# COMPARATIVE RAMAN SPECTROSCOPY STUDY OF THE COELOMIC FLUID OF GRAZING SEA URCHINS AND THEIR NATIVE SEAWATER: PROSPECT FOR A POTENTIAL INDICATOR OF ENVIRONMENTAL AGGRESSION

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**ABSTRACT. –** Sea urchins have limited ability to move, and they use the water-vascular circulation system to pump surrounding sea water in order to set body parts in motion. It is therefore thought that coelomic fluid, a body fluid of sea urchins which acts as its internal transport and immune system, contains metabolised pollutants. In the present study, we developed a method for detection of carotenoids in the coelomic fluid by Raman spectroscopy. Carotenoids were obtained from the coelomic fluid by ethanol extraction and their selective resonance Raman signal was enhanced employing the surface enhanced Raman scattering (SERS) technique. Carotenoids are ingested by sea urchins through their plant-based diet, metabolised and transported into the coelomic fluid, where they were detected for the first time via SERS. We further investigated the correlations of carotenoids signalling from the coelomic fluid with the local sea water to prospect a potential linkage with changes under environmental aggression. The antioxidative and immunomodulatory role of carotenoids, especially of $\beta$-carotene, was extensively studied in vertebrates (Chew and Park, 2004). Biological defence and increased antioxidant activity is associated with an increased carotenoids level and/or change in their species balance in native sea urchins. Additionally, we compared relative sulfate concentration of sea urchin coelomic fluid with local sea water using FT-Raman technique. We discuss the possibility for development of methods for rapid and cost effective monitoring of the native environmental waters via sea urchin carotenoids. Other co-existent pollutants which may enter the coelomic fluid via the digestive system or through water-vascular system of sea urchins are expected to correlate with the animal response via an increased antioxidant activity due to carotenoids. Thus, sea urchins may prove to be good sentinels of environmental water changes via their carotenoids signalling.

**Keywords:** environmental water, pollutants, sea urchins, Raman spectroscopy.

## 1. INTRODUCTION

*Paracentrotus lividus* is a common species of sea urchin in the study area, the southeastern Adriatic Sea. The biology and ecology of the species was

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extensively reviewed by Boudouresque and Verlaque (2013). Sea urchins are sedentary animals, meaning that their ability to move is limited, and they are exposed to local environmental conditions. According to latter review, the species is not sensitive to organic pollution, it can tolerate and accumulate heavy metals, but it is sensitive to ammonia pollution and oil spills. Since *P. lividus* is a benthic grazer, it is expected to accumulate pollutants incorporated in algae and marine plants from its surrounding.

The coelomic fluid fills the coelomic cavity of sea urchins. It is a cocktail of various types of cells (Arizza et al., 2007) and biomolecules (Liyana-Pathirana et al., 2002; Dheilly et al., 2013; Dev and Robinson, 2014). Since this fluid serves, *inter alia*, as the sea urchin`s immune system (reviewed by Ramirez-Gómez and García-Arráras, 2010), we have strong reason to believe that this fluid would reflect chemical and physical changes in the local environment through presence of molecular signs of stress and metabolised xenobiotics.

Carotenoids are a huge class of biomolecules with various biological properties, health benefits and commercial applications. They are usually synthesized by photosynthetic organisms like plants and algae (Vilchez et al., 2011). Sea urchins are not able to synthesize carotenoids *de novo*, but they possess enzymes which modify the carotenoids ingested through their plant-based diet. They are initially metabolised by the digestive system and then further distributed over the animal`s body (Symonds et al., 2007).

In the present paper, we show the first detection of carotenoids in *P. lividus* coelomic fluid using multiple Raman spectroscopy techniques. Coelomic fluid of this species has not been investigated yet with respect to carotenoids. Carotenoids have previously been identified in sea urchin *Strongylocentrotus droebachiensis* by means of thin layer chromatography. However, Raman spectroscopy has the advantages of rapidity, simple sample preparation and low per-analysis cost.

## 2. MATERIALS AND METHODS

Five sea urchin individuals of species *P. lividus* were collected from Bay of Gruž, Dubrovnik city, Croatia (42.662884 N, 18.077690 E). A photograph of the collection site is presented in Fig. 1. Collection was done from the shore using a hand held net, and the animals were taken from the depth of about 0.5 m. Additionally, local sea water was sampled in May 2016, and April and June 2017, using plastic 0.5 l bottles, from the same site and depth as were the sea urchins.

