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PERSPECTIVES

# The key role of ergothioneine in label-free surface-enhanced Raman scattering spectra of biofluids: a retrospective re-assessment of the literature

Stefano Fornasaro[1], Valter Sergo[1,2] and Alois Bonifacio[1]

1 Raman Spectroscopy Lab, Department of Engineering and Architecture, University of Trieste, Italy
2 Health Sciences Department, University of Macau, China

**Correspondence**
A. Bonifacio, Raman Spectroscopy Lab, Department of Engineering and Architecture, University of Trieste, Trieste 34127, Italy
Tel: +39 040 558 3768
E-mail: abonifacio@units.it

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**Label-free surface-enhanced Raman scattering (SERS) has recently gained attention in the field of liquid biopsy as a rapid and relatively inexpensive technique that could significantly ease clinical diagnosis and prognosis by investigating a biofluid sample with a laser. Indeed, SERS spectra provide information about a set of metabolites present in the analysed biofluid, thereby offering biochemical insight into specific health conditions. Ergothioneine plays a key role since it is one of the few metabolites in biofluids that are detectable by label-free SERS. In the past decade, many studies characterizing biofluids or other biological samples have unknowingly linked this amino acid with crucial metabolic processes, including inflammation, in a plethora of diseases. However, since the SERS spectrum of ergothioneine has been reported only recently, most past studies inadvertently assigned what are now recognized as the spectral features of this compound to other molecules. The purpose of the present review is to summarize and re-evaluate these studies in the light of the recent SERS characterization of ergothioneine so as to better recognize the role of ergothioneine in many clinical conditions.**

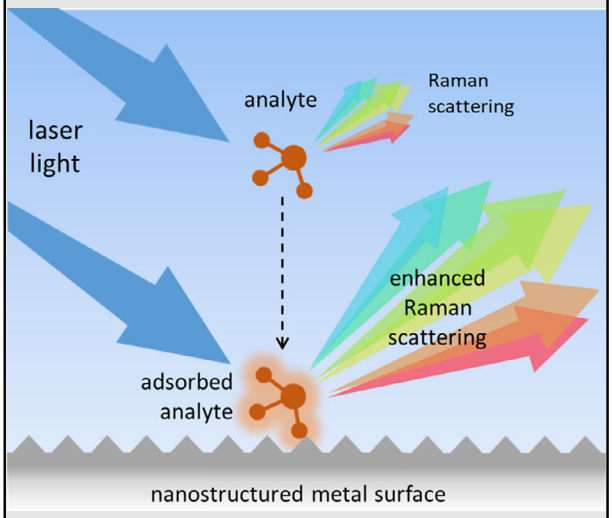
**Keywords:** biofluids; ergothioneine; liquid biopsy; plasma; Raman spectroscopy; SERS; serum

Label-free surface-enhanced Raman scattering (LF-SERS) spectroscopy has recently emerged in the field of liquid biopsy as a rapid and relatively inexpensive technique that could significantly ease clinical diagnosis and prognosis by investigating a biofluid sample with a laser [1].

Surface-enhanced Raman scattering refers to the dramatic enhancement of the inelastic scattering of laser photons (Raman signal) by molecules in close proximity to plasmonic nanostructures (i.e. the SERS substrates) (Box 1). This amplification allows for the detection of compounds present at low concentration in a biofluid, yielding measurable spectral signals, which provide information concerning the molecular composition of samples. Most biological molecules have unique SERS spectral signatures, so that the biochemical composition of complex biological specimens can be determined from their SERS spectra. SERS spectra can be easily obtained from liquid samples and can be performed by minimally trained personnel, with relatively inexpensive instrumentations and simple sample preparation protocols. These unique advantages have helped establish SERS spectroscopy as a very important tool in various branches of analytical

**Abbreviations**
Ag-NPs, silver nanoparticles; Au-NPs, gold nanoparticles; CHB, chronic hepatitis B; CSF, cerebrospinal fluid; EC, oesophageal carcinoma; ET, ergothioneine; HCC, hepatocellular carcinoma; NIR, near infrared; SAH, subarachnoid haemorrhage; SERS, surface-enhanced Raman scattering; UC, ulcerative colitis.

