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Identification of the chromophores in *Corallium rubrum* gem quality corals by HPLC/UV, ESI-MS and ^{1}H NMR spectroscopy

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ABSTRACT

Gem *Corallium rubrum* corals are calcitic biomaterials derived from the branched skeletons of *Cnidaria* marine invertebrates. Until now the structure of chromophores in *Corallium rubrum* is still under discussion. First attempts to identify pigments in red corals from Marseille revealed the presence of canthaxanthin. In 2008 Raman spectroscopy suggested that pigments are instead due to unsubstituted polyenes. More recently (2012) ECC theory in Raman spectroscopy stated that the spectrum of *Corallium rubrum* lies in between carotenoids and unsubstituted polyenes spectra. To confirm and complete the experimental and theoretical results, a group of gem quality specimens of colony skeletons of *Corallium rubrum* was tested. Rough coral samples were before demineralized with EDTA. HPLC combined with UV was used to separate the pure fractions, on which structural characterization was carried out by ESI-MS and ^{1}H NMR spectroscopy. Results have shown that the colouring pigment profile in *Corallium rubrum* seems more complex than hitherto considered. HPLC/UV chromatograms, confirmed by ESI-MS spectra, indicate the presence of two structurally modified carotenoids and of traces of astaxanthin and canthaxanthin. Owing to scarsity of material, ^{1}H NMR spectroscopy did not yield the expected results.

Keywords: *Corallium rubrum*; carotenoids; polyenes; HPLC/UV; ESI-MS; ^{1}H NMR.

INTRODUCTION

Among the gem materials derived from the Animal Kingdom corals have been the most appreciated over time and their value to man has been preserved throughout the centuries. Nevertheless, until the beginning of the 18th century, the origin of these marine gem materials was practically unknown. For centuries they were thought to be petrified vegetables. Only in 1723 the coral's origin within the Animal Kingdom was demonstrated by J.A. Peyssonel. Lacaze-Duthiers (1864) firstly described the morphology and histology of the red coral colonies. Now it is well known that corals are marine non-vertebrate animals. Therefore, gem corals are technically non-vertebrate biomaterials, as they derive from the branched skeletons of the colonies of marine invertebrates (polyps) belonging to the classes Hydrozoa and Anthozoa of the phylum Cnidaria (see Rolandi et al., 2005 for details on the zoological classification). The majority of gem corals belong to the class Anthozoa. According to the main taxonomical characteristics, the Anthozoans have been subdivided into two subclasses: Octocorallia or Alcyonaria and Hexacorallia or Zoantharia. The precious *Corallium rubrum* (Linnaeus, 1758) red corals belong to Anthozoa class, subclass Octocorallia, and live in the Mediterranean Sea and adjacent Eastern Atlantic Ocean on subtidal hard substrates (Costantini et al., 2009). The genus *Corallium* includes 19 species (Bayer and Cairns, 2003), of which the only shallow water species is *C. rubrum*. Up to now three typologies of red coral populations have been described: shallow water populations in a depth range between 15 and

60 m; intermediate water population sat a depth range of about 60-300 m; moderate deep-water populations below 300 m depth. Recently, live red coral colonies have been observed in the Strait of Sicily at depths of ca. 600-800 m (Costantini et al., 2009).

Corallium rubrum is a slow-growing gorgonian coral (Anthozoa class) and is the most famous and precious coral due to its lustre and colour (Figure 1), which varies from dark red (carbonetto or arciscuro) to red (rosso), orange-pink (secondo colore or salmone), bright rosè, pale fresh pink (pelle d'angelo), and sometimes white. It is a relatively long-lived species since longevity can easily reach more than 100 years (Marschal et al., 2004). Up to this time, the cellular mechanism for calcification in corals remains poorly understood: the mineralization processes involved in the growth of the coral skeleton are not yet well known as well as the nature of the colouring pigments included in calcareous skeletons. In particular, the nature of the chromophores in *C. rubrum* is still under discussion. According to McGraw (2006) the colour of the skeleton could be due to carotenoids but also to other natural compouds found in birds and fruits. Among all natural pigments, carotenoids are the most widespread colouring agents. They are a family of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) consisting of eight isoprenoid units, which comprise more than 700 different species of natural lipid-soluble pigments produced primarily within phytoplancton, algae and plants. In animals carotenoids have only a diet origin: they are adsorbed in the animal diet, transformed into other carotenoids and then incorporated into various tissues. Thus they are regarded as essential compounds for life, mainly due to the different roles they perform in photosynthesis. The interest in these pigments has expanded dramatically in the last years due to some of them exhibiting vitamin A activity and to the fact that they are antioxidant and may be beneficial for the prevention of several chronic human diseases. In any case, owing to their benefitial actions, carotenoids are of great nutritional importance.

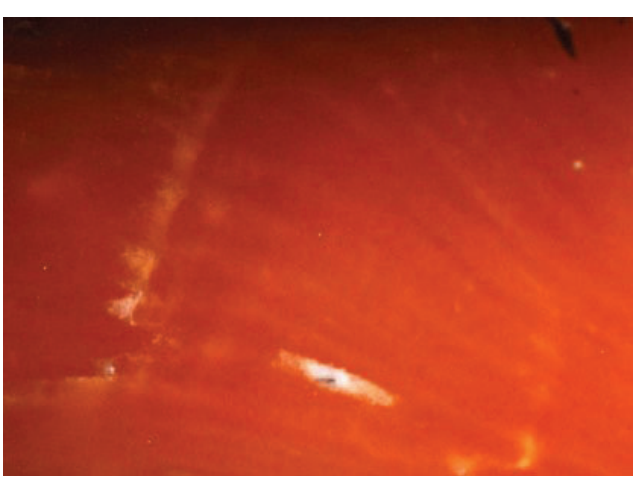

Figure 1. Surface texture of a polished *Corallium rubrum* skeleton showing sinuous canaltraces . 30x magnification.

The aim of this study was to extract, identify and characterize the chromophores in *Corallium rubrum* skeletons (gem quality corals) from a structural point of view, and to compare the results with the available previous experimental data (Raman spectroscopy, HPLC/UV and ESI-MS).

