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Liquid Chromatography-Electrospray-Mass Spectrometry as a Valuable Tool for Characterizing Biodegradation Intermediates of Branched Alcohol Ethoxylate Surfactants

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Alcohol ethoxylates (AEs) are surfactants widely used for domestic and industrial uses. Very little is known about the degradation pathway of BAEs, and structures of their related intermediates have not yet been fully characterized. We have used liquid chromatography-mass spectrometry (LC-MS) with an electrospray (ES) interface to fill in this gap. An example of BAEs, i.e., a 2-butyl branched $A_{12}E$ mixture with an average of five ethoxy units, was submitted to degradation, according to an OECD bioassay. After solid-phase extraction from samples of the bioassay solution taken at intervals, analytes were analyzed by LC-ES-MS. Elucidation of the metabolite structures were obtained by in-source collision-induced decomposition spectra. Initially, bacterial attack on the ethoxy chain (E-chain) produced species with the E-chain either shortened or, to a lesser extent, oxidized. Next, the major route for degradation produced abundant amounts of two new classes of rather stable intermediates via the ω, β-oxidation mechanism of the main alkyl chain. At the same time, a minor biodegradation route was that of the simultaneous attack of both alkyl chains by the ω,β-oxidation process. This route produced species which were rapidly converted by E-chain oxidation and β-oxidation mechanisms to very polar intermediates. After a rather long bacteria acclimation time, the major route was reactivated with shortening of the alkyl side chain via the ω,β-oxidation mechanism and the same intermediates as those formed through the minor route were produced.

Introduction

Nowadays, with increasing regulatory implementation of environmental legislation, there is active interest in the environmental fate of surfactants and their biodegradation intermediates (biointermediates) in order to assess their environmental compatibility. In particular, the effects of surfactant biointermediates on aquatic organisms is an area of continuing study, since very little is known on the aquatic toxicity of these species.

Alcohol ethoxylates (AEs) of the common structural formula CH_3-$(CH_2)_m$-O-$(CH_2CH_2O)_n$H ($m = 11-17$, $n = 1-22$) are widely used as detergents, emulsifiers, solubilizers, wetting agents, and dispersing agents in household products and industrial chemicals. The alkyl chain of commercial AE surfactants can be both linear (LAE) and branched (BAE). Usually, BAEs have a single branching in position 2 with respect to the ether bridge. It has been estimated that BAE accounts for ca. 25% of the commercial AEs in domestic detergents (*1*).

There is abundant evidence collated by Swisher (*2*) and corroborated by a very recent study (*3*) that the major, if not the only one, route for LAE biodegradation is that of cleavage at the ether bridge linking the alkyl chain with the ethoxy chain (central scission). Thereafter, biodegradations of fatty alcohols and polyethylene glycols (PEGs) proceed independently of one another.

The biodegradation pathway of BAEs has been much less extensively studied than that of LAEs. Branching of the alkyl chain in the vicinity of the central ether bridge seems to inhibit central ether cleavage. Results from some studies (*4*, *5*) conducted by combining elemental analysis and infrared spectroscopy have indicated that biodegradation of 2-alkyl branched AE in mixed cultures yields metabolites of two types: (i) ethoxylate shortened with the alkyl chain intact, and (ii) ethoxylate shortened with the alkyl chain oxidized and shortened. However, the structures of these metabolites were not fully characterized.

Leaving the intermediate molecular structures not completely defined is a limitation common to most of the studies devoted to explain the mechanistic pathway of surfactant biodegradation. This failure is due to the application of analytical protocols lacking of a sufficient structural elucidation potential. Definitive structure assignment of an unknown compound in a rather complex mixture can be obtained only by adopting sophisticated analytical techniques, such of those based on mass spectrometry (MS). On developing a liquid chromatography (LC)-MS-MS method for analyzing pollutants in sewage treatment plants, Schröder (*6*) identified AE metabolites generated from oxidation of the terminal carbon atom of the polyethoxylate chain by their collision-induced decomposition (CID) spectra. Neutral and acidic PEGs, partly coming from LAE biodegradation, were detected in a variety of aqueous environmental matrices by an analytical procedure based on LC-MS with an electrospray (ES) ion source (*7*).

