INTRODUCTION TO THE STUDY ON THE BIODETERIORATION OF THE POLY(VINYL) ACETATE RESIN MOWILITH 50 USING FTIR SPECTROSCOPY AND PYROLYSIS-GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT: *Evaluation of the alteration produced by microbiological attack on the poly(vinyl) acetate (PVA) resin Mowilith 50 has been carried out using FTIR spectroscopy and Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS). The latter proposed method includes the on-line derivatization of vinyl resins using hexamethyldisilazane during pyrolysis. Specimens consisting of thin films formed on glass slides from drying of an acetone solution of this PVA resin have been used. Analyses performed on the specimens on which different genera of bacteria and fungi were inoculated and allowed to grow, indicate that attack of microorganisms promotes the formation of acidic compounds. IR bands ascribed to carboxylic acids appearing in samples from inoculated specimens as well as an increase in the content of ethanoic acid detected by Py-GC/MS from specimens inoculated with fungi suggest that the effects of metabolic processes in these microorganisms are more significant than those of bacteria.*

KEYWORDS: Py-GC/MS, FTIR spectroscopy, vinyl resin, poly(vinyl) acetate, easel painting, hexamethyldisilazane, biodeterioration, bacteria, fungi

INTRODUCTION

Identification of the causes of deterioration and the mechanisms involved in these processes is one of the major problems facing analysts and conservators of easel painting. In parallel to physical and chemical deterioration factors, microbiological agents act on the different components of the painting, in particular, on those of an organic nature. Effects produced by these agents have been extensively described for traditional paintings (Rebrikova, 1978; Bergeaud et al., 1997, Nicolaus, 1999): lowering of the mechanical strength of the support due to the biocolonization by microorganisms, which are able to produce hydrolysis of the cellulosic and proteinaceous materials, fading and detachment of the paint layer, spots produced by coloring metabolic by-products such as fatty acids and glycerol formed as a result of the breakdown products of lipids due to fungi metabolic action or carotenoids such as beta-carotene, astaxanthin and lutein - which are the main components of the cyanobacterial photoprotective accessory pigment, scytonemin (Edwards et al., 1991). Fungal species such as *Trichoderma, Aspergillus, Penicillium* (Briski, 2001; Mayumi and Koyano, 1991), *Cladosporium, Fusarium* (Mayumi and Koyano, 1991), *Alternaria* (Mayumi and Koyano, 1991; Arai, 1990), *Paecilomyces,* and *Sordaria* (Arai, 1990), together with the *Bacillus* and *Streptomyces* genera of bacteria (Arai, 1990), have been identified in polychrome sculpture and canvas painting collections. These microorganisms utilize the organic compounds present in the painting as carbon source or excrete products from their metabolism using lytic enzymes (Janda, 2005). Concerning contemporary art, different methods have been reported for assessing the biodegradability of synthetic resins commonly present in paintings, sculptures and other contemporary art objects (Panhurst et al., 1972; Nugari and Priori, 1985; Salvadori and Nugari, 1988; Albertsson and Sigbritt, 1993; Heyn et al., 1995; Wagner et al., 1996; Solaro, 1998; Abdel-Kareem, 2000; Capittelli et al., 2004). In these works, optical and scanning electron microscopy (SEM), determination of the weight loss of specimens inoculated with selected microorganisms, characterization of structural changes by means of FTIR spectroscopy and Raman spectroscopy are generally used to characterize the biodegradability of the studied materials. In particular, the "Standard Practice for Determining Resistance of Synthetic Polymeric Materials to Fungi" (ASTM G21-96(2002)) and the "Standard Practice for Determining Algal Resistance of Plastic Films" (ASTM G29-96(2002)) - methods extensively used for this purpose - are based on measurement of the visual appearance, optical and electronic microscopic observation, as well as on the measurement of mechanical and electrical properties.