SKELETON GROWTH

From a compositional point of view corals are biocomposites with key components of minerals and organic macromolecules. Calcite and aragonite are the two polymorphs of $CaCO_3$ observed in the skeletons of corals. With few exceptions, calcite is commonly present in the skeletons of Octocorallia, while aragonite forms the majority of Hexacorallia. Some papers (Rahman and Oomori, 2008; 2009; Drake et al., 2013) analyzed several aspects of mineral-protein interaction to better understand the processes connected to the formation of calcite or aragonite in the coral skeletons. Rahman et al. (2011) demonstrated that only a reactive extracellular protein, ECMP-67, isolated from soft coral sclerites, might be able to regulate and maintain the preferred skeletal mineralogy of the marine animals even when the ocean chemistry composition would favor the deposition of a different calcium carbonate variety. Such protein greatly facilitates in Octocorallia corals the formation of "sclerites", which are small biomineralized individual structures (some tens μm in length) or spicules, composed of an organic matrix and of a calcitic fraction. All sclerites of this subclass are composed of calcite and the shape is species-specific. Within a species they generally differ according to the anatomical site at which they are formed (Rahman et al., 2013).

Spicules contain colour creating pigments and are separated from one another initially, but they become subsequently fused with one another to form the solid axial skeleton of the coral colony. The fusion of the spicules, at the end of growth, is so complete that the individual outlines are entirely lost. Spicules are produced by the scleroblasts of the mesoglea, which is included in the coenenchyme, an external tissue that contains polyps. Spicules and axial skeleton are generally qualified as "hard tissues", while polyps and coenenchyme are called "soft tissues".

Very little information about the biology of the biomineralization process (called "biocalcification" in the case of corals) in the genus *Corallium* is available. Proteins and polysaccharides play an important role in biologically controlled calcification and the skeletal organic matrix plays a central role in the entire process. A recent work (Rahman et al., 2014) identified 147 key functional skeletal

proteins in one of the Japanese precious corals, *Corallium konojoi*. It has been demonstrated that the Japanese coral skeletons contain "a complex group of proteins that guide the biomineralization process to form specific, genetically determined structures". 1D SDS-PAGE showed two strong protein bands with apparent molecular weights of 300 and 98 kDa. Both were observed to be highly glycosylated, implying that they "play active roles in biocalcification and axial skeleton formation". Nevertheless, "further studies on the functional properties of the proteins of this species are necessary to shed new light on the biocalcification process in precious corals and other octocorallians".

CHROMOPHORES IN CORALLIUM RUBRUM: BACKGROUND AND STATE OF ART

The nature of the chromophores in *Corallium rubrum* is still under discussion. Among natural pigments, carotenoids have been proposed by many Authors as the most probable compounds responsable of the brightness and variety of colours of the precious "red coral". Carotenoproteins and other organic complexes (polyenic molecules or parrodiene species or psittacofulvines) have also been considered in order to explain the great stability of the colour. Nevertheless, to our knowledge, till now no pigments could have been fully characterized both from soft tissues and hard tissues of *C. rubrum* coral so that the problem is still not completely resolved.

All the interest of *Corallium rubrum* for jewellery consists in the brilliant red colour and in the hardness of the axial skeleton, which can be easily worked and polished. The first analysis to detect the skeletal colour started in the nineteenth century. Ranson and Durivault (1937) indicated in the red iron oxide, in compounds of animal origin and in carotenoid substances the cause of the coral colour. Nevertheless, the Authors could not detect any carotenoid by micro-chemical techniques so that the presence of these organic compounds has long been debated. Fox (1972) analyzed orange to deep pink corals and treated them with warm, concentrated aqueous acetic acid, which dissolved the $CaCO_3$. A dirty orange-yellow suspension was yelded whence no pigment was extractable with hexane.

Attempts were made by Merlin and Delé-Dubois (1986) to extract pigments from *Corallium rubrum* and *Tubipora musica* corals and from several species of shells. Specimens were ground to a fine powder in a mortar, treated with hot aqueous NaOH and washed with distilled water and ethanol. The residue was treated with aqueous Na_2EDTA solution or acetone, acidified by 5% HCl until all the carbon dioxide of the calcareous skeleton was emitted. The final solution was analysed by RR spectroscopy. Polyenic pigments were shown to be responsible of a large range of colours. By comparison of the spectra, two kinds of polyenic structures could be considered: carotenoids, which bear lateral methyl groups, and polyacetylenic chains without lateral methyl groups. This last class of pigments seemed to occur quite frequently, but Authors concluded that for a complete understanding of the structure careful extractions, purifications and chemical analysis followed by new spectroscopic investigations were required.

Veronelli et al. (1995) analysed RR spectra of synthetic carotenoids and of a large number of bird's feathers on the basis of the effective conjugation coordinate (ECC) theory. Spectra can be classified in two classes: class A, showing v_1=1511-1528 cm^{-1} and v_3=1155-1158 cm^{-1}, and class B, showing v_1=1521-1539 cm^{-1} and v_3=1131-1139 cm^{-1}. The first difference between the two classes is the presence of a medium-intensity line v_6 at ca. 1007 cm^{-1} together with the occurrence of the v_3 line at ca. 1156 cm^{-1} in the class A. When the v_6 line disappers, v_3 shifts to ca. 1135 cm^{-1}. According to Saito and Tasumi (1983) and Ermakov et al. (2005) v_3 couples with the in-plane rocking mode of the CH_3 group attached to the polyene chain. In fact, the Raman spectrum of tetradesmethyl-β-carotene (Okamoto et al., 1984), from which the four methyl groups have been removed, does not show the line v_6, and v_3 shifts to ca. 1135 cm^{-1} as in the spectra of class B. Authors concluded that the pigmentation in parrots is not due to the presence of carotenoids at all, but to unmethylated polyene molecules showing the same Raman spectrum of class B.

Rolandi et al. (2005) characterized corals with gem potential determining physical properties (density and refractive indices) and recording FTIR and Raman spectra. FTIR analysis confirmed the hard coral skeletons to be formed from calcium carbonate: with few exceptions, Octocorallia were found to be calcitic, Hexacorallia aragonitic and Hydrozoa either calcitic and aragonitic. The Raman spectra of *C. rubrum* showed the v_3 peak at about 1130 cm^{-1}, as do unmethylated polyenes, but also the weaker v_6 peak at ca. 1007 cm^{-1}, attributed to rocking motion of the molecule's methyl side groups (Saito and Tasumi, 1983; Ermakov et al., 2005), usually present in the spectrum of carotenes, but absent in the spectrum of unsubstituted polyenes or parrodienes (Maia et al., 2010).