The object of this work has been that of evaluating the effectiveness of a method based on LC-ES-MS in characterizing and quantifying BAE metabolites. For this purpose, we have looked in detail at the progression of the biodegradation of a 2-butyl branched $A_{12}E$ ($BA_{12}E$) mixture with an average of 5 ethoxy units, where A stands for an alkyl group.

Experimental Section

Reagents and Chemicals. Individual $LA_{12}E$ with 1, 2, 3, 4, 5, 6, and 8 ethoxy units and $LA_{10}E_6$ (purities higher than 95%) were purchased from Fluka Buchs, Switzerland. The latter compound was used as internal standard. The $BA_{12}E$ surfactant mixture, designated as Nacolox, was kindly supplied by L. Cavalli. The hydrophobic moiety of Nacolox contains a total of 12 carbon atoms, a 2-butyl group as side chain, while the hydrophilic moiety is a poly(ethylene oxide) containing 2−16 ethoxy units (n_E) (average $n_E = 5$). The

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general formula is C_6H_{13}-CH(C_4H_9)-CH_2-O-($CH_2CH_2O)_nH$. Stock solutions of both individual standards and standard mixtures were prepared by dissolving known amounts of them in acetonitrile. Working standard solutions were obtained by further diluting stock solutions with acetonitrile. Trifluoroacetic (TFA), hexanoic, adipic, and 1,3,6-cyclohexane tricarboxylic acids were from Aldrich Chemical Co., Milwaukee, WI.

A_{12}ECs were individually prepared by chemical oxidation of the alcoholic group of the above LA_{12}Es with the Jones reagent, by suitably modifying a previously reported procedure (*8*). From this point onward, the letter C on the left and/or the right side of acronyms for BA_{12}E acidic intermediates will indicate carboxylation of the alkyl chain (A-chain) and/or the ethoxy chain (E-chain) of the AE surfactant molecule. Oxidation of the terminal alcoholic group of known amounts of AEs was performed by dichromate in an acidic aqueous solution at 25 °C for 20 min, instead of 60 °C for 2 h. Under the former conditions, hydrolytic decomposition of the ethoxy chain was minimized. Next, the reaction mixture was 100-fold diluted with water, a known amount of the $A_{10}E_6$ internal standard was added, and an aliquot of this solution was directly analyzed by LC-ES-MS, following conditions reported below. Evidence for the formation of AECs was obtained by matching their in-source CID spectra with one shown in a previous paper (*6*). After determining the reaction yield (ca. 70%), molar responses of the various AECs relative to that of the internal standard, that is molar response factors, were calculated. These factors were used to quantify AECs originated from BA_{12}E degradation.

For LC analysis, distilled water was further purified by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA). Acetonitrile "Plus" of gradient grade, was obtained from Carlo Erba, Milano, Italy. Other solvents were of analytical grade (Carlo Erba) and they were used as supplied.

Biodegradation Assay. According to the OECD confirmatory test (*9*), a 5-L test solution containing the necessary organic nutrients and inorganic salts was prepared. To this solution, 0.5 mL/L of a filtered fresh effluent of a mechanical-biological sewage treatment plant was added as the source of microorganisms. Thirteen micromoles per liter of the BA_{12}E homologue mixture was then dissolved in the test solution. The bioassay was conducted under continuous stirring in a constant-temperature room (21 ± 2 °C) and using 12 h dark-light cycle. Two hundred milliliter samples were withdrawn at intervals and analyzed in duplicate by the procedure reported below. When not immediately analyzed, samples were stored at 4 °C, after addition of $HgCl_2$ (20 mg/L), to inhibit bacterial activity.

A stock solution (which will be denoted as solution S) containing unknown amounts of all of the acidic biointermediates arising from degradation of BA_{12}E, except AECs, was prepared by submitting to degradation the surfactant mixture, as reported above. After about 2 weeks, the degradation process was stopped by addition of $HgCl_2$. Solution S was used for analyte recovery studies as well as for estimating molar response factors with the ES/MS detector of those acidic biointermediates for which standards were not available.