Vinyl resins are widely used for preparing pictorial binding media and protective coatings in contemporary art in Spain. Most of the commercial products used by Spanish artists are polyvinyl acetate (PVA) emulsions. A number of additives are frequently included in these commercial products, which improve chemical and mechanical properties of the polymer in the paint film. External plasticizer such as dibutyl phthalate as well as internal plasticization obtained by copolymerization of PVA with softer monomers such as acrylates or vinyl versatates (commercial mixtures of highly branched C9 and C10 vinyl esters), among others, have been reported (Learner, 1995).

Some works can be found in literature specifically dedicated to the study of biodeterioration of PVA resins used in wall and canvas painting (Giacobini 1957 (cited in Capitelli et al., 2004); Moriyama, 1993; Abdel-Kareem, 2000 and Cappitelli et al., 2002). In these works, optical and scanning electron microscopy (SEM, Cryo-SEM) observation, weight loss of specimens inoculated with selected microorganisms and FTIR spectroscopy were used for assessing changes in the specimens. Interestingly, Heyn et al. (1995) have reported significant microbial growth in PVA emulsions Mowilith DM5, Mowilith 20 and Mowiol 4-98. Measurement of pH and analysis of organic acid formation, mainly citric and ethanoic acid, was considered by HPLC. The microbial agents were, in this case, *Aspergillus versicolor, Aspergillus niger, Cladosporium herbarum, Cladosporium sphaerospermum, Engyodontium album, Penicillium aurantiogriseum, Penicillium chrysogenum, Trichoderma longibrachiatum, Debaryomyces hansenii, Rhodotorula mucilaginosa, Bacillus subtilis, Bacillus licheniformis* and *Rhodococcus fascians*. It should be noted that no works have been found reporting the study of biodeterioration of PVA resins due to bacteria attack.

On the other hand, Cappitelli et al. (2004) have extended the discussion, stressing the significant role of the additives included in resin-based products as main carbon source of the attacking microorganisms, suggesting that the polymer, more resistant than additive compounds, could be initially unaltered - but the biomass produced could generate unspecific enzymes (under so-called "co-metabolic conditions") which result in resin attack in a second step.

In this context, the present paper reports the first results obtained in a currently ongoing study on the biodeterioration of PVA emulsions used as paint media. The general aim of the present work was to evaluate the role of the pure PVA resin as potential carbon source of microorganisms. For this purpose, Mowilith 50, a PVA resin exempt of additives, has been selected. Specimens consisting of thin films formed on glass slides from drying of acetone solutions of Mowilith 50 have been employed for performing tests. Thus, a series of specimens where a number of fungi and bacteria were inoculated have first been analyzed by means of FTIR spectroscopy.

A second objective of this work has been to evaluate the suitability of Pyrolysis-Gas Chromatography-Mass Spectrometry (Py-GC-MS) as a complementary technique for evaluating the biodeterioration of PVA resins. For this purpose, a new method proposed by the authors, based on the on-line derivatization of samples with HMDS (Doménech-Carbó et al., in press) has been used. The proposed method has been successfully applied to the analysis of acrylic and poly(vinyl) resins used in artworks (Osete-Cortina and Doménech-Carbó, 2006). This method is based on the formation of trimethylsilyl ethers and esters, and has been proposed as an alternative to other commonly employed derivatization reagents such as tetramethyl ammonium hydroxide, to improve the detection of hydroxylated compounds and to avoid isomerization and unwanted alkylation reactions.

EXPERIMENTAL

Solvents, reagents and culture media

Analytical reagents and reference materials. The following reagents were used to treat the samples: hexamethyldisilazane (HMDS) (purity 99%) and acetone (Sigma-Aldrich, Steinheim, Germany).

Mowilith 50 solid poly(vinyl) acetate pure resin (Hoechst, supplied by Agar-Agar SL) has been the reference material used in this study.

Culture media: TSB media (Scharlau Chemie, Barcelona, Spain) were used for bacteria cultures and CM liquid medium (yeast extract 0.5 %, malt extract 0.5 % and glucose 1 %) was used for fungal cultures. Solid media were obtained by adding 2% Agar-Agar (Sigma-Aldrich Chemical, Milwaukee, USA) to the previously described liquid media.