**Box 1.** Surface-enhanced Raman scattering (SERS)




Raman spectroscopy is based on the Raman effect, i.e. the inelastic scattering of a monochromatic laser radiation by the vibrating molecules constituting a sample. The spectra are easily obtained and very informative in virtue of their close relation to molecular structures so that they can be effectively used as a sort of "unique" fingerprints to identify chemical species. However, the Raman effect is feeble, since only a tiny fraction of the incident light undergoes inelastic scattering, making the detection of analytes below the millimolar concentration extremely challenging. The SERS effect consists in the amplification of the Raman scattering in presence of nanostructured metal surfaces with particular properties, called SERS substrates. These substrates are usually made of gold or silver, in the form of a colloidal dispersion of nanoparticles or as solid metal surfaces presenting nano-sized features. The light scattered by analyte molecules directly adsorbed or in close proximity of these surfaces is greatly enhanced, mostly because of an increased local electromagnetic field due to highly localized "surface plasmons", i.e. collective oscillation modes for the surface electrons of the metal nanostructures. Provided that an analyte is successfully brought close to the nanostructured surface, driven by its own affinity for the metal or selectively bound by a recognition element, the intensity amplification of the scattered light can be huge. SERS spectra of single molecules have been reported, making SERS one of the most sensitive techniques in optical spectroscopy.

science [2-4], even if there is still room for improvement, especially regarding reproducibility and robustness [5]. Quantitative applications of SERS have been limited by the highly variable level of SERS enhancement, and typically require either external or internal standards. In the last decade, SERS has gained much attention for being able to provide a 'biochemical snapshot' of potentially clinically relevant information about the metabolic status of a sample, especially in cases where little is known about the molecules causing the studied condition [6-10]. SERS spectroscopy can contribute to bringing a new way of searching biomarkers, namely 'spectral signatures' or 'spectral features', which reflect the total biochemical composition of the investigated sample.

In the label-free approach, each SERS spectrum contains information about the molecules that freely adsorb on substrate surface, driven by the affinity with the surface itself. LF-SERS spectra of biofluids depend on both the relative concentration of the endo- and xeno-biotics present in a biofluid and their chemical affinity for the substrate's surface, the latter factor being the most relevant [11]. The surface of the substrate seems to act as a sort of 'spectroscopic filter' and greatly limits the types of biofluid components detected by SERS. Consequently, the LF-SERS spectrum of a biochemically complex sample is always an extremely simplified representation of that analysed sample [12]. For instance, all most intense bands in the LF-SERS spectrum of serum or plasma obtained with a NIR laser (excitation at 785 nm) on most commonly used substrates can be attributed to just about few metabolites out of more than 4000 metabolites identified in those biofluids [13], and a similar situation holds for other biofluids [14-16]. These bands have been identified as mostly related to purine metabolism (uric acid, hypoxanthine, xanthine), glutathione, proteins (for unfiltered, whole biofluids) [17,18], and, only recently, to ergothioneine (ET) [19], a dietary amino acid with a putative vitamin-like role. To date, biosynthesis of ET has only been demonstrated in specific bacterial and fungal species, including *Mycobacterium tuberculosis* and certain cyanobacteria [20]. Interestingly, the presence of ET spectral features can be found in LF-SERS spectra of cultures of *Mycobacterium* species [21], cyanobacteria [22] and dermatophytes fungi [23]. Mammals cannot biosynthesize ET and acquire it from both vegetable and animal dietary sources. In humans, ET is absorbed following oral consumption and it is not rapidly metabolized or excreted in urine, but rather accumulates selectively in most bodily cells and tissues, according to the differential expression of the ET-specific transporter SLC22A4. For a more complete overview of ET biology, the reader is referred to [20,24,25]. Up to date, the combination of fast high-resolution liquid chromatography (LC) technologies

with mass spectrometry (MS) is the golden standard for the identification and quantification of ET in dietary supplements, blood, and different human body tissues [26,27], due to enhanced sensitivity, higher selectivity and higher sample throughput. However, LC-MS methods are relatively time-consuming and require specialized personnel, significant sample extraction and preparation, and the use of calibration standards to gather quantitative information. For all of these reasons, versatile, less expensive and quick complementary/alternative techniques, such as LF-SERS, are desirable.