Sample Preparation. Analytes were extracted from the bioassay solution by solid-phase extraction (SPE) with a recently introduced form of graphitized carbon black, that is Carbograph 4 (surface area, 210 m^2/g; 120−400 mesh size, Lara, Rome, Italy). The preparation of the 0.5-g Carbograph 4 SPE cartridge, the extraction apparatus used, and the subsequent analyte extraction procedure have been described elsewhere (*10*).

After passage of 100 mL of the test liquor through the SPE cartridge, this was washed with 50 mL of distilled water. Next, a stepwise desorption procedure was performed to isolate neutral analytes from acidic ones, by suitably modifying a previously reported procedure (*7*). Neutral analytes were eluted by passing 1.5 mL of methanol through the cartridge followed by 12 mL of CH_2Cl_2/CH_3OH (80:20, v/v), at a flow rate of about 4 mL/min. The eluate was collected in a 1.4 cm i.d. glass vial with a conical bottom. The last drops of this solvent mixture were collected by further decreasing the pressure inside the vacuum flask. Acidic metabolites were removed from the sorbent bed and collected in a second vial by elution with 10 mL of CH_2Cl_2/CH_3OH (80:20, v/v) acidified with formic acid, 50 mmol/L.

Extracts were taken to dryness in a water bath at 40 °C, under a gentle stream of nitrogen. To avoid partial esterification of acidic analytes, the acidic extract was partially neutralized by addition of 15 μL of concentrated ammonia, before taking to dryness. The neutral residue was reconstituted with 150 μL of a water/methanol solution (80:20, v/v) to which 60 ng of the internal standard was added. The same procedure was followed to reconstitute the acidic residue, with the exception that the aqueous mixture was acidified with TFA, 0.2 mol/L. In both cases, one-third of the final extract was then injected into the LC column.

LC/ES/MS Analysis. The LC apparatus was the same as reported elsewhere (*7*). The analytes were chromatographed on an "Alltima" 25 cm × 4.6 mm i.d. column filled with 5-μm C-18 reversed phase packing (Alltech, Sedriano, Italy). For fractionating both neutral and acidic analytes, the phase A was glass distilled acetonitrile and the phase B was water. Both solvents were acidified with TFA, 1 mmol/L. The mobile phase composition was 15% A, which was first linearly increased to 50% in 35 min, and then to 100% in 20 min. The flow rate of the mobile phase was 1 mL/min, and 40 μL/min of the column effluent was diverted to the ES source, while the rest of the effluent was delivered to a Model 2550 UV detector (Varian) set at 210 nm wavelength. A Micromass "Platform" benchtop mass spectrometer (Manchester, U.K.) consisting of a pneumatically assisted ES interface and a single quadrupole was used for detecting and quantifying target compounds in the LC column effluent. This was introduced into the ES interface through a 40-cm length of 75 μm i.d. PEEK capillary tube. The MS was operated in the positive-ion mode by applying to the capillary a voltage of 4 kV. The source temperature was maintained at 70 °C. Full-scan LC-MS chromatograms were obtained by scanning the quadrupole from 43 to 900 m/z with a 4-s scan. Unless otherwise specified, the skimmer cone voltage was set at 30 V.

Results and Discussion

Intermediate Characterization. By itself, the ES source is capable of producing only adduct ions. This permits assignment of the molecular weight to a certain compound, but structural information is precluded. Yet, structure-significant fragment ions can be obtained by collision between adduct ions and residual drying gas molecules in the desolvation chamber. So-called in-source CID spectra can be easily obtained by increasing the potential difference between sample and skimmer cones. Providing the analyte is chromatographically separated from any other compound, these in source CID spectra closely resemble those obtained by the MS-MS technique (*11*, *12*).