Instrumentation

FTIR spectroscopy. IR absorption spectra were performed in attenuated total reflectance mode (ATR) with a Vertex 70 Fourier transform infrared spectrometer equipped with a FR-DGTS (fast recovery deuterated triglycine sulfate) temperature-stabilized coated detector. Number of co-added scans: 32; resolution: 4 cm^{-1}.

Pyrolysis-gas chromatography-mass spectrometry. Pyrolysis-gas chromatography-mass spectrometry were carried out with an integrated system composed of a CDS Pyroprobe 1000 heated filament pyrolyser (Analytical Inc. New York, USA), and a Gas Chromatograph Agilent 6890N (Agilent Technologies, Palo Alto, Ca., USA) coupled with an Agilent 5973N mass spectrometer (Agilent Technologies) and equipped with a pyrolysis injection system. A capillary column HP-5MS ((5%-phenyl)-methylpolysiloxane; 30 m, 0.25 mm i.d., 0.25 μm) was used. Pyrolysis was performed at 700 ºC for 10 s, using a precalibrated Pt coil type pyrolyser (CDS pyprobe). The pyrolyser interface and the inlet were set at 250 ºC. Samples were injected in split mode (split ratio 1:40). The GC column temperature conditions were as follows: initial temperature 50 ºC, held for 2 min and then increased at 5 ºC.min^{-1} up to 100 ºC, increased at 15 ºC.min^{-1} to 295 ºC, and held for 10 min. Helium gas flow was set to 1.2 ml.min^{-1}. The inlet pressure of carrier gas was 67.5 kPa. The electronic pressure control was set to the constant flow mode with vacuum compensation.

Ions were generated by electron ionization (70 eV) in the ionization chamber of the mass spectrometer. The mass spectrometer was scanned from m/z 20 to m/z 800, with a cycle time of one second. Agilent Chemstation software G1701CA MSD was used for GC-MS control, peak integration and mass spectra evaluation. EI mass spectra were acquired in the total ion monitoring mode, and the peak area (TIC) data were used for obtaining values of peak area percentage. The temperatures of the interface and the source were 280ºC and 150ºC, respectively. NIST and Wiley Library of Mass Spectra were used for identifying compounds.

The CLIMAS culture chamber (Génesis Instrumentación, Madrid, Spain) was used for incubation trials.

Derivatization of samples

Samples scraped from the specimens with the help of a scalpel were placed in a microquartz pyrolysis tube and then two small portions of quartz wool were introduced in both sides of the quartz tube for avoiding undesirable displacements of the sample. Afterwards, 5 μl of HMDS were added.

Preparation of test specimens

A series of test specimens were prepared by dissolving the PVA resin in acetone (50% w/v). The freshly prepared solution was applied by brushing in three successive thin layers on glass slides of standard size (24 x 80 mm). Then, the specimens were dried at room temperature during 2 months.

IR absorption spectra were directly obtained from the inoculated area in the specimen in attenuated total reflectance (ATR) mode. Blank analyses were performed on reference specimens where culture media without microorganisms were added to the specimens.

Samples for Py-GC/MS were taken scraping about 1 μg of the polymeric film from the inoculated area with the help of a scalpel. The same procedure was carried out to take blank samples.

Microorganism inoculation and incubation

The microorganisms studied were recognized biodeterioration agents, selected from an extensive review of the literature (Ciferri, 1999; Giacobini, and Firpi, 1981; Giacobini et al., 1988,

Giacobiniet al., 1991; Heyn et al., 1995; Abdel-Kareem, 2000), and choosing fungi and bacteria susceptible to originate processes of biodeterioration in synthetic resins. All species studied are ubiquitous saprophytes, abundantly distributed in the atmosphere, and came from collection stocks of the Spanish Collection of Type Cultures (CECT, Colección Española de Cultivos Tipo). The microorganisms used were:

Fungi: *Aspergillus niger* (An)(CECT-2088), *Penicillium chrysogenum* (Pc)(CECT-2306), *Trichoderma pseudokoningii* (Tp) (CECT-2937), *Cladosporium cladosporoides* (Cc) (CECT- 2110), *Fusarium oxysporum* (F) (CECT-2868), *Mucor rouxii* (Mr) (CECT-2655), *Chaetomium globosum* (Cg) (CECT-2701), *Rhizopus oryzae* (Ro) (CECT-2339), *Aureobasidium pullulans* (Ap) (CECT-2703).