Cvejic et al. (2007) determined the presence of carotenoids in natural samples of *C. rubrum* from Marseille, Riou, and investigated their chemical composition. Analysis was performed both on soft tissues and hard tissues, including spicules and skeleton, and extraction was done both with and without demineralization by EDTA. The extracts were analyzed by TLC, HPLC/DAD and HPLC/MS. Results showed that canthaxanthin (4,4'–diketo-β-carotene) was the major carotenoid present in skeletons, spicules as well as in soft tissues. Nevertheless, they concluded that there is the possibility that the colour can be due also to other natural pigments and therefore further work was needed to examine this hypothesis.

Smith et al. (2007) summarized the procedures that are useful to identify the colour origin of pink-to-red coral. The specific colour is influenced by the carotenoid's incorporation into the skeleton. In addition, carotenoids from complexes with other materials, most notably proteins, may significantly influence the colour exhibited.

Fritsch and Karampelas (2008), using Raman spectroscopy, proposed a mixture of unsubstituted polyenes as the most probable cause of colour of *C. rubrum*. Unsubstituted polyenes and carotenoids can be separated on the basis of their v_2 vibration, which is at about 1130 (± 15) cm^{-1} for unsubstituted polyenes and at approximately 1155 (±10) cm^{-1} for carotenoids. The 30 cm^{-1} shift in unsubstituted polyenes is due to the lack of methyl groups attached to the backbone carbon chain.

Brambilla et al. (2012) recorded the Raman spectra of *C. rubrum*, canthaxanthin and parrot feathers and proposed an interpretation with the help of quantum chemistry and of the effective conjugation coordinate (ECC) theory. It was stated that the Raman spectrum of *C. rubrum* is an interesting puzzle because it lies in between the spectra of carotenoids and psyttacofulvines. Evidence is found that the pigment does not posess a fully demethylated polyene chain (as in psyttacofulvins) nor a tetramethylated backbone chain (as in carotenoids). It was concluded that the pigment should consist of a sequence of approximately nine C=C bonds, and possible methylation states of the sp^2 chain was deduced: either one $–CH_3$ group near the end of the chain or two CH_3 groups probably symmetrically placed. The Raman spectrum does not provide any direct information on the chemistry of the two ends of the polyene chain.

MATERIALS AND METHODS

Collections

We analyzed 10 specimens of hard tissues (rough axial skeletons) of *Corallium rubrum* corals collected from several places of the Mediterranean sea: Torre del Greco (Figure 2), Sardinia, Sicily, Tuscany, France (Corsica and Marseille), Tunisia. We considered only specimens of gemmological interest.

Preparation of samples

Pigment extraction was performed on rough specimens, which were grinded into powder, then incubated in 1 M NaOH at 100 °C for 10 min. The solution was centrifuged (4000 g for 5 min), the pellet was washed with 1 M NaOH 15 min at 60 °C, centrifuged as above and dried. The powder was demineralized with 0.5 M chelating agent EDTA, which is wideley used for demineralization of invertebrate hard tissues (Cvejic et al., 2007 and references therein), for 48 h at 4 °C and dried.

Specimens were resuspended in chloroform and incubated at 4 °C for 5 days under vigorous shaking.

Figure 2. *Corallium rubrum* specimen (height 12 cm; width 13 cm) from Torre del Greco, used for the analyses.

After centrifuging at 4000g for 5 min, the supernatants were collected, dried, resuspended in acetonitrile/methanol (70/30 v/v) and loaded onto a C18 reverse phase HPLC column. Elution was carried out with the same solvent.

Analysis

Analysis was performed by means of High-performance liquid chromatography (HPLC/UV), Solution ^{1}H Nuclear Magnetic Resonance spectroscopy (^{1}HNMR) and Electrospray-ionization mass spectrometry (ESI-MS).

HPLC system with UV detection

We used a Jasco HPLC apparatus which consisted of a micro vacuum degasser, a binary pump and a UV detector. UV absorbance was monitored at 475 nm. All data processing as well as continuous online quantification and conditions control were performed by using a Jasco ChromNAV station software.

Conditions

Mobile phase consisted of Acetonitrile/THF/Water+0.1% Phosphoric acid (75/25/5 v/v). A 250 mm x 4.6 mm Lichrospher 100-RP18 (particle size 5 µm) column (Merck) and a Lichrocart 4-4-RP18 (10x4.6 mm particle size 5 µm) guard column were used. Flow-rate of 0.9 ml/min. Injection volume was 50 ml.

ESI-MS

A hybrid triple quadruple - time of flight mass spectrometer (QTOF, AB Sciex) equipped with a nano-electrospray source was employed for ESI-MS experiments. Samples

were injected by borosilicate-coated capillaries (Thermo Scientific) with 1μm internal diameter.

Conditions

HPLC fractions and molecular standards were lyophilized and resuspended in 80% acetonitrile, 1% formic acid before injection. Measurements were performed at room temperature with a spray voltage of 1100 V and a declustering potential of 80 V. The spectra are averaged over an acquisition time of 1 minute.

Reagents and standards

Ultrapure water was generated with Millipore water purification system, including reverse osmosis, activated carbon and ion-exchange cartridges. The chemical used were of analytical grade and the solvents of HPLC grade.

Trans-astaxanthin (3,3'-dihydroxy-4,4'-diketo-β-carotene) and trans-canthaxanthin (4,4'-diketo-β-carotene) (Figure 3), purchased by Sigma Aldrich, were used as standard compounds for comparison and quantification of colouring pigments.

Solution ^{1}H NMR Apparatus

A Varian Mercury AS-400 spectrometer operating at 11.7 T was used. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) using the residual solvent peak as an internal reference or TMS where added. ^{1}H NMR spectra were recorded in $CDCl_3$ at room temperature (recycle delay: 3 s, 1024 scans).