The addition of a relatively large concentration of a strong acidic agent, such as 1 mmol/L TFA, to the LC mobile phase served to reach two objectives. One was that of suppressing ionization of the acidic metabolites so that they were eluted from the LC column as untailed and well-retained peaks. The second objective was that of obtaining CID spectra displaying well-detectable signals for MH^+ adduct ions, in addition to related fragment ions. When decreasing the TFA

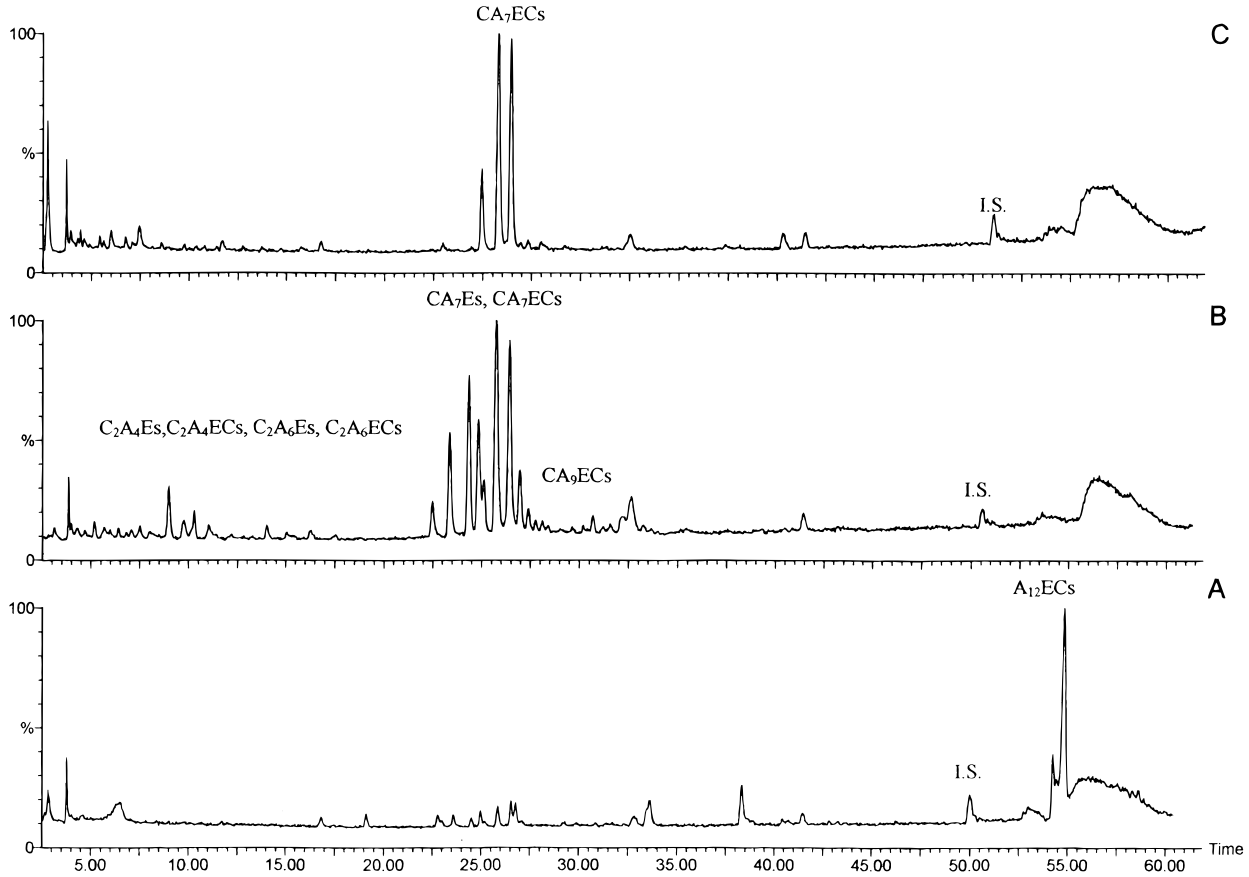

FIGURE 1. LC-ES-MS chromatograms of the acidic extracts of samples from the treated sewage-inoculated solution biotransforming a 2-butyl branched $A_{12}E$ surfactant mixture on (A) day 5, (B) day 19, and (C) day 37. For explanation of acronyms above the peaks, see the text.

