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Ultrasound extraction and thin layer chromatography–flame ionization detection analysis of the lipid fraction in marine mucilage samples

M. Mecozzi*, M. Amici, G. Romanelli, E. Pietrantonio, A. Deluca

Istituto Centrale per la Ricerca Scientifica e Tecnologica Applicata al Mare, Via di Casalotti 300, 00166 Rome, Italy

Abstract

This paper reports an analytical procedure based on ultrasound to extract lipids in marine mucilage samples. The experimental conditions of the ultrasound procedure (solvent and time) were identified by a FT-IR study performed on different standard samples of lipids and of a standard humic sample, before and after the sonication treatment. This study showed that diethyl ether was a more suitable solvent than methanol for the ultrasonic extraction of lipids from environmental samples because it allowed to minimize the possible oxidative modifications of lipids due to the acoustic cavitation phenomena. The optimized conditions were applied to the extraction of total lipid amount in marine mucilage samples and TLC–flame ionization detection analysis was used to identify the relevant lipid sub-fractions present in samples.

Keywords: Ultrasound extraction; Extraction methods; Lipids

1. Introduction

The sonication of biological samples in organic solvents represents an alternative technique to the Soxhlet method which is usually used for the extraction of the lipid fraction from different matrices, because ultrasound allows a remarkable reduction of extraction times from 16 to 18 h required by applying the Soxhlet technique to a few minutes [1–4]. The reduced times of extraction depend on a complex system of physical and chemical reactions called acoustic cavitation which produces high energy and a high contact between solvent and solute [5]. However, the application of ultrasound to the extraction of lipids from environmental samples must be carefully evaluated because during the sonication step, reactive transients such as radicals and the pyrolytic conditions produced by the acoustic cavitation [6,7] can be responsible for undesired oxidative modifications of the extracted lipids.

With the aim to apply ultrasound to the extraction of lipids from marine mucilages, we studied by FT-IR spectroscopy, the effects of the sonication in two solvents (methanol and diethyl ether) on standard samples of humic acids, *n*-alkanes, fatty acids, cholesterol, alcohols, esters and chlorophyll which are the natural constituents of the lipid fraction in marine samples [8].

The optimized experimental conditions found were applied to the extraction of lipids from marine

*Corresponding author. Tel.: +39-06-6157-0458; fax: +39-06-6156-1906.

E-mail address: mecoma@rdn.it (M. Mecozzi).

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mucilages coupled to TLC–flame ionization detection (FID) chromatography to identify the different lipid classes.

2. Experimental

2.1. Sampling

Marine mucilages were sampled in the Northern Adriatic Sea during the summer of 2000 and in different places of the Tyrrhenian Sea during summer 1999 and 2000. All the samples were stored frozen at −20 °C until the purification process was performed.

2.2. Mucilage pre-treatment

All the samples were dialysed (Spectrum 1000 dialysis membrane) to eliminate inorganic salts and then lyophilized prior to sonication and TLC–FID analysis. Samples were stored in dark glass containers. The same procedure was used for marine sediments.

2.3. Fourier transform infrared spectroscopic study on sonicated standard samples of lipids

Standard samples of humic acid and different lipid compounds were dissolved in methanol and diethyl ether (5%, w/v). The solution was sonicated at room temperature in an ultrasonic cleaning bath Elma operating at 35 kHz. The sonication consisted of six replicate treatments, 15 s each, for a total sonication time of 90 s. After each sonication, 1 ml of solution was collected, added to 100 mg of KBr. After the evaporation of the solvent, the pellet of KBr was used for recording the FT-IR spectrum.

A Fourier transform infrared spectrophotometer Jasco Model 410, equipped with an EasiDiff diffuse reflectance accessory, was used. All the spectra were collected after 520 scans at 4 cm^{-1} resolution using the cosine function as apodization process.

Spectral correlation analysis [9] was applied to verify the modification caused by ultrasound. It was performed on the digitized ASCII files of the FT-IR spectra by using laboratory-developed Microsoft Basic software.

2.4. Determination of the lipid fraction by sonication and TLC–FID analysis

The extraction of the lipid fraction was performed by sonication of the samples in the above ultrasonic cleaning bath. Mucilage sample (300–500 mg) was added to 6–10 ml of diethyl ether and sonicated for 30 s. After the separation of the supernatant by centrifugation, the sonication with diethyl ether was repeated twice on the residue and the extracts were collected; the solvent was evaporated under vacuum and the residue was used for the gravimetric estimation of the lipid fraction. Then the residue was re-dissolved with 1 ml of chloroform for the TLC–FID analysis.

TLC–FID analysis was performed by an Iatroscan thin-layer chromatograph (Model Mark V) equipped with a flame ionization detector and connected to a personal computer for collecting the chromatograms as ASCII files.

For the analysis by TLC–FID, the extracts (5 μl) were spotted at the origin of the silica rod (Chromarod) using a microliter syringe. Then the development of the rods to resolve the main lipid classes, was performed by an elution phase consisting of hexane–diethyl ether–acetic acid, 97.8:2.0:0.2, v/v. The constant humidity was reached by placing a saturated NaCl solution in the tank [10].