Bacteria: *Streptomyces cellulofans* (Sc)(CECT 3242), *Bacillus amyloliquefaciens* (Ba)(CECT 493), *Arthrobacter oxydans* (Ao)(CECT 386.), *Burkholderia cepacia* (Bc) (322).

Lyophilized collection stocks were hydrated in CM (fungi) and TSB (bacteria) and incubated for 48-72 h in a culture chamber (28°C). Afterwards, they were inoculated on solid CM medium (fungi) and TSA (bacteria). After 48 h, cultures were used to prepare dense microbial suspensions in distilled water, which were inoculated on specimens containing the studied PVA materials after centrifugation to eliminate any possible remains of culture media.

Several drops (20 µl) of the suspension containing the microorganisms were applied with the help of a micropipette on the support, covering an area (at approximately 20 mm^2.$drop^{-1}$) of the solidified polymeric film, and then the inoculated test specimen was placed in the incubator.

Previous experiments established the optimal conditions for incubating the test specimens. The experimental conditions in which the incubation was carried out were 28ºC for temperature and 60% for relative humidity. Specimens were incubated for 30 days.

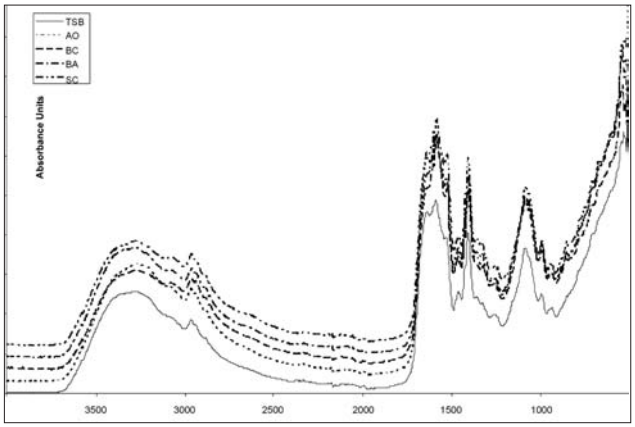

Figure 1. IR spectrum of sample from the blank specimen and that inoculated with the four bacteria studied

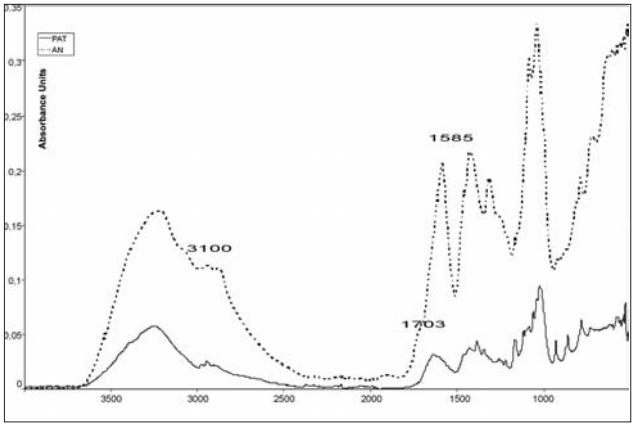

Figure 2. IR spectra of samples from the blank specimen and that inoculated with Aspergillus niger