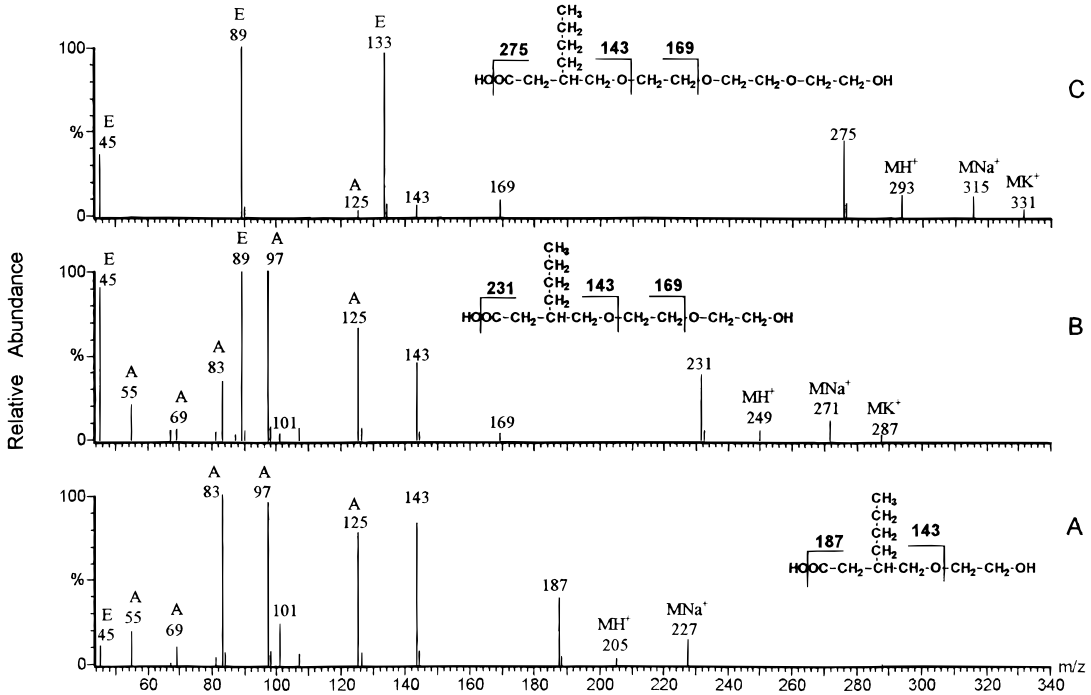

FIGURE 2. CID spectra of three intermediates originated from biodegradation of the 2-butyl branched $A_{12}E$ surfactant mixture. Postulated structures of these metabolites are shown on their respective spectra. Signal at *m/z* 101 originates from propene loss (−42) of the fragment ion at *m/z* 143. Signals for ions resulting from fragmentation pathways of the carboxylated alkyl chain (after H_2O loss) and the ethoxy chain are labeled, respectively, with letters A and E.

concentration, spectra of analytes displayed intense signals for Na^+, K^+, and NH_4^+ adduct ions, which overwhelmed that for MH^+ adduct ions. This effect was accompanied by signal weakening for fragment ions. With very few exceptions, the