2.5. Estimation of lipid phosphorus in mucilage samples by colorimetric determination of P

The method described by Aspila to detect total P in sediment samples was used for the estimation of organic (lipid) phosphorus content in the extracts coming from marine mucilages [11]. The residue of the diethyl ether extract obtained after evaporation, was placed in an oven at 550 °C for 2 h. The residual ash was added with 25 ml of 1 *M* HCl and shaken at room temperature for 18 h.

For the determination of P, 0.5 ml of ascorbic acid solution (10%, w/w, in 4 *M* H_2SO_4) and 0.5 ml of the ''mixed reagent'' [2% (w/w) ammonium heptamolybdate tetrahydrate, 0.2 (w/w) potassium antimony tartrate, 3 *M* H_2SO_4] were added. The absorption was measured at 880 nm in 50 mm quartz cell against a spectrophotometric blank consisting of

deionized water and using a standard solution of KH_2PO_4 in the range 0–5 μM/l for the instrumental calibration. The limit of detection attainable by applying this procedure was 0.3 $\mu g/g$ of P. The determination of P was performed by means of a Varian DMS 200 double beam spectrophotometer (spectral resolution 0.1 nm).

2.6. Chemical reagents

All the reagents were of analytical reagent grade Carlo Erba (Milano, Italy) and only ultra-pure deionized Milli-Q water (Millipore, Bedford, MA, USA) was used.

3. Results and discussion

3.1. A re-examination of the ultrasound extraction of lipids from environmental samples

Most papers reporting the ultrasound extraction of lipids from biological samples show neither the possible oxidative effect of ultrasound on the different lipid fractions nor the effect due to the solvent used which could minimize or increase the oxidative power of ultrasound. With the aim to clarify these two aspects, the first step of our study was to identify the most suitable solvent for the ultrasound extraction of lipids in marine samples.

As the most common solvents for the ultrasound extraction of lipids in biological samples are diethyl ether and methanol [1–3], we collected the FT-IR spectra of a humic standard sample in these solvents. The sonication time ranged between 0 and 90 s because previous studies have shown that a treatment consisting of three replicated sonications (30 s each) of samples by using diethyl ether gives recoveries of total lipids comparable to those obtained by using the Soxhlet extraction with ethyl ether [1]. In Figs. 1 and 2 we report the FT-IR diffuse reflectance spectra of a standard sample of humic substance sonicated in ethyl ether and methanol respectively and compared with the unsonicated sample. The spectra of the extracts of the humic sample in the two solvents before the sonication show the relevant differences existing in the chemical composition of the extracts depending on the different values of the partition

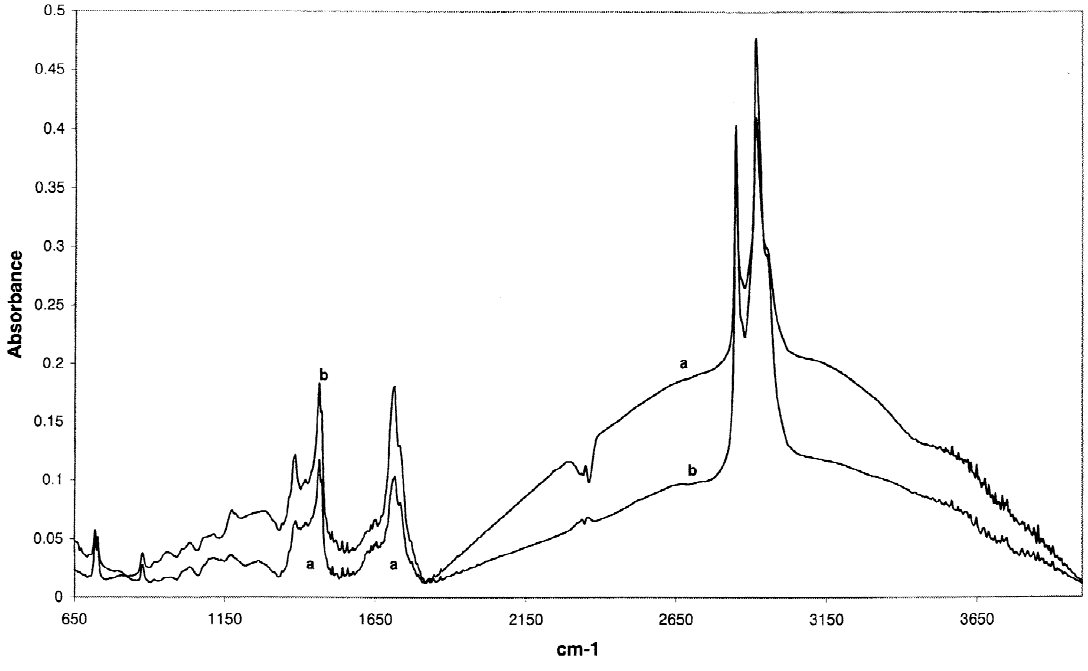

Fig. 1. FT-IR reflectance spectra of a standard sample of humic substance before (a) and after 90 s of sonication (b) in diethyl ether.