RESULTS AND DISCUSSION

The aim of our work was to confirm experimentally the previous theoretical Raman spectroscopic results (Brambilla et al., 2012) and at the same time to identify the structure of the biochromes in *Corallium rubrum*. As reported by Cvejic et al. (2007), spicules and skeletons are responsible for the red colour of the coral. This finding is supported also by Rahman et al. (2014), who observed that the colouration of the separated branches of *C. konojoi* "disappeared almost completely soon after the sclerites were isolated". We did not look for soft tissues specimens (with coenenchyme and spicules), but decided to investigate the intensive red coloured axial skeletons of the colony corals, which are used for gem materials and were more available, with the purpose to verify if the extraction of the colouring pigment could be possible also from the hard tissues. Owing to the organic solvents used for the analyses, because carotenoids are liposoluble, proteins mostly underwent denaturation. Only proteins covalently bound with carotenoids could have been detected, which are characterized by an UV absorption band at 280 nm. Nevertheless, our UV spectra (Figure 4) showed a band between 400 and 500 nm, matching the range of carotenoids (LaFountain et al., 2010), but any band at 280 nm appeared, implying that carotenoids are not covalently bound with proteins.

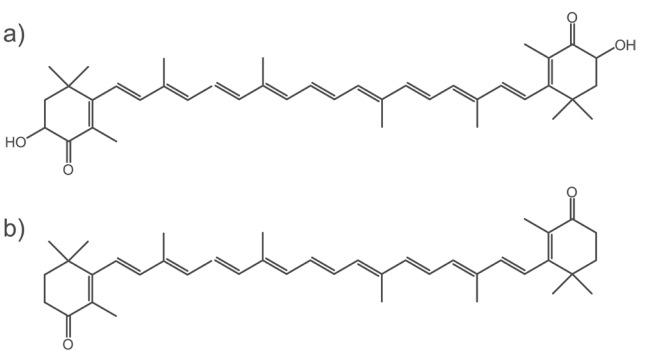

Figure 3. Chemical structure of a) astaxanthin; b) cantaxanthin.

We tried to characterize the chemical composition and structure of the colouring pigments in *Corallium rubrum* (Linnaeus, 1758) gem quality corals by means of high-performance liquid chromatography (HPLC system with UV detection), electrospray-ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (^{1}H NMR spectroscopy).

Carotenoids and psyttacofulvines are the most common colouring pigments in animals. Carotenoids are methylated polyenic chains, while psittacofulvins are fully de-methilated polyenic chains, therefore "unsubstituted polyenic" pigments. Carotenoids are the most common biochromes in aquatic animals and appear also in birds, with some exceptions however: parrots (class Aves, order Psittaciformes) use psittacofulvins as feather colorants.

Carotenoids and psittacofulvins share similar light-reflectance characteristics (Veronelli et al., 1995) and have similar solubility properties (Hudon and Brush, 1992). Carotenoids are acquired from dietary sources and then delivered to peripheral tissues for pigmentation (McGraw and Hill, 2001), while the physiological or anatomical origins of psittacofulvins are unknown (Stradi et al., 2001). However, according to McGraw and Nogare (2004), these pigments cannot originate from the diet.

In our opinion, the characterization of the colouring pigment was connected to the possibility of determining either the number and position of the $–CH_3$ groups linked to the polyene backbone chain and the chemical structure of the two ends of the chain. As the Raman spectroscopy cannot reveal the presence and the structure of the end-rings of the backbone chain of carotenoids, we decided to try to use the solution ^{1}H NMR spectroscopy to investigate the chemical composition of the chromophores.

The NMR spectroscopic diagnostic signals identifying carotenoids are to be found in the range between 6.3-6.7 ppm (diagnostic for polyene chains) and in the range between 1-2 ppm (diagnostic for $–CH_3$ groups).Theoretical information given by Raman spectroscopy have shown

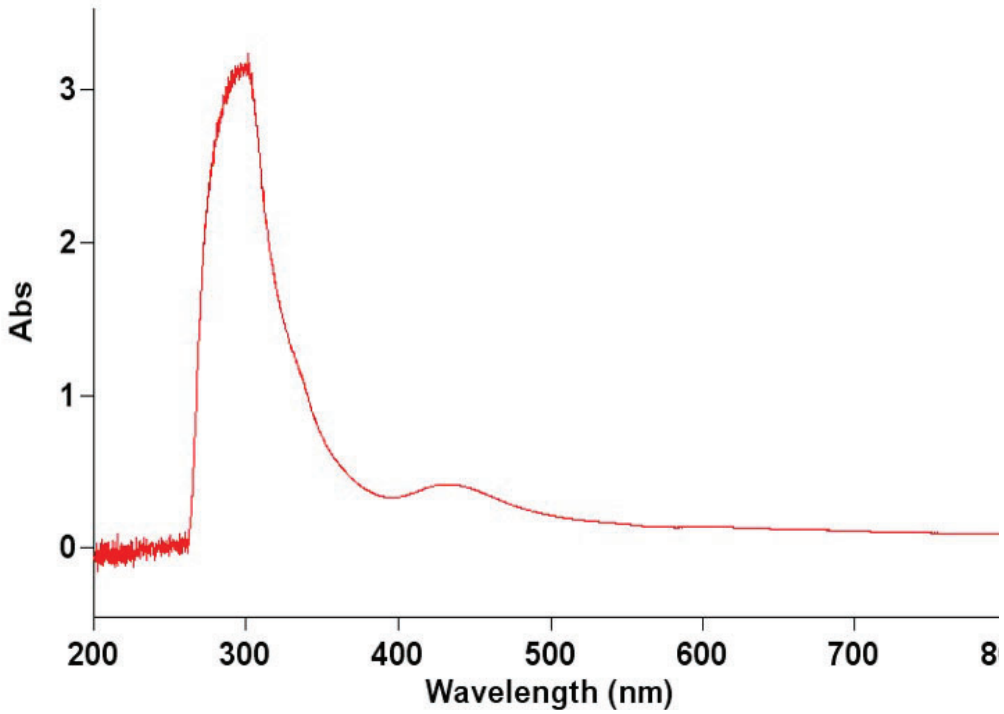

Figure 4. UV Absorption spectrum of *Corallium rubrum* in chloroform solution.