Coelomic fluid was withdrawn from the coelomic cavity of the animals using syringes. Each sea urchin provided about 2 to 3 ml of coelomic fluid. Coelomic fluid withdrawn from first two sea urchins (about 2 ml each) was discharged into 5-ml sample vials containing 2 ml of phosphate buffer (pH 7), and immediately cooled to 4 °C. The fluid from remaining three sea urchins was discharged into respective 10 ml vials for each individual, and the remaining volume till 10 ml was filled with analytical grade ethanol (> 99.5 % purity).

Handling of coelomic fluid from withdrawal to discharge into vials was done fast, in order to avoid premature agglutination inside syringes.

Ethanol extraction lasted 7 days, at ambient temperature and in darkness. Thereafter, respective subsamples were taken from the extraction vials for conventional Raman and for Surface-Enhanced Raman Scattering (SERS) analyses. Raman analyses were done directly in 1 ml glass cuvettes, and SERS solutions of both native fluid (in phosphate buffer) and ethanol extracts were prepared by adding 10 µl of each coelomic fluid sample to 500 ml µl of colloidal silver nanoparticles (AgNPs).

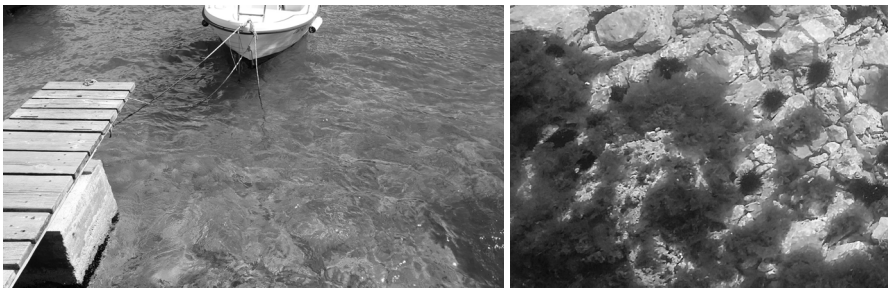

**Fig. 1.** ***Photographs of the collection site, showing the shallow rocky sea bottom covered with algae and inhabited by sea urchins.***

Raw sea water was filtered on a Milipore vacuum filtration system. The entire volume (0.5 l) was passed through a carbon fibre filter with 0.45 µm pore size. Sea water samples were kept at 4 °C during storage. SERS solutions were prepared by addition of 10 µl of filtered sea water into 500 µl of AgNPs.

Details on preparation and characterisation of AgNPs can be found in a paper by Cintă Pinzaru et al. (2016). In short, AgNPs were prepared by the Lee-Meisel method: silver nitrate reduction by trisodium citrate under boiling temperature. Nanoparticle quality was checked by UV-Vis absorption spectroscopy.

Raman spectroscopy analyses of coelomic fluid extracts were done on a Renishaw InVia confocal Raman microscope, using accessory equipment for liquid samples. Spectra were acquired using a Cobolt diode pumped solid state (DPSS), air cooled laser emitting at 532 nm. The system was operated through WiRE 3.4 software, and the spectra were acquired with 0.5 $cm^{-1}$ spectral resolution.

Raman spectroscopy analyses of native coelomic fluid and filtered sea water samples were done on a DeltaNu Advantage 532 compact, dispersive Raman spectrometer. A 532 nm laser line was employed for sample excitation, and spectra were recorded with 8 $cm^{-1}$ spectral resolution through NuSpec software.

Fourier transform Raman spectroscopy (FT-Raman) spectra were collected with a Bruker Equinox 55 FT-IR spectrometer with an integrated FRA 106S Raman module. A Nd:YAG laser emitting at 1064 nm with the output power of 350 mW was used for FT-Raman spectra excitation. Spectra were acquired with

500 accumulations and spectral resolution of 4 $cm^{-1}$. Origin 6.1 software was used for processing of spectral data.

## 3. RESULTS

### 3.1. Surface-enhanced Raman scattering

Applying SERS with 10 µl of native coelomic fluid added to 500 µl AgNPs results in strong signal dominated by the bands at 1563 and 1369 $cm^{-1}$, along with other medium or weak bands, as showed in the Fig. 2A.

The two strong bands observed in the SERS feature of coelomic fluid are believed to the due to the skeletal structure of echinochrome, the naphthoquinoid pigment from sea urchins. Echinochrome is known to be responsible for the antibactericidal activity in European edible sea urchin (*E. esculentus*) (Service and Wardlaw, 1984). Although the vibrational properties of echinochrome (2,3,5,6,8-pentahydroxy-7-ethyl-1,4-naphthoquinone, PubChem CID: 164644) are not reported in the literature, naphthalene-dione fused rings stretching modes of naphthoquinone and 2-methyl naphthoquinone derivative and their radical anions have been extensively investigated (Singh et al., 2010). The additional five hydroxyl groups in echinochrome are not expected to drastically change the skeletal vibrational structure relative to that of ethyl-1,4-naphtoquinone.