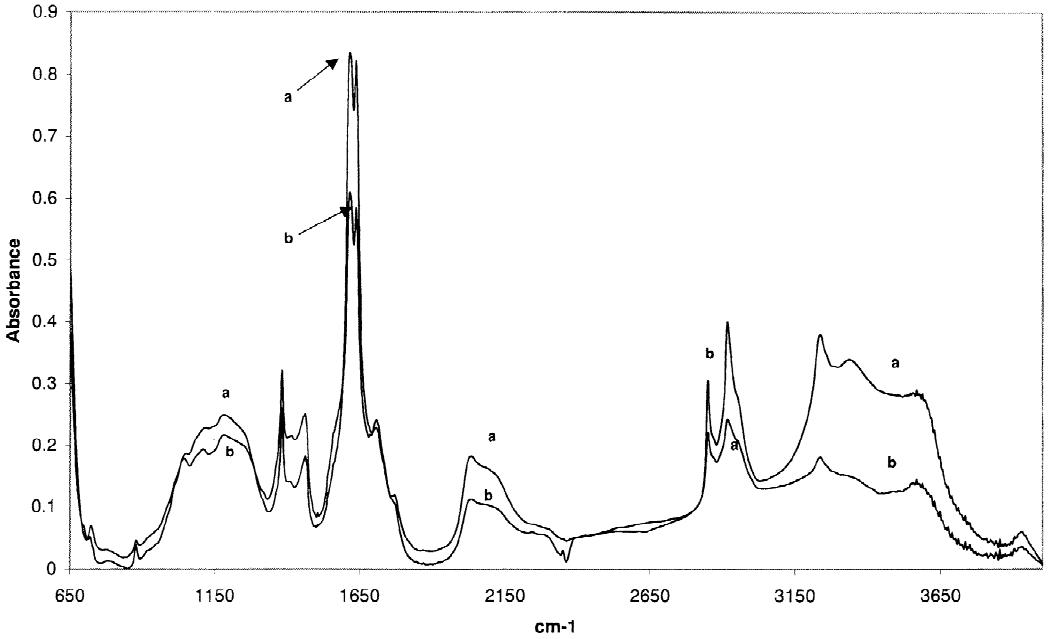

Fig. 2. FT-IR reflectance spectra of a standard sample of humic substance before (a) and after 45 s of sonication (b) in methanol.

coefficients of the lipid compounds in the two solvents. Other differences are present between the unsonicated and sonicated samples and these differences depends on the effect of ultrasound. The spectra obtained in diethyl ether show that no appreciable differences between the two samples until 60 s of sonication and the differences become more significant after 90 s of sonication (Fig. 1). These differences are represented by the variation of intensity of the bands of the –C=O group of esteric and acid compounds between 1710 and 1740 cm^{-1}, aliphatic –CH (between 2850 and 2950 cm^{-1}), alkenic –CH (between 3010 and 3050 cm^{-1}) and hydroxyl group of acid and alcoholic groups (between 3200 and 3500 cm^{-1}).

As far as the sonication in methanol, the modifications of the spectrum involves the variation of the intensity of the same bands observed in the case of the sonication with ethyl ether but these modifications become significant after 45 s of sonication (Fig. 2) and tend to increase with the increasing times of sonication. A large decrease involves the bands between 1700 and 1740 cm^{-1}, the typical band of the –C=O group of acid and esteric compounds; the reduction of these bands could depend on the decarboxylation of these compounds as already observed in the sonication of humic substance [12]. Contemporary, other new bands are present within 1500 and 1000 cm^{-1}. These bands depend on the structural modifications of the lipid fraction which can involve both oxidative and non oxidative (hydrolysis and isomerization) processes. Due to the high heterogeneity of the lipid fraction, these new bands can be hardly identified but, however, they remain the evidence of the modifications caused by ultrasound in methanol. The oxidative degradation of organic compounds depend on the interaction between ultrasound and solvent which produces heat (i.e. pyrolitic conditions) [5] and radicals [13]. As a consequence of the oxidation caused by ultrasound, new compounds such as peroxides, aldehydes oxy and hydroxy derivatives are introduced in the organic extracts but the identification of these compounds is a less interesting goal than the identification of the lipid compounds which are degraded by ultrasound more easily. So the second step of our study was to test the relative stability of different lipid standards and to identify the limit of application of ultrasound procedure with diethyl ether. For this second part of our study, chlorophyll, cholesterol, docosane,

phthalic acid, ethyl oleate and methyl arachidate, cetil alcohol and a mixture of stearic and palmitic acid were chosen as references of the different classes present in the lipid fraction of marine samples [8]. They were dissolved in ethyl ether, sonicated at different times, as already shown for the humic standard and their FT-IR spectra were collected. The spectra were compared by spectral correlation analysis applied both on the overall IR range (650–4000 cm^{-1}) and in the ''fingerprint region'', to have a direct evidence of possible degradation produced by ultrasound. The results are reported in Figs. 3 and 4.