## ET in LF-SERS of biofluids

Bands that are now recognized as due to ET appeared in the LF-SERS spectra of several biofluids, reported by various groups (see Fig. 1 for some examples). In spite of the variety of substrates, protocols, and instruments used by different groups, the overall spectral pattern of ET (with minor variations) is clearly recurrent, suggesting that the adsorption mode of this molecule is conserved, and highlighting the important role of this amino acid in LF-SERS spectra of biological samples.

Interestingly, some recent studies characterizing biofluids with LF-SERS have linked ET with crucial metabolic processes, such as inflammation and antioxidant capacity, in several disease conditions (e.g. gingivitis [28], hepatocellular carcinoma [29], large B-cell lymphoma [30] and myeloma [31]). On the other hand, many other studies, most of which were published before the SERS spectrum of ET was reported, inadvertently assigned ET bands to other molecules or left them unassigned. For instance, the intense band around 483 $cm^{-1}$ in SERS spectra of serum and plasma (sometimes overlapping with a uric acid band at slightly higher Raman shifts) has been reported as due to glycogen [9,32]. Other ET bands have been misinterpreted as due to other amino acids such as tryptophan, phenylalanine or serine, to proteins such as collagen (although collagen is not expected to be found in these biofluids in significant amounts), to lipids, to vitamins such as ascorbic acid or riboflavin, and to metabolites such as acetoacetate [6,7,8,9,33,34]. Moreover, SERS bands due to ET have been often mistaken as due to haemoglobin [35-40]. In these studies, haemoglobin was obtained from erythrocytes, where ET is present in high concentrations and has been detected by SERS [19,41]. Since ET has a very high affinity for Ag substrates, small amounts of this metabolite, still present in haemoglobin samples after the purification steps from the erythrocytes lysates, were apparently sufficient to yield the intense, characteristic ET SERS bands instead of those of haemoglobin (whose SERS spectrum, reported in a previous study [42], has completely different features).

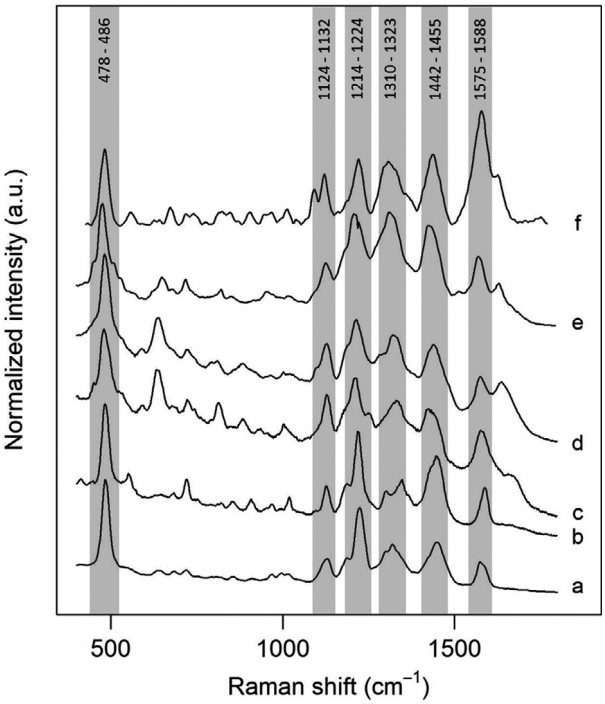



**Fig. 1.** Example of how characteristic ET bands (highlighted with grey areas) are observed in LF-SERS spectra of a variety of human biofluids of healthy donors. SERS spectrum of (a) ET [19] is compared to SERS spectra of (b) erythrocytes lysate [19], (c) serum [29], (d) gingival crevicular fluid [28], (e) seminal plasma [50] and (f) cerebrospinal fluid [32]. All spectra were obtained with an excitation at 785 nm, and using Ag nanoparticles as substrates, with the exception of (f), for which Au nanoparticles were used. Ranges of Raman shifts reported for ET bands in different biofluids are reported on the top and ET bands are highlighted with grey areas. Spectral data of (e) and (f) were obtained from fig. 2d in [50] and fig. 4A in [32], respectively, by using the open software web plot digitalizer [57].