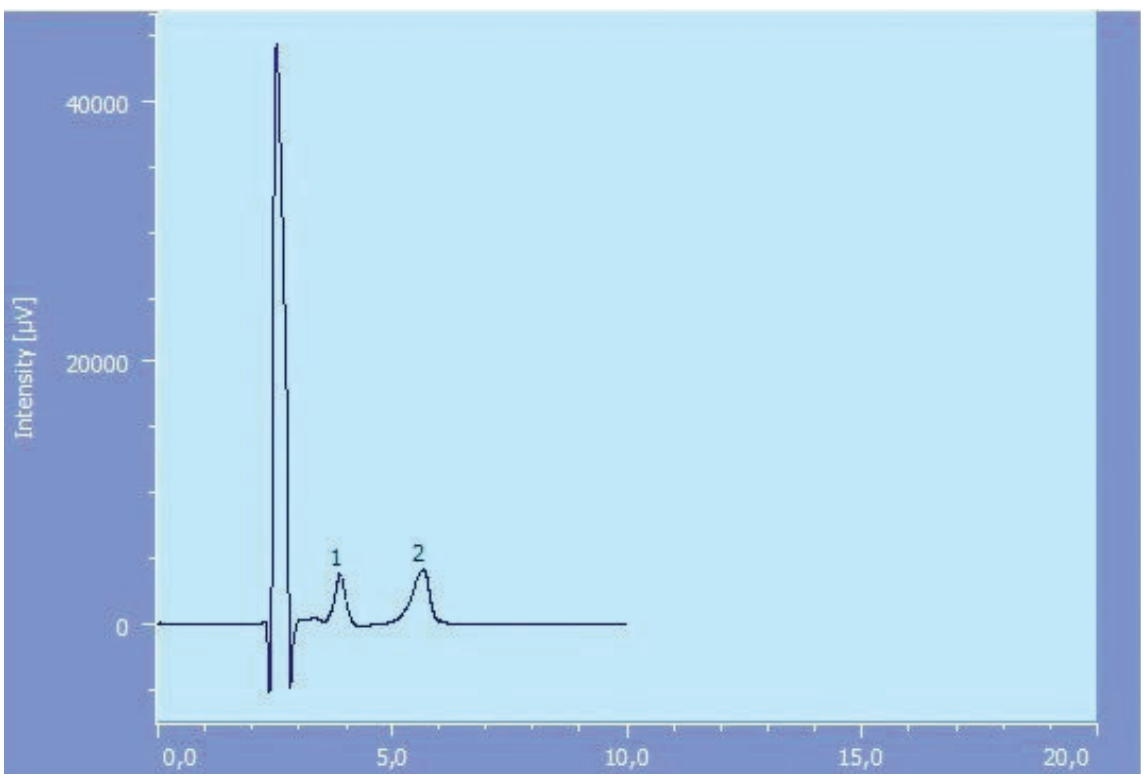

Figure 5. HPLC/UV chromatogram performed on astaxanthin and canthaxanthin used as reference standards: peak 1-10 ng astaxanthin (t_R=3.86 min); peak 2-10 ng canthaxanthin (t_R=5.67 min).

that the colouring pigment in *C. rubrum* should contain one or two $–CH_3$ groups in the polyene backbone chain and therefore it could be a carotenoid, even if partially demethylated. The first NMR spectra collected on some coral specimens showed signals in the over-mentioned ranges. Nevertheless, due to the presence of signals of other organic compounds in the same range, the obtained NMR spectra needed more resolution.

For that reason, before collecting further NMR spectra, specimens were purified by means of HPLC complementary method with the purpose to obtain more resolved and diagnostic signals (as reported in the following literature: Cvejic et al., 2007; Holtin et al., 2009; LaFountain et al., 2010; LaFountain et al., 2013).

HPLC/UV spectra were recorded by comparison with reference stardards (Figure 5) of synthetic astaxanthin (t_R=3.86 min) and synthetic canthaxnthin (t_R=5.67 min). The analysis performed on all samples after demineralization gave diagnostic chromatograms showing two intense peaks respectively at t_R=2.87 min and t_R=3.28 min (Figure 6). Only the sample of Torre del Greco, characterized by a more intense red colour (Hue 7.5 R, value/chroma between 4/8 and 4/10 of the Munsell Book of Color, Glossy Collection) and of which a great quantity of material was available (see Figure 2), showed two additional weaker peaks featured at t_R=3.84 min and t_R=5.60 min. According to the retention time value, it could be deduced that the sample from Torre del Greco (Figure 7) contained traces of astaxanthin and possibly of canthaxanthin. In some spectra of the same sample the astaxanthin peak is not shown, while it is enhanced the intensity of the peak at t_R=2.87.

ESI-MS analysis was also carried out to try to determine the accurate mass of the colouring pigment. In all the coral samples two distinct signals, respectively featured at 579.2 and 506.9 mass-to-charge ratio (Figure 8), indicate the possible presence of modified forms of carotenoids. In fact, the first signal could be ascribed to astaxanthin without one water molecule (-18 Da), which seems to be confirmed by the lower retention time (2.87 min) showed by the HPLC chromatograms, caused by the change of polarity due to the loss of water. The second signal could be thought as a lycopene (536.87 *m/z*) lacking of two methyl groups in the backbone chain, or as a similar modified carotenoid. Comparing the ESI-MS spectra of synthetic astaxanthin and canthaxanthin with the fractions eluted by HPLC/UV of the coral sample from Torre del Greco (Figure 9), the presence of astaxanthin is confirmed by the signal at 597.4 mass-to-charge ratio, corresponding to the protonated form of the carotenoid, together with traces of oxidized canthaxanthin (581.34 *m/z*).