RESULTS AND DISCUSSION

FTIR Spectroscopy

Table 1 summarizes the main IR absorption bands identified in the set of specimens studied. As can be seen, bacteria have not produced relevant changes in the structure of the specimens, and the IR absorption spectra were quite similar to that from the blank specimen (Figure 1). Similarly, *Trichoderma pseudokoningii* and *Penicillium chrysogenum* exhibited IR absorption spectra close to that from the blank specimen, apart from the increase in the absorbance values across the complete interval of wavelengths studied. Noticeable differences in the IR spectra are, however, observed in specimens inoculated with the fungi *Fusarium*, *Cladosporium cladosporoides*, *Chaetomium globosum*, *Rhizopus oryzae*, *Aureobasidium pullulans* and *Mucor rouxii* and, especially, *Aspergillus niger* (Figure 2). The appearance of the band at 1709 cm^{-1} is ascribed to the stretching vibrations of –C=O groups from carboxylic acids. The increase in the absorbance value of the IR band at 1585 cm^{-1}, which is associated to the stretching vibration of metal (alkaline) carboxylates (Van der Weerd et al. 2005) probably results from the free acids released from the resin due to the fungal activity. This band is partially overlapped with the bending vibration band from the associated -OH groups. Additionally, an increase in the absorbance value of the IR band at 1247-37 cm^{-1} is also observed. This increase is due to overlapping of the in-plane bending vibration of -OH group ascribed to polysaccharide molecules from the culture medium and the asymmetric stretching vibration of the carboxylate group (C(=O)-O), which is associated to the ester groups present in Mowilith 50 resin. The appearance of acidic compounds evidenced by this FTIR study, as a consequence of the fungal metabolic activity, is in good agreement with previous results obtained with HPLC by Heyn et al. (1995).

Pyrolysis-Gas Chromatography/Mass Spectrometry

Analysis of PVA emulsions used in Fine Arts by direct Py-GC/MS has been previously reported in the literature (Learner, 1995 and 2001). Under these conditions, ethanoic acid together with benzene are the main products formed as consequence of the breaking down of the PVA polymer chains during pyrolysis via a side group elimination mechanism (Learner, 2001); as a result, these compounds have been suggested as a marker of PVA emulsions for analytical purposes by this author. On-line derivatization with HMDS has as major advantage the ability to form the trimethylsilyl ester of carboxylic acids, in particular, of ethanoic acid - thus improving the efficiency in the formation of this pyrolysis product and avoiding the appearance of a fronting peak as usually occurs with strong polar organic compounds such as carboxylic acids. Figures 3 and 4 show the pyrograms corresponding to specimens inoculated with the fungus *Mucor rouxii* and the bacterium *Streptomyces cellulofans.* Both pyrograms are dominated by the peak corresponding to the trimethylsilyl ester of ethanoic acid (retention time: 2.869 min), in good agreement with that previously reported in the specialized literature (Learner, 2001). Unfortunately, the benzene peak is overlapped by the more intense peak from the derivatization reagent, hindering its identification and quantification in some cases. Nevertheless, a series of peaks can be recognized at higher retention times which can be associated to recombination and condensation reactions of the original benzene molecules formed during pyrolysis, namely, 1,4 dihydronaphthalene (13.116 min), naphthalene (13.582 min) and phenylbenzene (16.694 min).

On the basis of the identified compounds, a comparative study has been carried out attempting to recognize the changes in composition

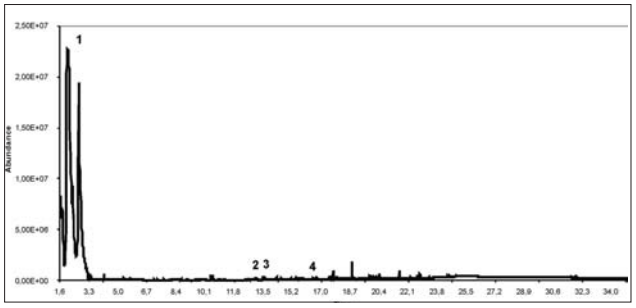

Figure 3.- Pyrogram of sample from the specimen inoculated with the bacterium Streptomyces cellulofans. Peaks: (1) ethanoic acid, TMS ester; (2) 1,4 dihydronaphthalene; (3) naphthalene; (4) phenylbenzene

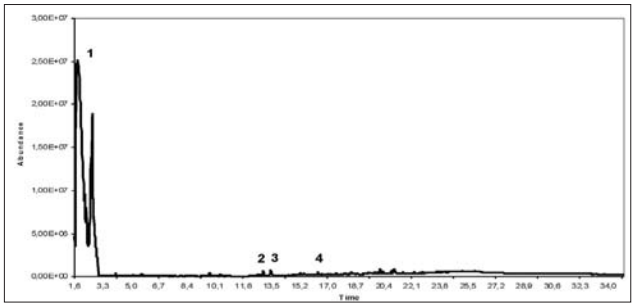

Figure 4.- Pyrogram of sample from the specimen inoculated with the fungus Mucor rouxii. Peaks: (1) ethanoic acid, TMS ester; (2) 1,4 dihydronaphthalene; (3) naphthalene; (4) phenylbenzene