The different behaviors of the lipid standards are evident. Some compounds such as saturated hydrocarbons (docosane), saturated fatty acids (mixture of palmitic and stearic), aromatic acids (phthalic), saturated esters (methyl arachidate) cetilic alcohol and cholesterol show negligible modifications of their structural characteristics because the value of the spectral correlation coefficients is always close to 1. Moreover, in some cases such as saturated fatty acids and alcohols, the coefficients of spectral correlation analysis performed on the overall spectrum and in the fingerprint region are overlapped.

For other types of lipid compounds, the use of ethyl ether has some drawbacks which must be taken into account. The values of the coefficient of the spectral correlation coefficient for chlorophyll (Fig. 3) and ethyl oleate (Fig. 4), show relevant structural

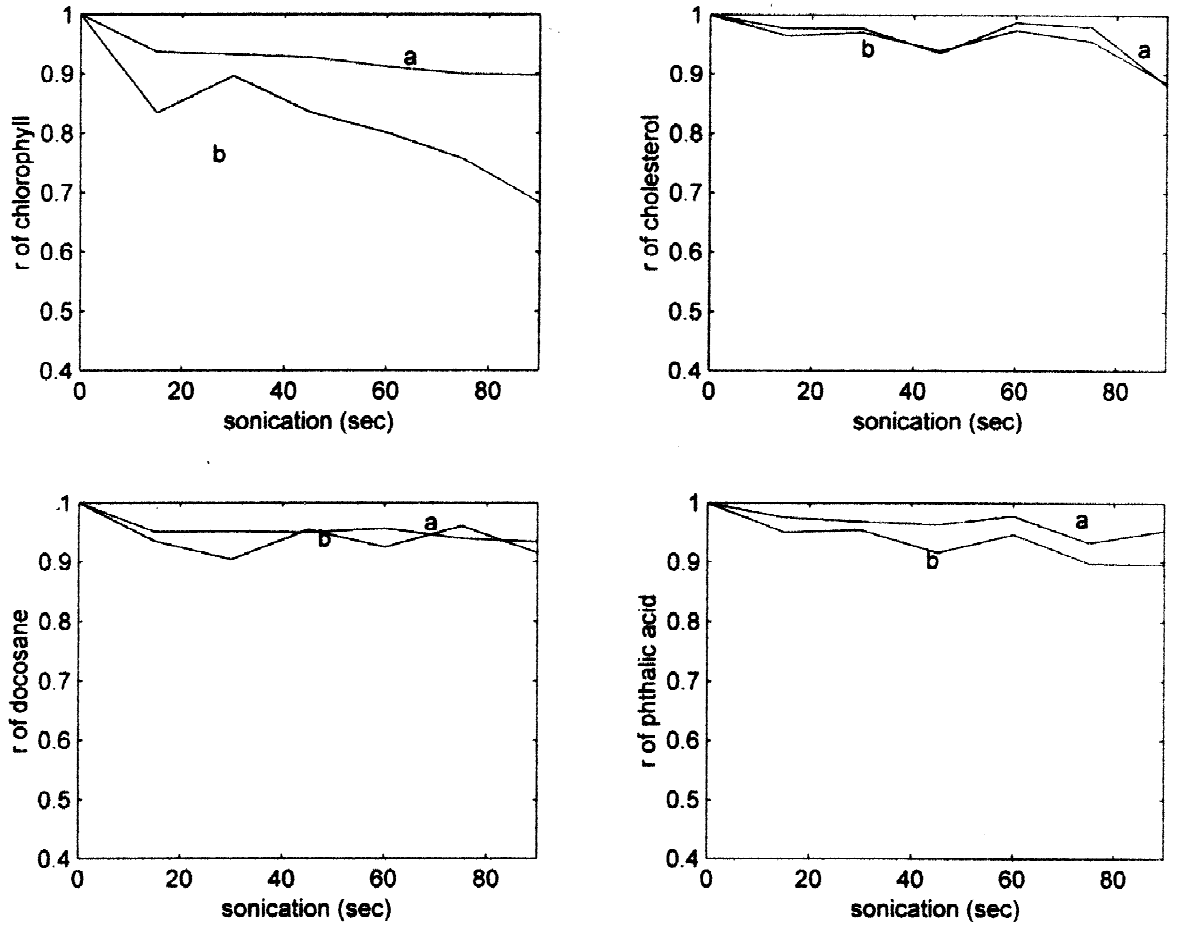

Fig. 3. Values of the correlation coefficient (*r*) for spectral correlation analysis for the overall spectrum (a) and for the finger print region (b) of different lipid standards at different time of sonication in diethyl ether.