In this scenario, the recent characterization of ET SERS spectral features as key components in the LF-SERS spectra of biofluids is a potential game changer for the biochemical interpretation of the LF-SERS data. The purpose of the present review is to summarize and re-evaluate these studies in the light of the recent SERS characterization of ET, to better recognize the role of this natural antioxidant compound in many clinical conditions.

## Relevance of ET in diagnostic studies using blood, serum, and plasma

The analysis of biological fluids (i.e. liquid biopsy) seems particularly suitable for the detection of many

types of diseases because biofluids are in direct connection with organs of the human body and are generally easily collected [43]. Blood components like serum and plasma are the primary clinical specimen of interest as they contain biomarkers that are useful for disease diagnostics [44]. Cancer represents the main group of diseases for which LF-SERS has been tested as a noninvasive and rapid tool in areas such as disease sub-type classification, response to treatment, and diagnosis of disease relapse [1]. Most investigations reported very high degrees of accuracy (usually higher than 90%), using advanced multivariate chemometric algorithms. However, it should be noted that even if such algorithms work perfectly without knowing the molecular origin of each band, a proper interpretation of LF-SERS bands can be very helpful in assessing the validity of the results by considering them from a biochemical perspective. Moreover, a correct interpretation of LF-SERS data might help in expanding the biochemical insight on a particular disease, suggesting further studies or research directions. Regrettably, this aspect is often overlooked in LF-SERS studies, as highlighted in the previous section, with ET contributions specifically considered only in some very recent reports. Indeed, a leitmotiv of this review is that understanding such spectral signatures better will assist the analyst considerably in exploiting them for diagnostic purposes.

For instance, preliminary studies have suggested that LF-SERS spectroscopy on serum/plasma samples can be used to distinguish patients with several types of hepatocellular carcinoma (HCC) from patients with chronic hepatitis B (CHB), normal individuals, or patients with oesophageal carcinoma (EC) [8,29,33,45]. Recent serum data published by Gurian et al. reported how ET bands were relatively less intense in HCC patients when compared with healthy controls [29] . A relative decrease in the SERS intensity of ET bands for liver cancer patients with respect to controls can also be found in the works by Xiao et al.[8] (Fig. 2) and Liu et al. [38,45], although with a different band interpretation (Fig. 3).

These observations are consistent with the notion that lower blood levels of ET are associated with the incidence of several disorders. Whether this decline in ET is a cause or consequence of disease remains to be established. Similar examples for different disorders can be found in the studies by Liu et al. [7], in which LF-SERS was combined with multivariate analysis methods to analyse the serum from patients with lung adenocarcinoma (LAC), and by Li et al. [9], in which the serum from patients with prostate cancer was analysed. In both cases, it is possible to re-assign some detected differences

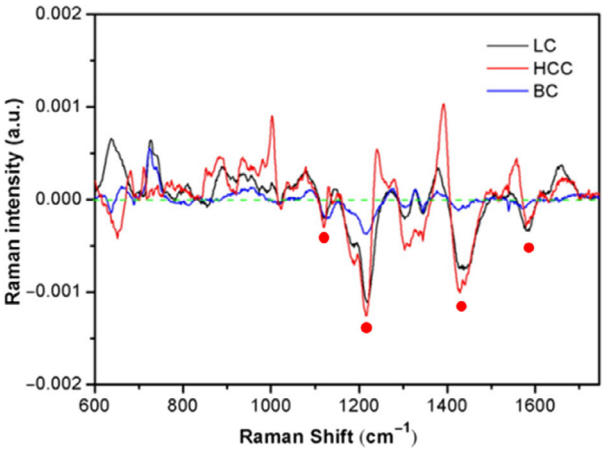



**Fig. 2.** Intensity variation of ET bands in serum as determined using difference spectra, from groups of subjects with different health conditions. Difference spectra calculated from the mean SERS spectra of serum samples from lung cancer (LC), hepatocellular carcinoma (HCC) and breast cancer (BC) subjects minus that of the control group. Reproduced with permission from reference [8]. The negative bands marked with red dots can be attributed to ET, implying that ET levels are lower in the serum of HCC and LC than in controls. Spectra were obtained from serum samples by using Au nanorods as substrate upon 785 nm excitation.

to ET features, with ET levels slightly higher in SERS spectra from the healthy volunteers.