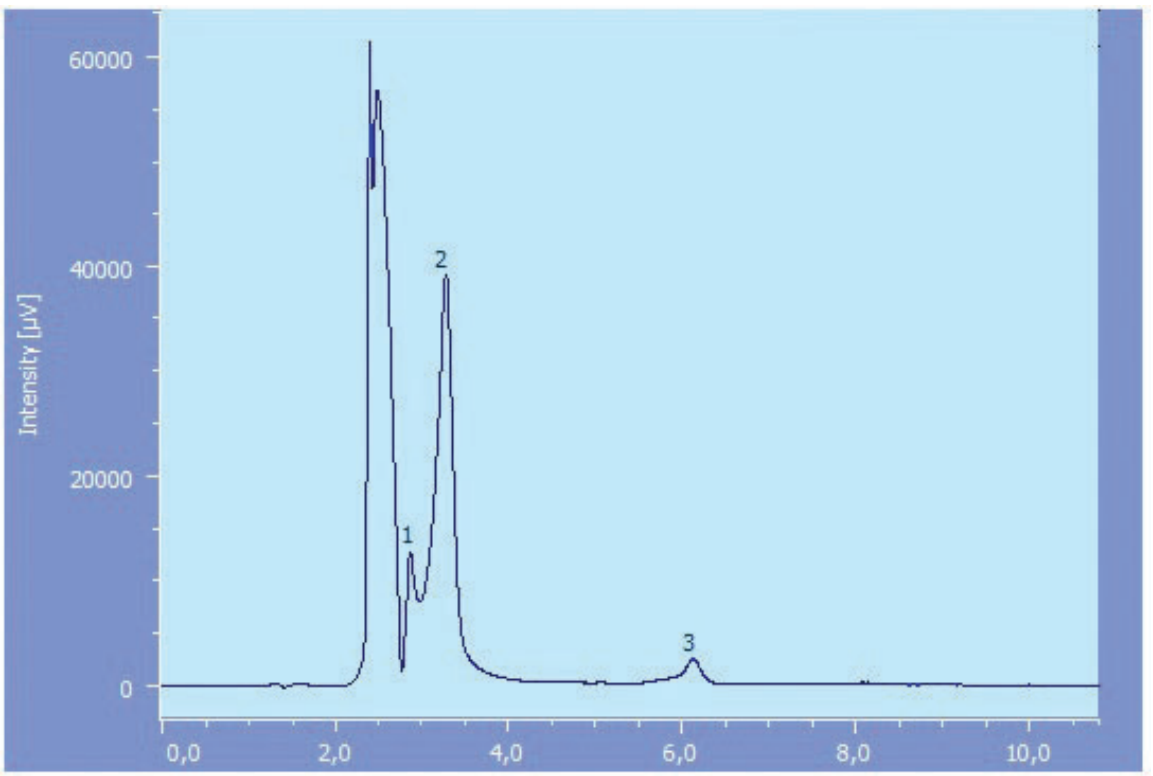

Figure 6. HPLC/UV chromatogram performed on the demineralized skeleton of a *C. rubrum* specimen, showing peak 1-t_R=2.87 min; peak 2-t_R=3.28 min.

The peak at 579 *m/z* is to be seen also in the ESI-MS spectrum of standard astaxanthin after a night at room temperature (Figure 10a). To verify that the peak at 579 *m/z* of Torre del Greco sample matches the molecule of dehydrated astaxanthin, ESI-MS/MS fragmentation spectra of this peak of both standard and sample have been recorded (Figure 10). Similar products were obtained (Figure 10 c,d), indicating that the molecule is the same.

The solution ^{1}H NMR spectroscopy was again performed only on the coral sample from Torre del Greco, of which a great quantity (about 20 g) was available. The sample was previous HPLC purified. Though the measurement times have been longer than those of the standard samples (10 min), so as to collect enough scans (1024) to be able to detect very weak features, in the range typical of carotenoids we could only record some signal traces, which do not allow us to ascribe them to carotenoids or to unsubstituted polyenic systems. We presume that such poor result has been due to the small quantity of the tested material. According to the calculations done on the base of the quantity of pigment (about 700 ng) used for the HPLC/UV technique and on the base of the quantity of pigment (2 mg) of the standard samples used for NMR spectroscopy, we deduced that to identify the pigment structure with the NMR method it would be necessary a quantity of material of an order of magnitude three times higher. As we have to deal with gemological material, which is very expensive and not easy to find, it can be desumed that at the moment the NMR method is not the most suitable to solve the problem.

CONCLUSIONS

Our findings can be summerized as follows:

The retention times from HPLC/UV chromatograms show the presence of unknown colour creating organic compounds and of traces of the astaxanthin carotenoid;

ESI-MS spectra recorded on the fractions eluted by HPLC/UV reveal the presence of modified carotenoids and of traces of astaxanthin as colour creating pigments;

The ^{1}H NMR spectra do not allow to identify the chemical structure of the biochromes, due to the small quantity of the pigment in the samples.

The UV absorption matches the range of carotenoids and the mass-to-charge ratio values of the ESI-MS spectra suggest the presence of modified carotenoids (dehydrated astaxanthin and possibly partially de-methylated llycopene as colour creating compounds). If so, the results obtained by Brambilla et al. (2012) on the base of the effective conjugation coordinate (ECC) theory seem to find a confirmation: at least one colouring pigment does not posess a tetramethylated backbone chain. Unfortunately, due to the scarsity of the material, we could not identify their chemical structute by NMR spectroscopy. Taking into