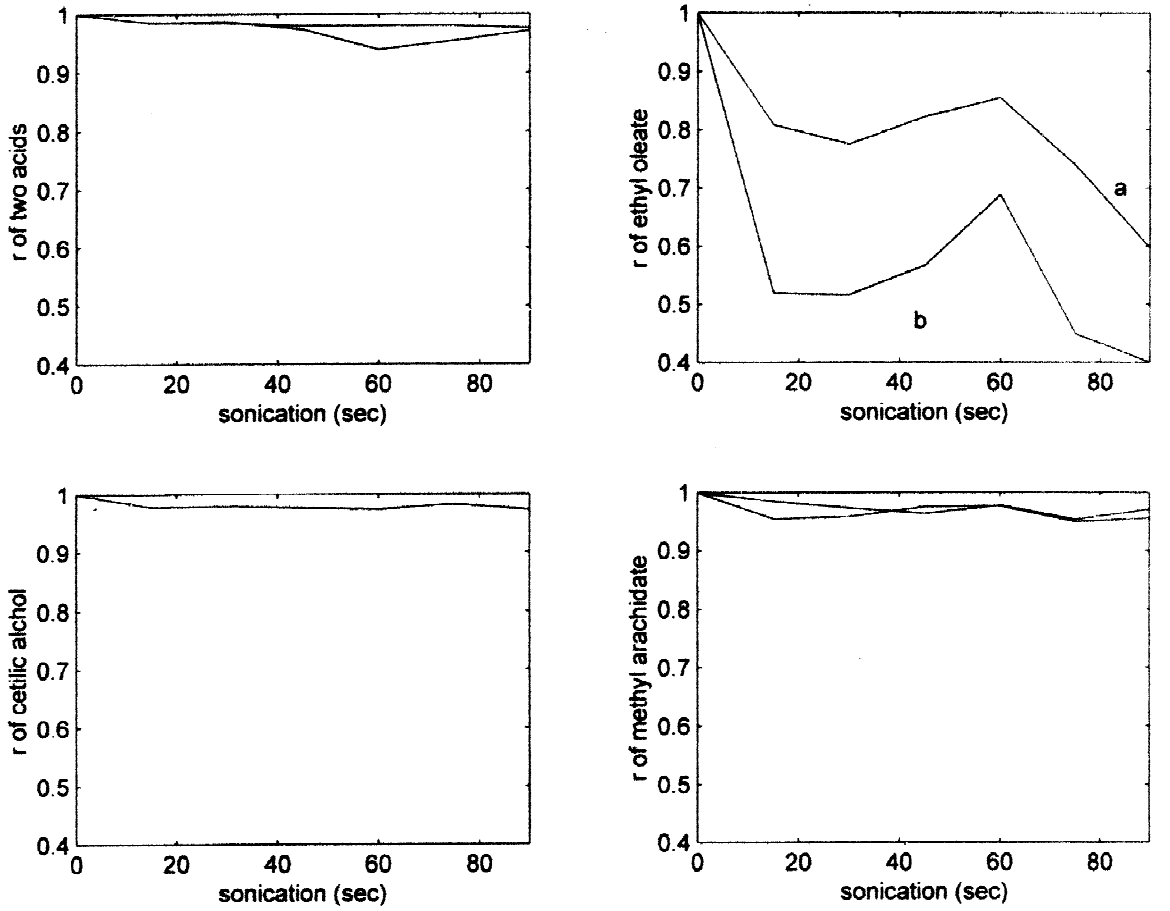

Fig. 4. IR values of the correlation coefficient (*r*) for spectral correlation analysis for the overall spectrum spectral for the overall spectrum (a) and for the finger print region (b) of different lipid standards at different times of sonication in diethyl ether. The ''a'' and ''b'' indices are reported for ethyl oleate only because the curves of the spectral correlation analysis of unsonicated and sonicated of the other sample solutions are overlapped.

modifications with respect to the unsonicated sample. As far as the ethyl oleate is concerned, this modification involves the increased absorption of the bands between 1500 and 1000 cm^{-1} as already shown by the spectra of humic sample in diethyl ether and the presence of a new band at 3450 cm^{-1} due to the presence of the –OH hydroxyl group. This band could be the result of the oxidation of an olefinic group into an alcoholic group. In contrast, the band at 1740 cm^{-1} does not seem involved in oxidative reactions (Fig. 5), because the appearance of two other bands at 1700 and 1715 cm^{-1} it is not observed [13]. Moreover, the absence of oxidative reactions of the –C=O esteric group is confirmed by the result of the coefficient of the spectral correlation analysis performed on a saturated esteric compound such as methyl arachidate (Fig. 3). So we can suppose that also other esteric lipid compounds such as diglycerides, triglycerides and phospholipids are not involved in oxidative modification by the action of ultrasound in the experimental condition used.

