b), as evidenced by the downward shift in the νC=C band from 1644 to 1590 cm^{-1}, associated with the interaction of the S atom with the metal surface. The absence of bands attributed to νS-H indicates that a subsequent ionization of EGT is also occurring during the interaction. The increase in aromaticity associated with the thione to thiol tautomer in EGT and the prominent band at 485 cm^{-1}, attributed to single bond νC-S coupled to ring NCS and NCN bending vibrations, also reinforces this assumption. The weakening of the νC=S vibration appearing at 984 cm^{-1} in the Raman spectrum of the solid (Figure 1b) also corroborates this tautomerization.

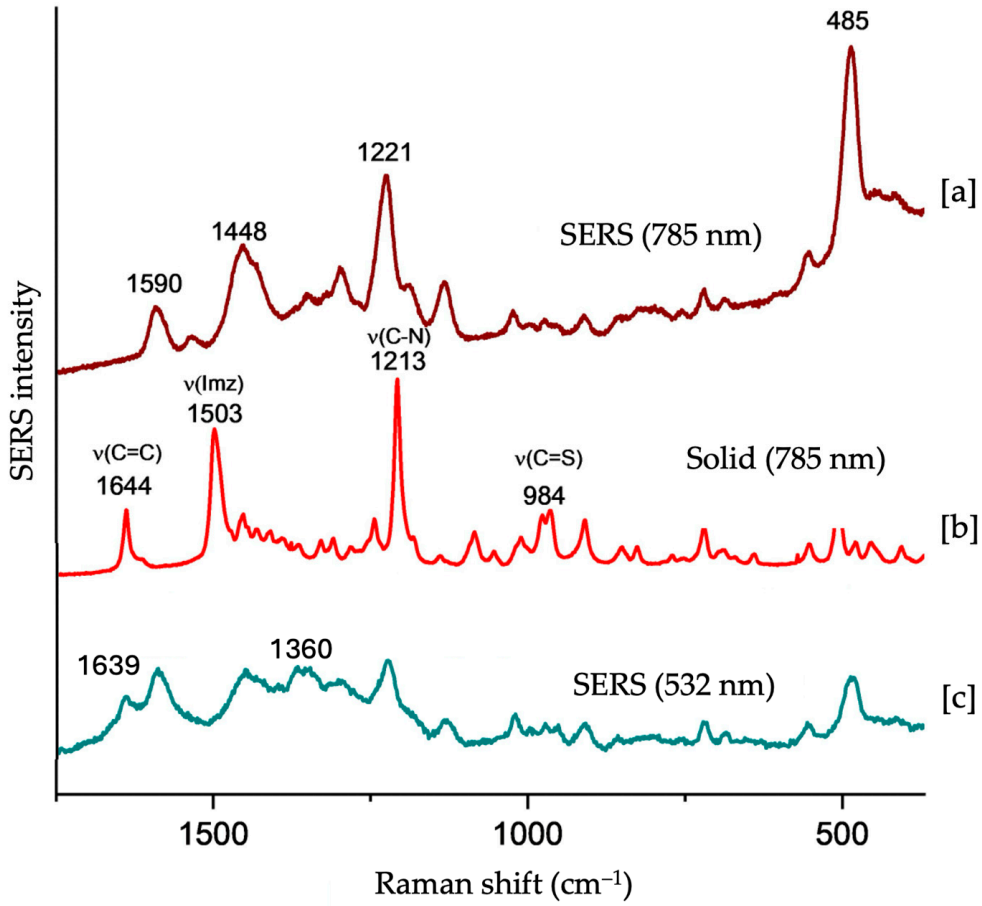

Figure 1. SERS spectra of EGT (2×10^{-6} M, pH 6.5) obtained at different excitation wavelengths: (**a**) 785 nm and (**c**) 532 nm. (**b**) Normal Raman spectrum of solid EGT excited at 785 nm.