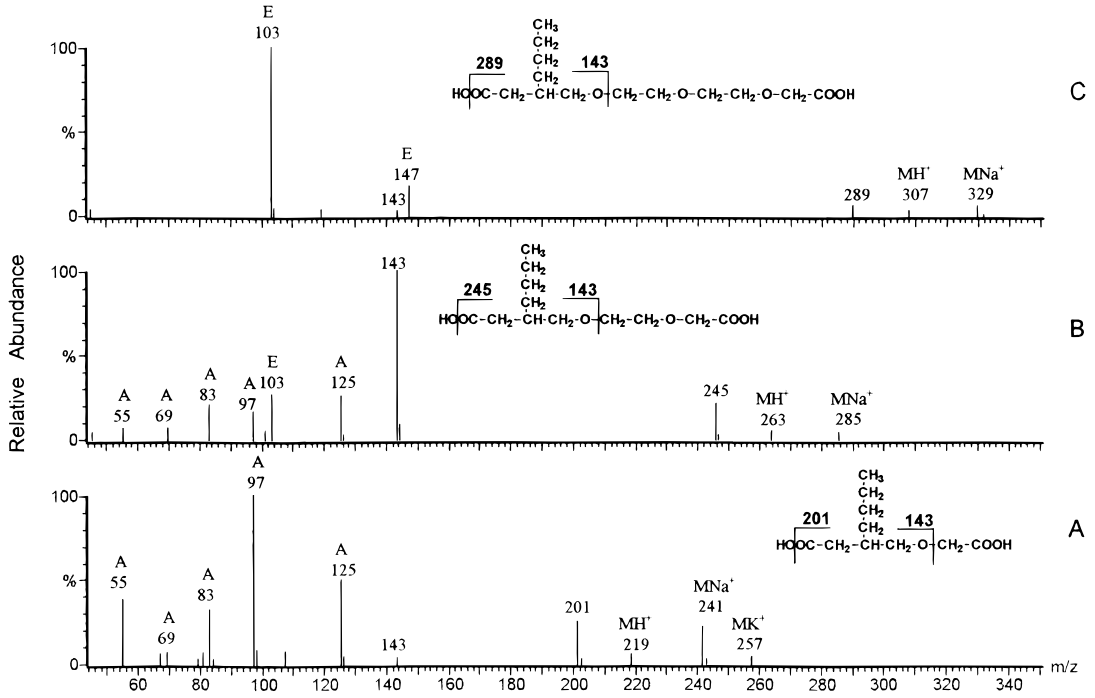

FIGURE 3. CID spectra of three metabolites arising from biodegradation of the 2-butyl branched $A_{12}E$ surfactant mixture. Postulated structures of these metabolites are shown on their respective spectra. Signals for ions resulting from fragmentation pathways of the carboxylated alkyl chain (after H_2O loss) and the carboxylated ethoxy chain are labeled, respectively, with letters A and EC.