Concerning the oxidation of –C=C– double bonds, it should be observed that some differences exist between the double –C=C– bond of a linear fatty chain such as ethyl oleate and the double –C=C– bond of a cyclic condensed structure such as cholesterol. The plot of the spectral correlation coefficient ''*r*'' for ethyl oleate diminishes down to a

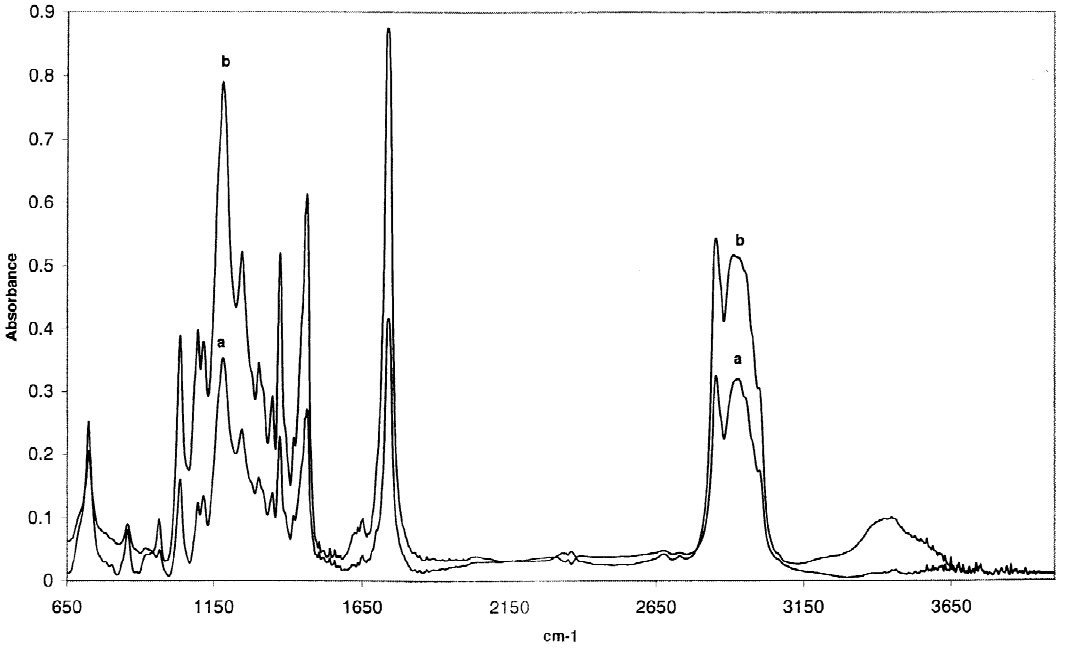

Fig. 5. FT-IR spectra of the unsonicated (a) ethyl oleate sample and of the same sample after 90 s of sonication (b) in diethyl ether. *x*-axis: nm^{-1}; *y*-axis: .

value close to 0.5 after 15 s of sonication (Fig. 2), while the value of ''*r*'' for cholesterol remains higher than 0.9 after 90 s of sonication (Fig. 4) showing that the oxidation rate of ethyl oleate is faster than cholesterol. The different rates between cholesterol and ethyl oleate can depend on the different steric hindrance with respect to the attack of reactive transients produced by the acoustic cavitation. Cholesterol with its condensed cyclic structure, has a higher steric hindrance than a linear structure of a lipid compound such as ethyl oleate and so it is less reactive than the former.

In conclusion we can claim that the use of diethyl ether as solvent is more reliable than methanol for the ultrasonic extraction of lipids. By using diethyl ether as solvent, only chlorophyll and compounds having the unsaturated –C=C– bond in a linear fatty chain can be modified by complex reactions, while in methanol, the experimental results show that other groups are involved in oxidative degradations faster than in diethyl ether. In any case, as the sonication between 20 and 50 kHz of unsaturated fatty chains produces other fatty structures by means of polymerizations [14,15], the extraction by sonication at room temperature in diethyl ether is still reliable for the determination of the total lipid fraction in biological samples and for their qualitative characterization. Vice versa, the use of methanol as solvent for the ultrasound extraction is not recommended because of the complex and faster oxidative reactions than those observed for diethyl ether.

3.2. Determination of lipids in marine mucilage samples

We applied the described ultrasound technique to the determination of lipids present in marine mucilage samples. The phenomenon of mucilage appearance in the Italian seas consists of the presence of relevant gelatinous aggregates of organic matter suspended along the water column. The appearance of mucilages is a casual occurrence either in time and dimension of aggregates [16–18] and sometimes the phenomenon has reached such an intensity as to cause serious damages to tourism and fishery industries in the Adriatic Sea.

With respect to the possible origin and causes of the phenomenon, some experimental evidences have

shown that the high N/P ratio with the consequent low availability of some marine nutrients can increase the hyperproduction of organic matter as mucilage aggregates [18] though mucilage aggregates have been observed both in eutrophic and oligotrophic waters [17]. Peculiar climatic conditions also seem to play a fundamental role in mucilage formation because the last appearances of aggregates in the Adriatic Sea occurred in hot springs and summers following mild winters, when reduced rates of the marine currents and wave motions enhance the anoxic conditions of the water column [16,19,20].