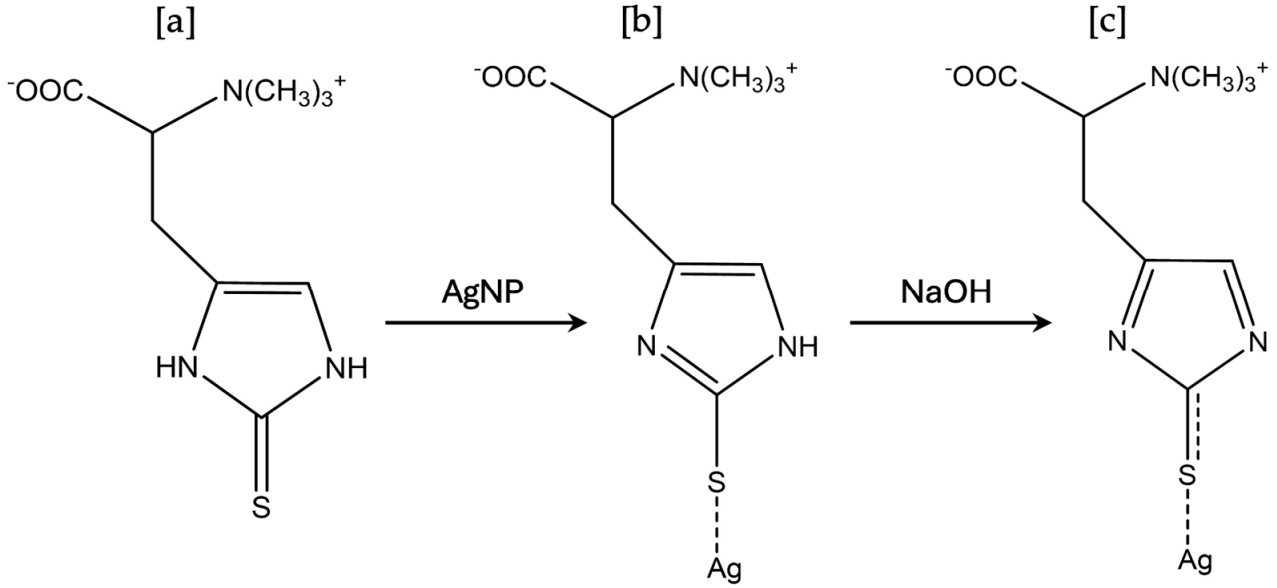

Figure 2. Different structures of the imidazole group of EGT: (**a**) 1,3-dihydro-2H-imidazole-2-thione structure in aqueous solution or solid state; (**b**) 1H-imidazole-2-thiolstructure on Ag surface at pH under 6.5; and (**c**) imidazole-2-thione structure on Ag surface and pH above 6.5.

This adsorption model implies a perpendicular orientation of the Imz ring on the metal surface, enhancing the in plane ν_1–ν_4 modes, in accordance with the SERS selection rules [25]. In addition, the strong intensification of the ν_1 band at 485 cm^{-1} is attributed to a direct interaction between EGT and Ag through the S atom.

Although the thiol form predominates on the Ag surface, evidence from the SERS spectrum obtained using 532 nm excitation suggests the coexistence of the thione form. The latter spectrum (Figure 1c) reveals the intensification of two bands at 1639 and 1360 cm^{-1}, characteristic of the thione tautomer, which could also be adsorbed on the surface.

SERS spectra at different pH are shown in Figure 3. As the pH rises, a second ionization of the Imz ring occurs (Figure 2c), further shifting the ν_4 band to 1534 cm^{-1}, corresponding to νC=N vibrations. In addition, a strong new band appears at 1293 cm^{-1} also due to the ionization of the Imz ring [44]. The ν_1 band only undergoes a marginal shift from 488 cm^{-1}, at pH 3, to 483 cm^{-1}, at pH 11. This is due to the fact that the δNCS, δNCN and νCS vibrations responsible for this band remain largely unaffected by either ionization or tautomerization, given their distance from the N-C=C-N moiety of the opposite part of the Imz ring. Furthermore, the firm adsorption of the sulfur atom on the Ag surface further stabilizes this region of the molecule.