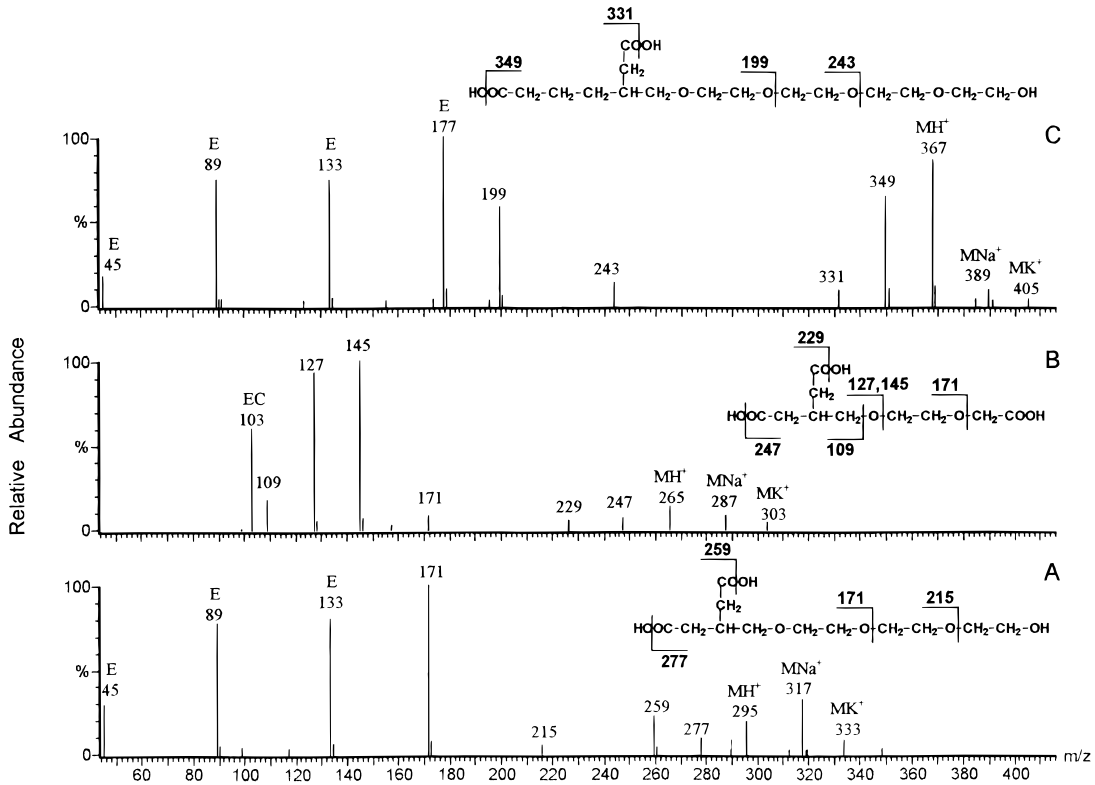

FIGURE 4. CID spectra of three metabolites formed from biodegradation of the 2-butyl branched $A_{12}E$ surfactant mixture. Postulated structures of these metabolites are shown on their respective spectra. Fragment ions at *m/z* 145 and 127 (Δ*m/z* 18) displayed in spectrum B are, respectively, formed after loss of one or two H_2O molecules from the carboxylic groups on the alkyl chain. Signals for ions resulting from fragmentation pathways of the ethoxy chain, and the carboxylated ethoxy chain is labeled, respectively, with letters E and EC.

CID process of cationized molecules does not generate structure-significant ions.

Figure 1 shows three selected LC-MS CID chromatograms obtained by analyzing acidic extracts of samples of the biodegradation test solution of the $BA_{12}E$ mixture. These samples were taken at increasing times from the beginning of the biodegradation experiment. Peaks appearing in the chromatogram A at more than 53 min were formed by coelutions of homologues of one metabolite class. Mixed CID spectra taken from these peaks were matched with those

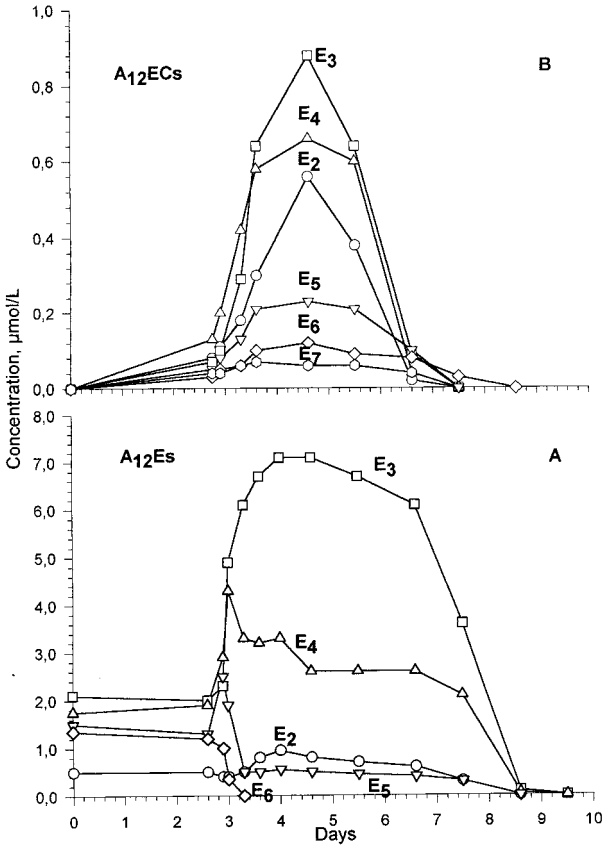

FIGURE 5. Concentration vs time plots of 2-butyl branched $A_{12}E$ metabolites arising from initial (A) shortening of the ethoxy chain and (B) carboxylation of the terminal carbon atom of the ethoxy chain. (○) $A_{12}E_2$ or $A_{12}E_2C$; (□) $A_{12}E_3$ or $A_{12}E_3C$; (△) $A_{12}E_4$ or $A_{12}E_4C$; (▽) $A_{12}E_5$ or $A_{12}E_5C$; (◇) $A_{12}E_6$ or $A_{12}E_6C$; (⬡) $A_{12}E_7C$.