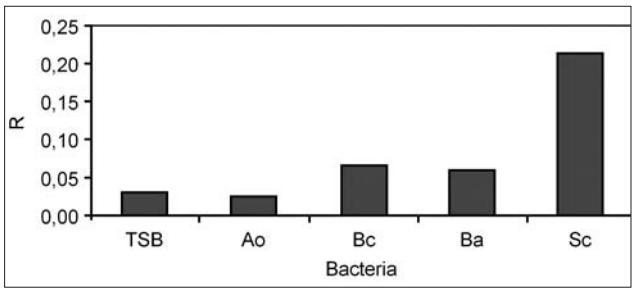

Figure 5. Bar chart summarizing the R values from the set of bacteria in study

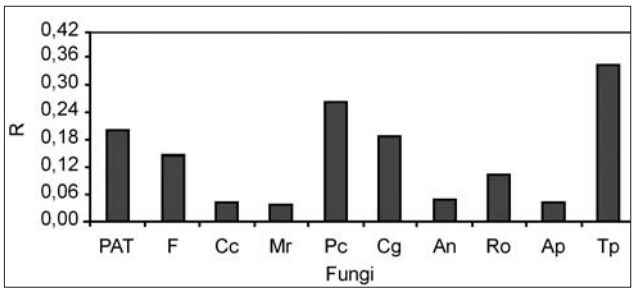

Figure 6. Bar chart summarizing the R values from the set of fungi in study.

due to microbial attack and to confirm the results obtained by FTIR spectroscopy. The methodology proposed is based on calculation of the parameter *R* defined as the ratio of the summation of the normalized peak area of the polycyclic aromatic compounds recognized in the samples analyzed (namely, naphthalene, 1,4 dihydronaphthalene and phenylbenzene) to the normalized value of peak area of the trimethylsilyl ester of ethanoic acid:

$$R = \frac{N}{N_a}$$

Where *N* is the summation of the normalized value of peak areas of polycyclic aromatic compounds, and N_a is the normalized value of peak area of the trimethylsilyl ester of ethanoic acid.

$$N_i = \frac{A_i}{\sum_1^n A_i} x\,100$$

Normalization of peak area values has been carried out according to the following equation:

Where *n* is the total number of compounds occurring in the pyrogram, and which are considered for normalization, and A_i is their peak area obtained by integrating the pyrogram.

Assuming that the formation of ethanoic acid and benzene molecules represent simultaneous processes taking place during pyrolysis as consequence of breaking down of the chains of PVA polymer, a decrease in the *R* values from blank to the inoculated sample indicates that biodegraded samples could experience a reduction in the efficiency of the pyrolytic processes of polycyclic condensation from the formed benzene molecules. Likewise, it could be supposed that the content of ethanoic acid in the biodegraded samples was increased. Contrarily, an increase in the value of the parameter *R* could be ascribed to increased efficiency of the pyrolytic processes of polycyclic condensation.

Figure 5 shows the bar chart summarizing the *R* ratios obtained in the samples inoculated with bacteria. It first of all should be noted that values of peak area obtained for the trimethylsilyl derivative of ethanoic acid were notably higher than those from polycyclic aromatic compounds. Thus, values obtained for *R* are in the range of 0.001 to 0.09. This result confirms, as expected, that the condensation of polycyclic compounds is produced to a significantly lesser extent than formation of ethanoic acid and benzene during pyrolysis. On the other hand, *R* values found for inoculated specimens were, in general, slightly higher than that from the blank specimen, apart from that corresponding to the specimen inoculated with *Streptomyces cellulofans*, which evidenced a more remarkable increase in *R* value. This increase in the efficiency of the pyrolytic processes of polycyclic condensation seems to denote an increase in the amount of benzene molecules formed during pyrolysis, which tentatively could be correlated to breaking down as well as to changes occurring in the cross-linking of the polymer as a consequence of the metabolic activity of the bacterium - resulting in an increase in benzene content versus that of ethanoic acid.