Lorentzian fit of the 1500-1640 $cm^{-1}$ range from the SERS spectrum of coelomic fluid featuring the main band at 1563 $cm^{-1}$ showed a complex contribution of seven components (Fig. 2B). Among them, two distinct bands attributable to carotenoids are observed at 1524 and 1532 $cm^{-1}$. The carotenoid band at 1524 $cm^{-1}$ could be tentatively assigned to echinenone, while the band at 1532 $cm^{-1}$ could be originated from fucoxanthin species. Given their low intensity in SERS (combined with the resonance Raman effect), it is assumed that the concentration of carotenoids in coelomic fluid sample is extremely low.

SERS spectra of coelomic fluid extracts in ethanol featured even more defined carotenoid bands (Fig. 2C). The three main carotenoid Raman bands, caused by major atomic vibrations within the molecule, are termed $\nu_1$, $\nu_2$ and $\nu_3$. The $\nu_1$ band, positioned at 1527 $cm^{-1}$, is the strongest band, and it is caused by C=C double bond stretching from the conjugated polyene chain of carotenoids. Multiple carotenoid species may contribute to the band profile. The $\nu_2$ band at 1157 $cm^{-1}$ arises from single C-C bonds in the polyene chain, while the $\nu_3$ band, which is mainly caused by methyl rocking, was clearly split into two peaks at 1000 and 1013 $cm^{-1}$ (Tschirner et al., 2009; Cintă Pinzaru et al., 2015).

On the other hand, conventional Raman spectra of native coelomic fluid featured a high fluorescence background, and no carotenoid bands could be observed (Fig. 2A). Similarly, the ethanol extracts yielded normal Raman spectrum of stronger ethanol bands and a weaker carotenoid signal (Fig. 2C).

Comparison of the SERS signal of filtered seawater from the sea urchin environment with that from the coelomic fluid showed a slightly different

carotenoids contribution (bands at 1523 and 1533 $cm^{-1}$) (Fig. 2D). These results suggests that the environmental water contribution to the SERS output of coelomic fluid is not exclusive, chemical metabolites being present in the latter. The fit components revealing the carotenoids bands are quite different.

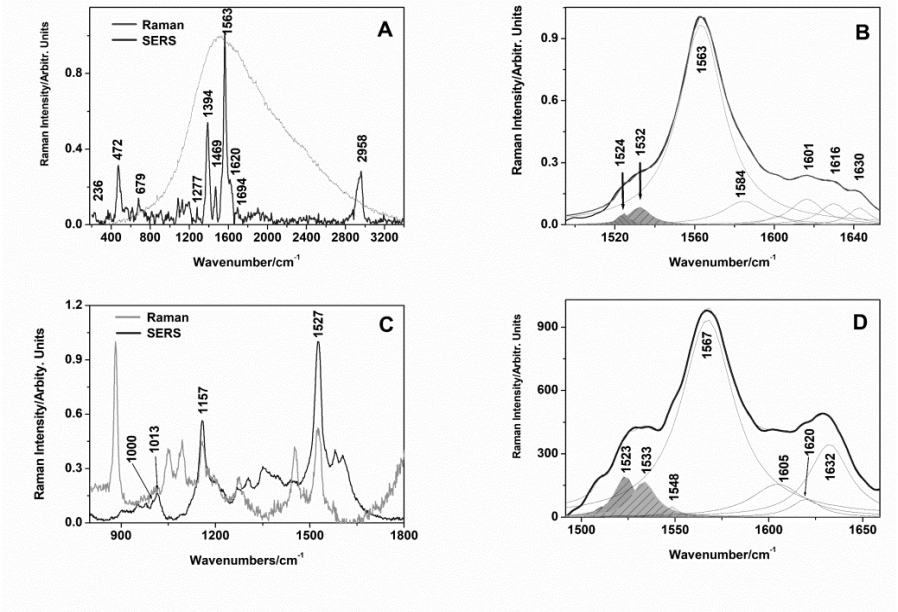


**Fig. 2.** ***A) Raman and SERS spectra collected from native P. lividus coelomic fluid; B) Lorentzian fit of the 1500-1650 $cm^{-1}$ range, with the main echinochrome band at 1563 $cm^{-1}$ showing carotenoid components at 1524 and 1532 $cm^{-1}$; C) Raman spectra of ethanolic extract from P. lividus coelomic fluid, showing the ethanol bands along with the carotenoids signal, as indicated; D) Lorentzian fit of the filtered sea water SERS band at 1567 $cm^{-1}$ showing carotenoid components at 1523 and 1533 $cm^{-1}$. Excitation: 532 nm.***

## 3.2. Fourier transform Raman spectroscopy

FT-Raman spectra of *P. lividus* coelomic fluid compared to sea water from its native environment is presented in Fig. 3.