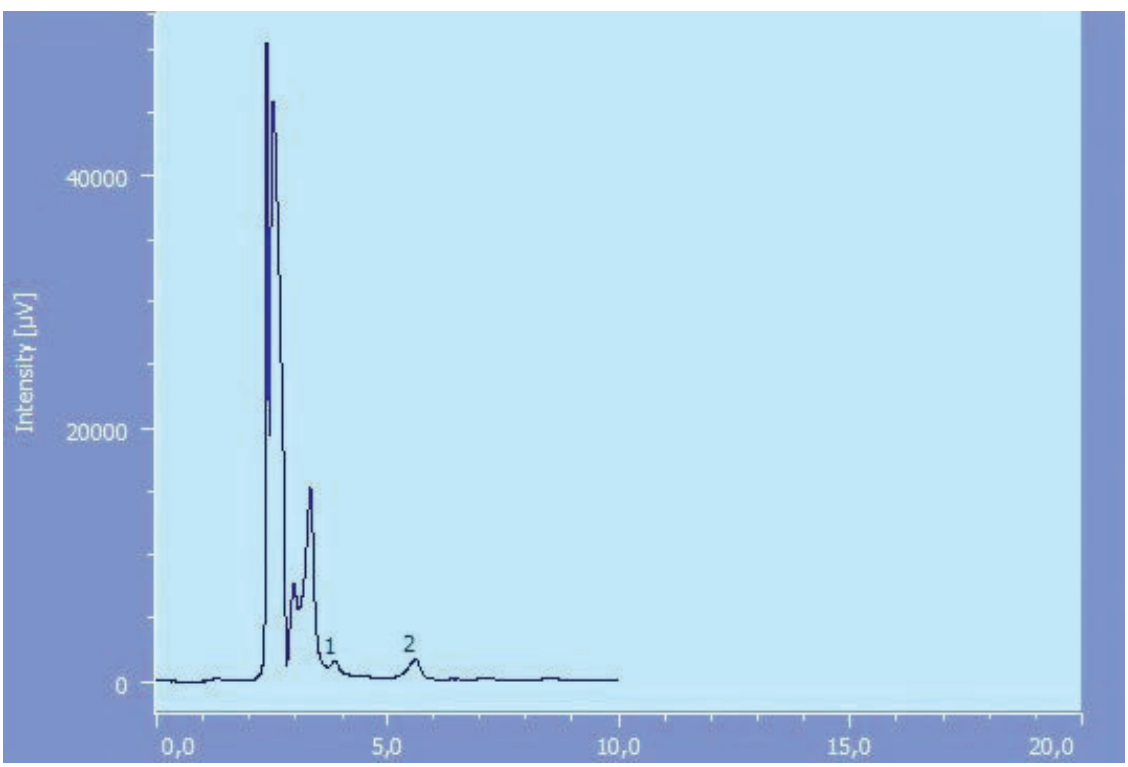

Figure 7. HPLC/UV chromatogram performed on the demineralized skeleton of the coral specimen from Torre del Greco, showing peak 1-t_R=3.84 min; peak 2-t_R=5.60 min.

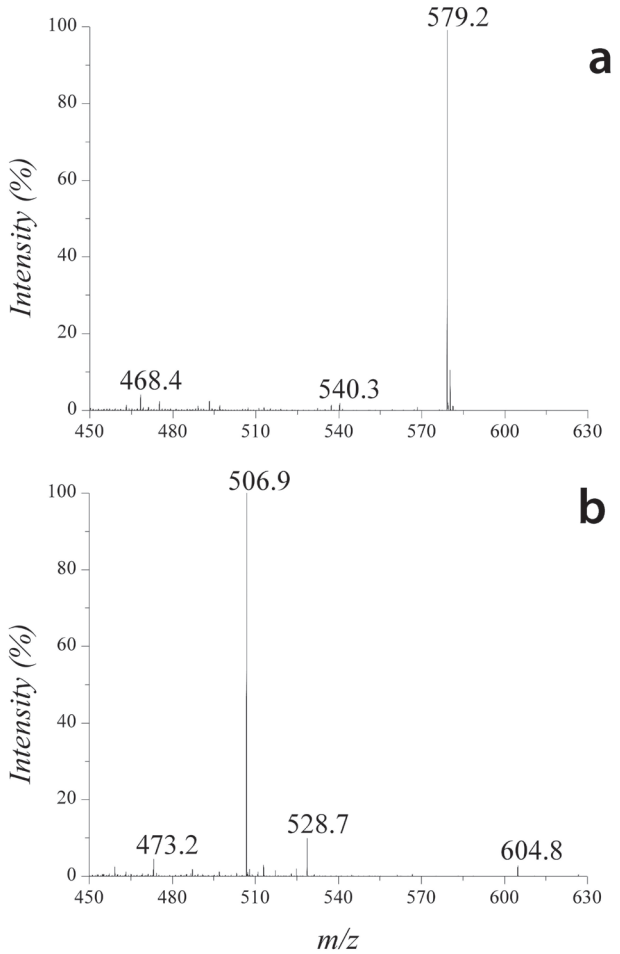

Figure 8. ESI-MS spectra of HPLC elution peaks 1 (a) and 2 (b) of the sample Torre del Greco. The most intense peaks are labelled by their mass-charge ratio (*m/z*).

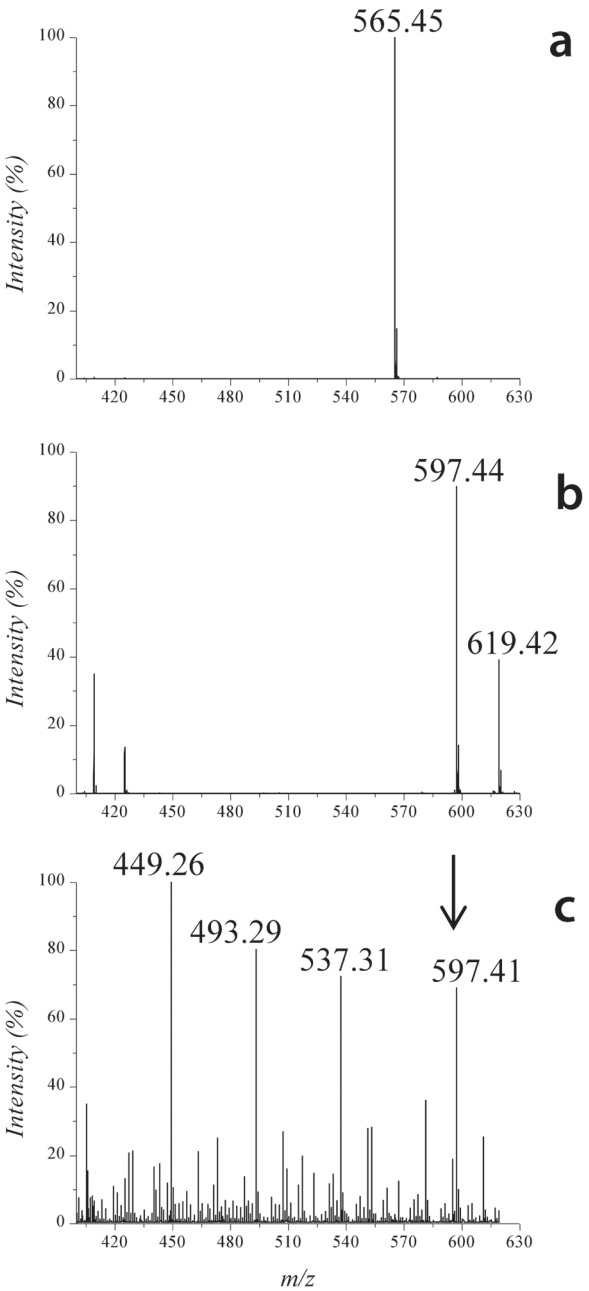

Figure 9. ESI-MS spectra of synthetic astaxanthin and canthaxanthin compared with the fraction eluted by HPLC/UV of the sample from Torre del Greco. The peak at 581.34 *m/z*, before that of astaxanthin (597.41 *m/z*), could be ascribed to oxydized canthaxanthin.