On the other hand, in the report by Li et al., in which serum samples from healthy people, liver cancer patients, post-operative liver cancer patients and liver cirrhosis patients were analysed, the SERS intensity of the ET bands seem relatively lower in the healthy group [34]. In an analogous contribution by Feng et al. differentiating the blood plasma of nasopharyngeal cancer patients from that of healthy subjects with, ERG related bands appeared greater in nasopharyngeal cancer plasma samples [46].

## Relevance of ET in diagnostic studies using other biofluids

An important concept when considering liquid biopsy is that all biofluids are in some way related to blood. It is therefore not surprising that LF-SERS spectra of many of them can share the same spectral features, with ET contributions being distinguishable in the LF-SERS spectra of seminal plasma, cerebrospinal fluid (CSF), and gingival crevicular fluid (GCF).

In a first study by Chen et al. [47], LF-SERS was used to obtain biochemical information from normal and abnormal samples of seminal plasma. The relatively high amount of ET in this biofluid is well-documented

in the literature, corresponding rather closely with the blood values [48,49], and is clearly detectable in the LF-SERS spectra [50]. However, ET levels seem to be unaltered with respect to the considered conditions.

Kim et al. [32] analysed human lumbar cerebrospinal fluid (CSF) samples using AuNPs-functionalized paper strips and NIR laser to identify and predict cerebral vasospasm and hydrocephalus complications induced by subarachnoid haemorrhage. Interestingly, an increase in SERS intensities associated with ET bands can be appreciated in the vasospasm group when compared to the other groups. However, these findings need to be interpreted with caution since contamination with blood during CSF collection can falsely increase ET levels *via* red blood cells (RBCs) lysis. Haemolysis is very common in practice (not only in CSF studies), especially upon incubation of blood with metal colloids [51], and can interfere with SERS analysis of biofluids by releasing analytes into the serum or plasma that are in high concentration within the RBCs, thereby giving false concentrations of those analytes, and leading to misinterpretation of results. For that reason, haemolysis should be always considered when collecting SERS of biofluids.

More recently, an exploratory study by our group reported the first SERS characterization of gingival crevicular fluid (GFC), using AgNPs-covered paper strips and NIR laser. ET was identified as one of the key markers of the spectral difference between healthy dental elements and elements with periodontal disease, being relatively higher in the healthy ones [28].

## ET in LF-SERS of other biological samples

Ergothioneine distribution is especially high in tissues that are considered to have the potential for oxidative stress and it may also be accumulated in the central nervous system [20]. Li et al. were able to distinguish healthy brain tissue from glioma brain tumours at different stages using silver nanorods as substrates for LF-SERS [52]. The spectra obtained from aqueous extract of the tissue exhibited signs of ET contributions, especially in the samples from advanced gliomas (Fig. 4).

Similarly, tissue samples isolated from human nasopharyngeal specimens were studied by Feng et al. with LF-SERS [53]. The authors found that the band shapes and relative intensities of the normal tissue were clearly different from the LF-SERS spectra of

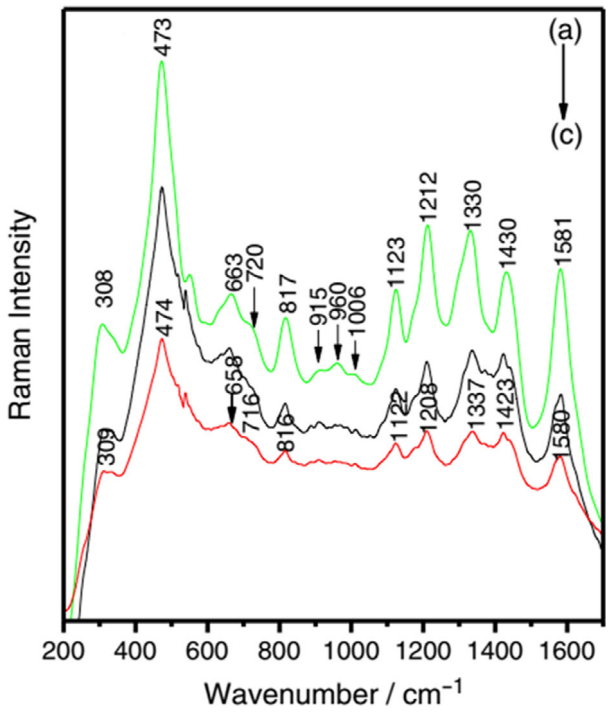



**Fig. 3.** Average SERS spectra of samples obtained from erythrocytes lysates, featuring ET bands. Spectra were collected from samples from normal subjects (a), patients with liver cancer after surgery (b) and patients with liver cancer (c). Reproduced with permission from reference [38]. Differences in intensity indicate that ET levels are lower in cancer patients than in normal subjects. Spectra were obtained by using deposited Ag nanoparticles as substrate upon 785 nm excitation.