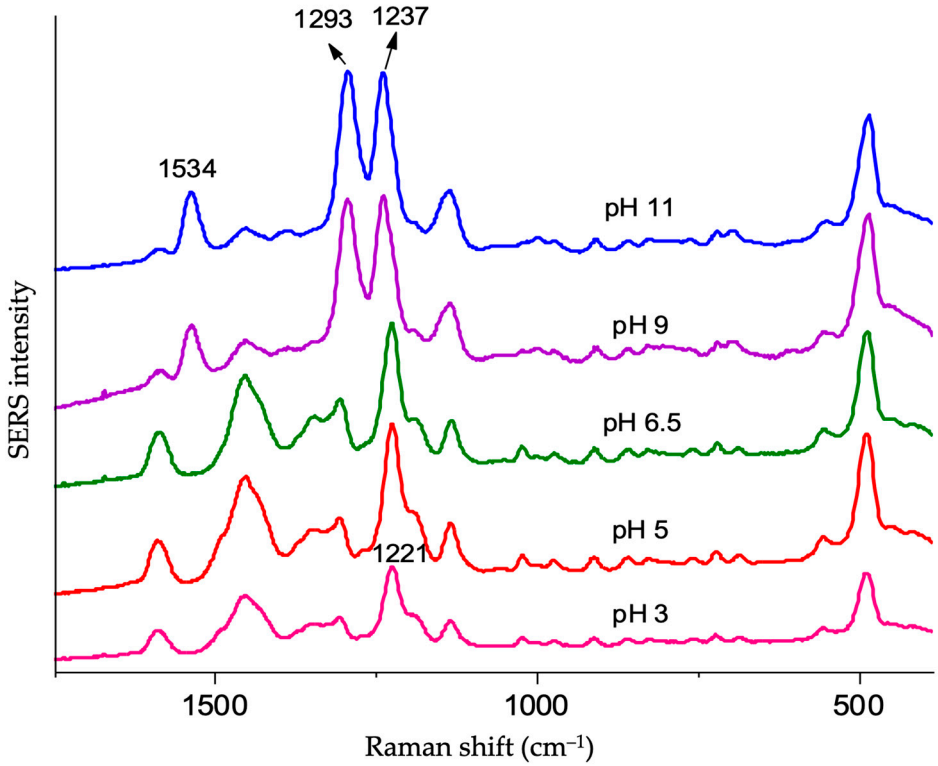

Figure 3. SERS spectra of EGT (5×10^{-6} M) recorded at different pH values. Laser excitation at 785 nm.

The EGT spectra at different concentrations are displayed in Figure 4. The observed increase in band intensity at 1535 and 1297 cm^{-1} when moving from a high concentration (2×10^{-5} M) to a lower concentration (2×10^{-6} and 2×10^{-7} M) points out a possible ionization of the EGT at lower concentrations. This is likely due to chemical changes at the metal interface. At the lowest analyzed concentration (2×10^{-7} M), the ν_4 band is shifted to 1575 cm^{-1}, attributed to incomplete coverage of the metal nanoparticles.