As far as the production of mucilage aggregates is concerned, field observations have shown that several organisms (phytoplankton, cells, diatoms, benthic macroalgaes and also bacteria) can produce mucilages [16–20] but the information related to the chemical composition is still generic and only the contribution of polysaccharides to the chemical structure of the mucilage aggregates is well recognized [19,20]. For this reason several studies are in progress to characterize the chemical composition of mucilage aggregates and to identify the causes of this phenomenon. In a previous study we performed a characterization of several marine mucilage samples by applying IR and UV–Vis spectroscopy and elemental analysis [21]. The results showed relevant structural similarities between mucilages and the insoluble fraction of marine humic substance depending on the common presence in all the examined samples of polysaccharide, protein and phenolic fractions. However this study did not give information on the presence of lipid compounds. Because the complete knowledge of the qualitative composition of mucilages allows to hypothesise the pathway involved in the synthesis of organic matter in aquatic environment [21,22], we determined the total lipid amount by gravimetry and used TLC–FID because this technique is known to be very attractive to analyze the qualitative composition of the lipid fraction present in marine samples [23–26]. In Table 1 the total amount of the lipid fraction present in the mucilage samples is reported. Lipids are a small fraction of the organic matter of mucilages that never exceed 3% (w/w) and in some samples the lipid fraction is quite negligible. So, the reduced contribution of lipids to the mucilage composition seems to exclude that the humification process depends on a pathway such as the ''polyunsaturated lipid concentration model'' where the formation of the organic matter in marine environment occurs by the polymerization of polyunsaturated fatty acids and lipids [22]. Maybe a humification process based on the contribution of all the possible classes of natural organic matter (polysaccharides, proteins, phenolic compounds and lipids) is more likely [21,22].

Table 1
Total lipids extracted from mucilage samples by sonication in diethyl ether (three sonication, 30 s each)

Mucilage sample	Total lipids % w/w
Procida 2000	0.33
Circeo D. 2000	0.74
Circeo S. 2000	2.56
Giglio 1999	0.55
Elba 2000	0.10
Scario 2000	<0.10
Sapri 2000	<0.10
Formia 2000	3.13
Elba 1999	0.12

Before analyzing the lipid sub-fractions by TLC–FID, a preliminary estimation of the phospholipid amount in mucilages was set up by detecting the organic P content in the diethyl ether extracts by a colorimetric technique. This technique was selected because FID is not specific for organic phosphorus compounds [27]. The levels of P resulted always lower than 0.3 μg/g of P (as sample) and we can conclude that phospholipids do not give a significant contribution to the lipid fraction of marine mucilages.

In Figs. 6 and 7 we report the chromatograms of some lipid extracts from mucilages compared with the lipid extract from a standard humic sample and with the lipid extract from a marine sediment respectively. Silica stationary phases are suitable to separate saturated hydrocarbons, aromatic hydrocarbons, wax esters, fatty acids, alcohols [28] and the comparison with the standard solutions of these lipids confirms that the lipid fraction of mucilages, though not very relevant, is qualitatively heterogeneous. In fact, in Fig. 6 the TLC–FID analysis shows the presence of saturated hydrocarbons (R_F close to 2.5), aromatic hydrocarbons (R_F close to 3.5), fatty acids (R_F ranging between 6.5 and 7.0). At last,

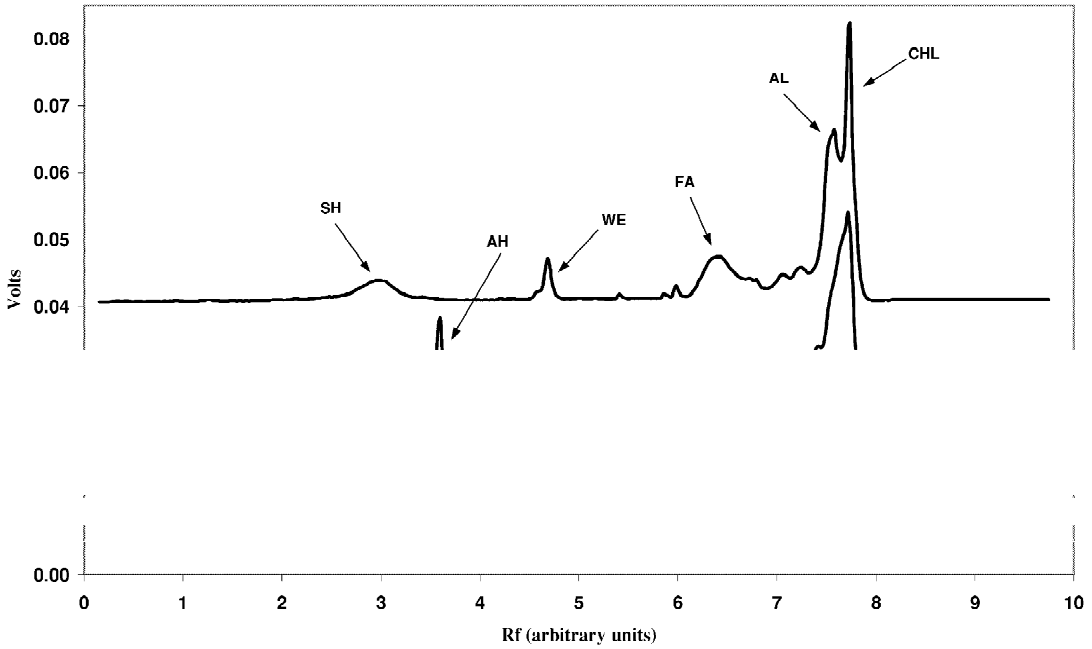

Fig. 6. TLC chromatograms of the lipid fraction extracted from two marine mucilage samples (lower and intermediate chromatograms, Circeo and Formia respectively) compared with the lipid fraction extracted from a humic standard sample (upper chromatogram). Saturated hydrocarbons (SH), aromatic hydrocarbons (AH), wax and sterol esters (WE), fatty acids (FA), free alcohols (AL), free cholesterol (CHL).