The water band at 3245 $cm^{-1}$ in environmental seawater signal has been used to calculate the relative intensity of the sulfate to water Raman ratio, to estimate the concentration of sulfate in coelomic fluid. Data shown in Fig. 3 indicate that sulfate in coelomic fluid is higher than in environmental sea water. The compared ratio R of different sea water samples is slightly different, however, consistent with small fluctuation over time.

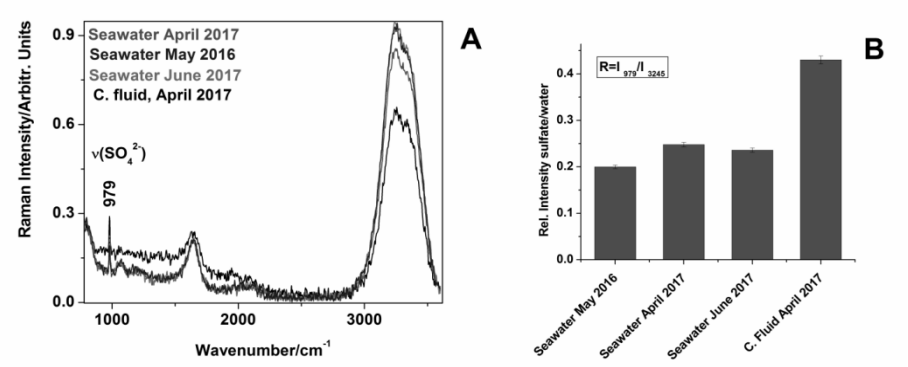


**Fig. 3.** ***A: FT-Raman spectra of P. lividus coelomic fluid compared to the raw sea water signal collected from urchin environment. The sulfate Raman band at 979 cm$^{-1}$ and the water band at 3245 cm$^{-1}$ were used to calculate the ratio R of sulfate to water plotted in B. Excitation: 1064 nm.***

## 4. DISCUSSION AND CONCLUSIONS

We have shown that is possible to detect carotenoids and sulfates both in sea urchin coelomic fluid and local environmental water using Raman spectroscopy techniques. It was difficult to record Raman bands of carotenoids using conventional Raman technique and the 532 nm laser line, because of their low concentration and strong fluorescence of polycyclic aromatic species (echinochrome) which completely covered the carotenoids signal even for the resonance Raman conditions. However, FT-Raman spectroscopy using the NIR laser line at 1064 nm was able to overcome the fluorescence issue, but the weak carotenoid signal was not detectable in the fluid. Additional information was revealed via the sulfate band from the FT-Raman spectrum: its intensity relative to water was found higher in coelomic fluid than in native seawater. SERS was able to enhance Raman signals from carotenoids present at very low concentration. In addition, the water band at 3425 cm$^{-1}$ and colloidal AgNPs lattice bands between 100 and 300 cm$^{-1}$ may serve as internal standards, allowing relative quantification of dissolved molecular species.

The carotenoid profile of *P. lividus* coelomic fluid preserved in phosphate buffer, partially uncovered by Raman spectroscopy in the present study, is similar to the profile of *S. droebachiensis*, where Liyana-Pathirana et al. (2002) reported fucoxanthin as the most abundant carotenoid and minor quantities of xanthophylls (astaxanthin, canthaxanthin).

Based on the corroborated results of these experiments, we suggest that the carotenoid profile of the coelomic fluid and their relative concentration could be used as indicators of environmental water status. Further monitoring studies are certainly needed. First step in developing such indicators would be to calculate their average values and establish the natural range and seasonal variation. Thereafter, statistically relevant number of samples could draw the "normal values", and any significant

deviation will indicate that the individual is under stress, and may in turn provide grounds for deeper research into the status of the environment.

This study shows that analysis of coelomic fluid is not straightforward: detection of carotenoids required the use of SERS, and detection of sulfate in coelomic fluid required the use of FT-Raman spectroscopy with an infrared laser line. However, in both cases we have achieved our goal, proving that coelomic fluid reveals a Raman signature of seawater and a SERS signature of metabolic carotenoids. These findings suggest that optimized, sensitive detection methods based on Raman spectroscopy could be employed in fast tracking any occurrence environmental chemistry changes in native waters inhabited by sea urchins.

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