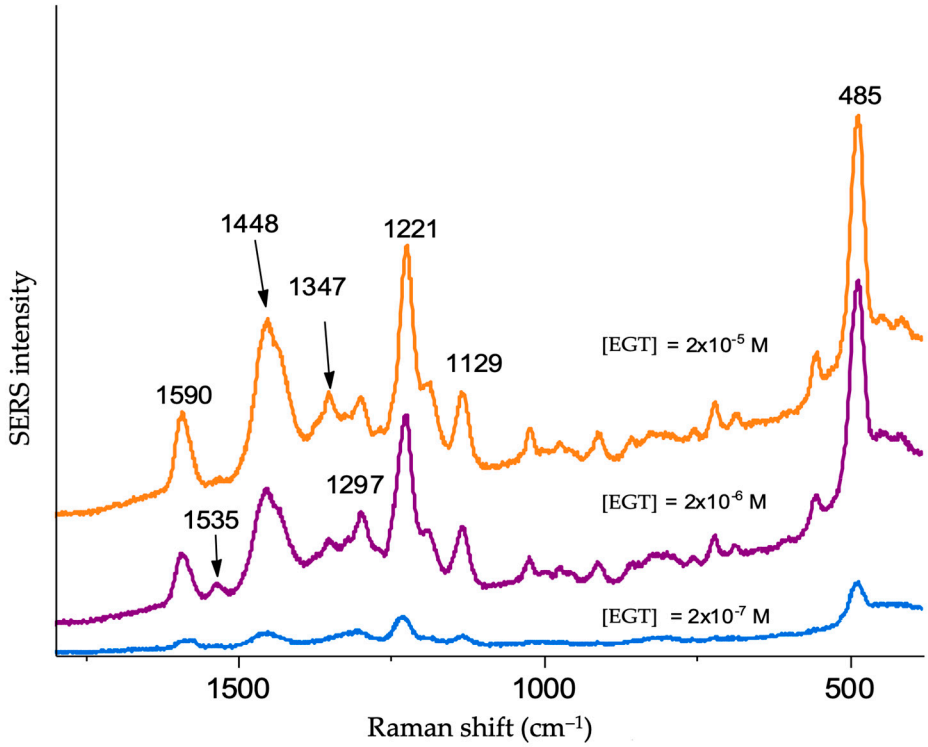

Figure 4. SERS spectra of EGT at pH 6.5, recorded at different concentrations. Laser excitation at 785 nm.

Figure 5 displays the SERS spectra obtained in situ from PCO, PER and POS fruiting bodies and the EGT extracted from POS fruiting bodies. POS was selected for this study based on literature reports suggesting that it contains relatively high levels of EGT compared to other fungal species [12]. Additionally, it is one of the most commercially available and widely cultivated edible mushrooms, making it a relevant and accessible species for investigation [45]. As can be seen, the EGT bands are dominant in the fungi spectra, and they are very similar to the reference SERS spectrum for EGT (Figure 4). However, subtle shifts and intensifications are observed: (a) a shift towards lower frequencies of the bands attributed to the imidazole ring; (b) a slight intensification of the bands at around 1448 and 1304 cm^{-1} accompanied by noticeable variability in the intensity ratio between the peaks at 1343 and 1304 cm^{-1}. This modification is likely influenced by differences in the molecular environment, adsorption orientation and interaction of EGT with the substrate, which modulate the relative enhancement of C-N stretching vibration; (c) the appearance of the band at 1637 cm^{-1}, due to imidazolic νC=C. The observed changes in SERS indicate that EGT can be found in fungal structure under both thiol and thionic forms, with predominance of the thiol one, but with an increase in the thione form in fungi. The predominance of the thiol form is attributed to its biological activity and its role as activator of several enzymatic processes [46]. Specifically, the role of the thiols is of great importance in serving as electron donors for thiol-dependent enzymes in redox-mediated metabolic and signaling processes. They also protect cellular macromolecules from oxidative stress and participate in the reduction in oxidative post-translational modifications [47]. Moreover, the comparison between EGT extracted from POS fruiting bodies and the SERS spectra recorded in situ from the PCO, PER and POS fruiting bodies, confirms that all bands are attributable to EGT. To further validate this assignment, a negative control spectrum of the EGT-depleted extract was recorded (Figure S3), showing the absence of characteristic EGT bands and thus supporting the specificity of the spectral features observed.