account ESI-MS spectra, it was also found that the colour of the skeleton of *C. rubrum* corals is to a lesser extent linked to the presence of traces of the astaxanthin carotenoid.

The presence of unsubstituted polyenes cannot be excluded, but it seems less probable. In fact, taking into account the weaker v_6 peak at ca. 1007 cm^{-1} attributed to the rocking motion of the molecule's methyl side groups (Saito and Tasumi, 1983; Ermakov et al., 2005), which appears in the Raman spectra of *Corallium rubrum*, the hypothesis of the presence of partially de-methylated carotenoids, laying in between carotenoids and psyttacofulvines, seems the most plausible.

It must also be underlined that the supply of carotenoids in corals is an established fact. It is clear that *Corallium rubrum* corals have the capacity to incorporate and accumulate from dietary sources carotenoids and metabolically derived carotenoids in the body. It is therefore more plausible to suppose that they have the physiological ability to carry on them to the skeleton and to bind them to the calcitic calcium carbonate, instead of to produce de novo fully de-methylated compounds as biochromes in the hard tissues of the colonies.

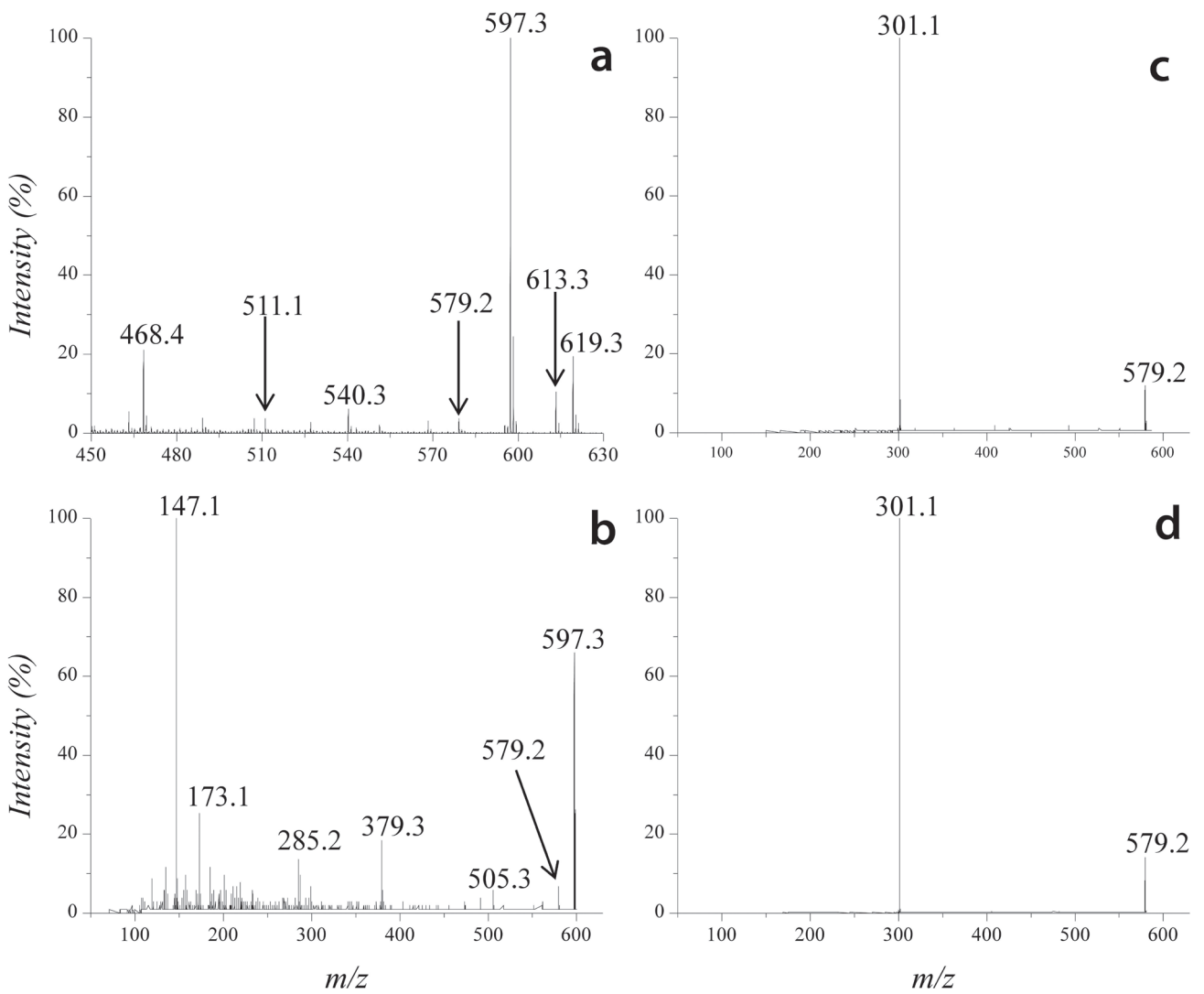

Figure10. (a) ESI-MS spectrum of standard astaxanthin. (b) ESI-MS/MS fragmentation spectrum of the peak 597.3 from panel A. (c) ESI-MS/MS fragmentation spectrum of the peak 579.2 from panel A. (d) ESI-MS/MS fragmentation spectrum of the peak 579.2 from panel A of Figure 9 (sample Torre del Greco). The most intense peaks are labelled by their mass-charge ratio (*m/z*).

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