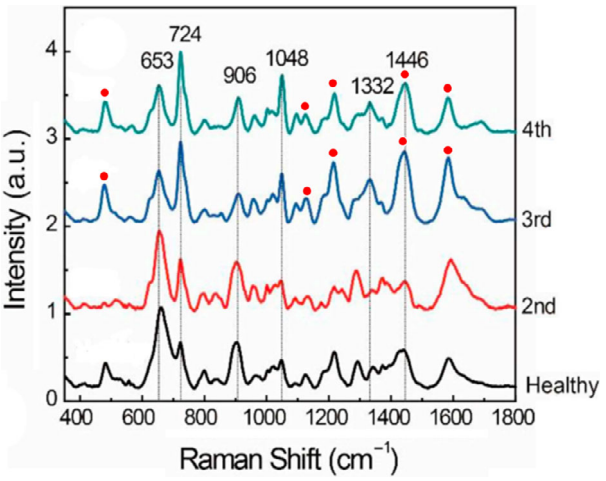



**Fig. 4.** SERS spectra of normal brain tissue and gliomas at different stages. Reproduced with permission from reference [52]. The bands marked with red dots can be attributed to ET, implying that ET levels are higher in the glioma samples than in healthy controls. Spectra were obtained from tissue extracts by using Ag substrates upon 785 nm excitation. SERS spectra of normal brain tissue (Healthy, black line) and SERS spectra of gliomas at different stages (coloured lines).

the carcinoma tissue section, with ET bands more present in the SERS spectra of cancer tissue. The same group succeeded in differentiating oesophageal cancer tissue from normal control tissue [54]. Also in this case, cancer tissues showed higher relative intensity for the ET related bands.

Besides tissues, ET SERS bands have been detected in samples obtained from bacteria as well. In fact, several studies assessed whether LF-SERS could be used for rapid identification and discrimination of medically relevant bacteria by revealing their metabolic fingerprint. This approach usually involves LF-SERS measurements on the supernatant acquired after a washing–centrifugation process [55,56]. Recent work by Cheng et al. demonstrated a new detection method to identify mycobacteria based on the SERS spectra of their secreted metabolites, among which those due to ET are clearly visible [21].

## Conclusions and perspectives

Ergothioneine bands in SERS studies of a variety of biofluids and other biological samples were misinterpreted and assigned to other biomolecules. Such ET bands often contributed to the spectral changes observed between different clinical conditions, suggesting a role of this amino acid in pathology. With some exceptions, most studies on serum or plasma reported a higher relative amount of ET in control samples with respect to those of patients affected by a disease. Evidence in this sense is particularly consistent for liver diseases, for which 4 out of 5 studies on samples obtained from blood (serum or erythrocytes) reported lower ET levels in patients than in healthy individuals. This would be consistent with the hypothesis, further corroborated by the higher ET levels detected by LF-SERS in tissue extracts, that ET actively accumulates in cancer tissue by depleting blood of ET. It should be noted that all of the mentioned studies were proof of concept, being carried out on rather small populations. Nevertheless, they have held promise for clinical utility and suggest that ET might be used, together with other metabolites detected by LF-SERS, as a diagnostic marker for some diseases. The knowledge about ET is limited in SERS field as much as SERS remains an unknown technique to most researchers in the field of ET. Hence, we hope that this review will increase the awareness of the importance of ET in LF-SERS of biofluids, as well as of the utility of SERS as a tool to investigate the biological role of ET. Among the steps necessary to consolidate the role of SERS in ET research, the development of a reliable quantitative SERS-based method to quantify ET, and validation studies with a direct comparison with ET levels quantified by LC-MS methods, would be of utmost importance.

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