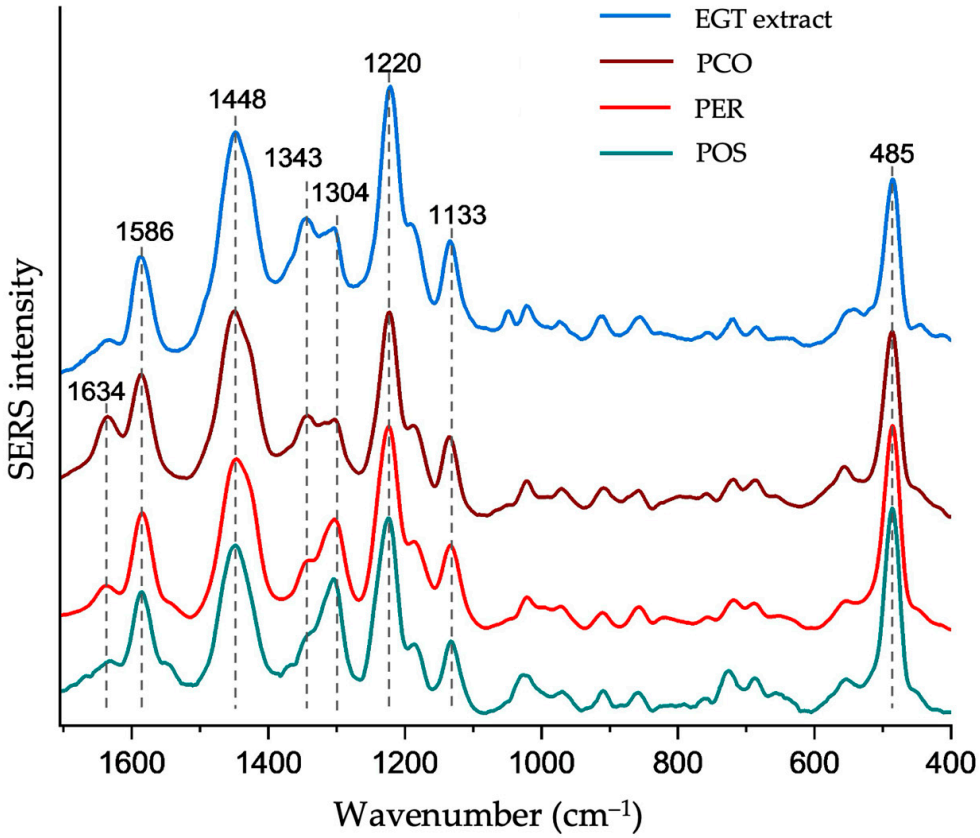

Figure 5. SERS spectra of lyophilized EGT extract from POS fruiting bodies, and dried PCO, PER and POS fruiting bodies. Laser excitation at 785 nm.

4. Conclusions

In conclusion, SERS has been shown to be particularly effective due to its high sensitivity, molecular specificity, and minimal sample preparation requirements. The technique amplifies Raman signals from EGT molecules adsorbed on metallic nanoparticles, enabling the detection of very low concentrations and structural analysis of this molecule. Additionally, SERS has provided distinct spectral fingerprints, allowing for the differentiation of EGT from other compounds in complex samples. This study demonstrated that the SERS spectroscopic technique, due to its non-destructive nature and its applicability, is a remarkable method for the selective detection of EGT in a complex matrix such as fungal tissues. EGT is an ideal candidate for SERS, as in the presence of Ag nanoparticles it gives rise to different pH- and concentration-dependent tautomeric forms. This method for detecting EGT directly in complex matrices such as mushroom extracts can be a valuable tool for quality control, authentication and standardization of mushroom-based products. In the context of food processing or raw material selection, in situ detection enables fast screening without the need for labor-intensive extraction and purification steps.

While the pharmaceutical industry might require more quantitative and highly regulated analytical protocols, the ability to monitor the presence and distribution of EGT at the microscale could also support the development of EGT-enriched formulations or bioavailability studies. Thus, the field of research focusing on the detection of molecules using SERS spectroscopy is set to remain an exciting and evolving area of study in the coming decades. It has the potential to address an increasing number of intriguing questions at the intersection of biology and medicine.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemosensors13060213/s1, Figure S1: Extinction spectrum and TEM image of the Ag nanoparticles synthetized by reduction with hydroxylamine; Figure S2: Raman and FTIR spectra of (2S)-3-(2-sulfanylidene-1,3-dihydroimidazol-4-yl)-2-(trimethylazaniumyl)propanoate (ergothioneine); Figure S3: SERS spectra of dried POS fruiting bodies (green line), lyophilized EGT extract from POS fruiting bodies (blue line) and remaining POS fruiting bodies after EGT extraction (negative control, red line). Laser excitation at 785 nm; Table S1: Band assignment of SERS and Raman spectra of standard EGT. Ref [48] was cited in Supplementary Materials.

Author Contributions: Conceptualization, F.P., S.S.-C., V.Z. and O.F.; methodology, F.P., S.S.-C., V.Z. and P.M.; software, F.P. and S.S.-C.; validation, F.P., S.S.-C. and V.Z.; formal analysis, F.P., V.Z. and S.S.-C.; investigation, F.P., V.Z. and S.S.-C.; data curation, F.P., S.S.-C., V.Z. and O.F.; writing—original draft preparation, F.P., S.S.-C., V.Z., A.Z., P.M. and O.F.; writing—review and editing, F.P., S.S.-C., V.Z., A.Z., P.M. and O.F.; visualization, S.S.-C.; supervision, S.S.-C., O.F. and A.Z.; funding acquisition, S.S.-C., P.M. and V.Z. All authors have read and agreed to the published version of the manuscript.

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