Figure 6 shows the bar chart summarizing the *R* ratios obtained in the samples inoculated with fungi. Similarly to for the findings in bacteria, the values of this parameter are low (0.037-0.49) - indicating that the content in polycyclic aromatic compounds is notably lower than that of ethanoic acid. Comparison of *R* values of the blank specimen and the inoculated specimens suggests that fungal activity in general results in behavior different to that of bacteria, apart from *Penicillium chrysogenum* and *Trichoderma pseudokoningii* specimens where, as in the prior case, an increase in *R* value was found. The rest of the fungi exhibited *R* values lower than that from the blank specimen. This behaviour tentatively can be ascribed to the decrease in efficiency of polycyclic compounds formation as well as to the increase in the amount of ethanoic acid formed during pyrolysis. In this last case, an hypothetical extra supply of ethanoic acid owing to the metabolic activity of fungi could justify this increase, in addition to the decrease in the efficiency of polycyclic aromatic compounds. Results obtained from FTIR spectroscopy sustain this latter hypothesis.

CONCLUSIONS

Results obtained by FTIR spectroscopy and Py-GC-MS suggest that the metabolic activity of most of the fungi tested in this study results in the formation of carboxylic acids. This result is in good agreement with other data previously reported in the literature (Heyn et al., 1995). As an hypothesis, it could be postulated that this compound could be formed from the PVA polymer through a side group elimination pathway, in a way similar to that occurring during pyrolysis.

The proposed method based on the on-line HMDS derivatization with Py-GC-MS has proven to be not completely successful for recognizing the changes occurring after microbial attack with the studied bacteria and fungi. Overlapping of peaks of benzene and derivatization reagent obstructs quantification of the benzene content, thereby hindering direct assessment of the polymer composition after microbial attack from the analysis of the primary pyrolysis products. Assessment by means of secondary pyrolysis products introduces an additional uncertainty factor in the obtained data.

Finally, the presence of intense features characteristic of the compounds corresponding to the culture media in the FTIR spectra suggests that they have not been completely consumed during incubation of the microorganisms, so that PVA resin has been used to a low extent as carbon source. In this sense, new series of experiments will be performed where the microorganisms will be induced to grow under more drastic conditions, so that they are impelled to use PVA resin as carbon source by completely consuming the nourishment provided by the culture medium.

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Versión española

TITULO: ***.Introducción al estudio del biodeterioro de la resina de acetato de polivinilo Mowilith 50 usando la espectroscopia FTIR y la pirolisis-cromatografía de gases-espectrometría de masas.***

RESUMEN: *La evaluación de la alteración producida por el ataque microbiológico en la resina de acetato de polivinilo (PVA) Mowilith 50 ha sido llevada a cabo usando un espectroscopio (FTIR) y Cromatografía de gases y pirólisis/espectrometría de masas (Py-GC/MS). El último método propuesto incluye la derivatización en linea de las resinas de vinilo usando hexametil- disilazano durante la pirólisis. Se han utilizado muestras consistentes en películas delgadas formadas sobre láminas de vidrio procedentes del secado de una solución de acetona de esta resina de acetato de polivinilo. Los análisis realizados sobre las muestras en los cuales fueron inoculados y guiados para crecer diferentes géneros de bacteria y hongo, indican que el ataque de microorganismos promueve la formación de componentes ácidos. Las bandas infra-rrojas atribuidas a los ácidos carboxílicos que aparecen en las muestras de las probetas inoculadas así como el incremento del contenido de ácido etanoico detectado por Py-GC/MS de las probetas inoculadas con hongos sugiere que los efectos de los procesos metabólicos en estos microorganismos son mas significativos que los correspondientes a bacterias.*

PALABRAS CLAVES: *Py-GC/MS, FTIR espectroscopia, resinas de vinilo, acetato de polivinilo, pintura de caballete, - disilazano, biodeterioro, bacteria, hongos*