overlapped peaks due to free cholesterol and alcohols (R_F between 7 and 8) are present. With the exception of methyl esters (R_F between 5 and 6, Fig. 7) not observed in mucilage samples, most fractions are present also in the diethyl ether extracts of a standard humic sample (Fig. 6) and of a sediment sample (Fig. 7). Moreover, a peak belonging to wax and sterol esters is observed in one sample reported in Fig. 7.

Figs. 6 and 7 give other significant information about the composition of lipids in mucilages. Alcohol and cholesterol type compounds represent the most relevant classes according to the lipid composition of the extracellular products of marine phytoplankton [8,29] while fatty acids and hydrocarbons (both saturated and aromatic) are generally less abundant but not negligible. The observed heterogeneity of the lipid fractions confirm that the qualitative composition of the marine mucilage samples depends on the natural (animal and vegetal) lipid contributions as already observed for other constituents of the organic matter such as proteins, phenolic compounds and carbohydrates [21]. These results support also our previous hypothesis that the mucilage formation follows the pathway called ''biopolymer degradation and polymerization model'' where all the chemical fractions of the organic matter (i.e. proteins, polysaccharides, phenolic types compounds and now lipids) give a contribution to the synthesis of the organic matter of marine mucilages [22].

4. Conclusion

Our results show that ultrasound is a reliable tool for the fast extraction of the lipid fraction present in environmental samples. The reliability of the ultrasound technique was obtained by the identification of the best solvent, diethyl ether, which minimizes the oxidative degradation of the constituents of the lipid fraction. The proposed ultrasound technique was applied as an extraction step in the TLC–FID

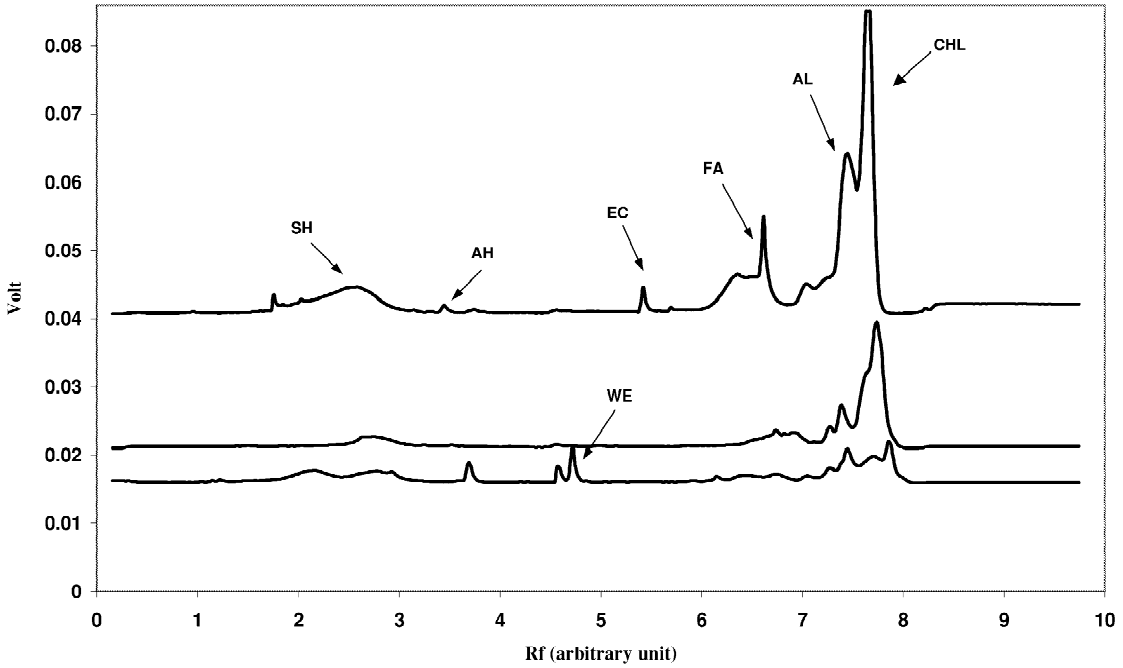

Fig. 7. TLC chromatograms of the lipid fractions extracted from two marine mucilage samples (lower and intermediate chromatograms, Procida and Giglio respectively) compared with the lipid fraction extracted from a marine sediment (upper chromatogram). Saturated hydrocarbons (SH), aromatic hydrocarbons (AH), wax and sterol esters (WE), methyl esters (EC), fatty acids (FA), free alcohols (AL), free cholesterol (CHL).

analysis and characterization of the lipid fraction present in marine mucilage samples.

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