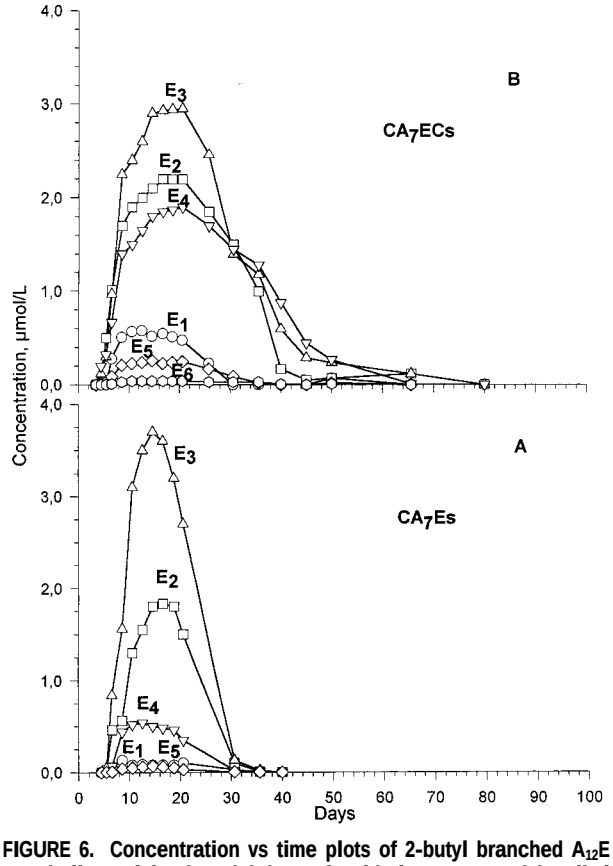

FIGURE 6. Concentration vs time plots of 2-butyl branched $A_{12}E$ metabolites arising from (A) the ω,β-oxidation process of the alkyl chain and (B) the ω,β-oxidation process of the alkyl chain plus carboxylation of the ethoxy chain. (○) CA_7E_1 or CA_7E_1C; (□) CA_7E_2 or CA_7E_2C; (△) CA_7E_3 or CA_7E_3C; (▽) CA_7E_4 or $CA_{12}E_4C$; (◇) CA_7E_5 or CA_7E_5C; (⬡) CA_7E_6C.

of synthesized AECs. This comparison gave sufficient evidence for the presence in the extract of $A_{12}EC$ metabolites with n_E ranging between 2 and 16, which originated in the initial stage of the $BA_{12}E$ degradation.

Figure 2 shows three background-subtracted CID spectra taken from the apices of peaks with retention times of 22.2, 23.1, and 24.2 min, respectively. When setting the cone voltage at a low value, that is 20 V, so as to inhibit the CID process, each of the three spectra were dominated by signals for MH^+, MNa^+, and MK^+ adduct ions. These signals were tentatively assigned to three biointermediates coming from the ω, β-oxidation process of the main A-chain of $BA_{12}Es$, that is CA_7Es with $n_E = 1-3$. Definitive confirmation of the molecular structure of this biointermediate class was obtained by the CID process, after raising the cone voltage at 30 V. The formation of acylium ions by H_2O loss indicated that all of the three compounds contained one carboxylic group in the alkyl moiety (*13*). The series of fragment ions at m/z 125, 97, 83, 69, and 55 resulting, respectively, from neutral losses of one ethylene unit and three methylene units indicated this moiety was composed by eight carbon atoms. Finally, cleavage of the C−O bond produced $[(CH_2\text{-}CH_2\text{-}O)_n + H]^+$ fragment ions indicative of the ethoxy chain length. CA_7E species with $n_{EO} > 3$ could be easily speciated by looking at the different E-chain fragmentation pathways displayed on their CID spectra.

Another class of biointermediates were eluted with retention times similar to those of CAEs. CID spectra taken from apices of peaks at 23.7, 24.7, and 25.3 min produced by three of these compounds are shown in Figure 3. On the basis of their molecular weights, which were deduced after individualization of related protonated and cationized molecules, this series of compounds were tentatively identified as CA_7ECs with $n_E = 1-3$, that is as biointermediates formed by oxidation of both the E- and the A-chains. Again, definitive evidence for the molecular structures of this class of compounds was achieved by interpretation of their CID spectra. Substantially, this series of spectra differed from those of CAEs in that signals for cleavage of the carboxylated E-chain produced $[CH_2{=}CH\text{-}O\text{-}(CH_2\text{-}CH_2\text{-}O)_n\text{-}CH_2\text{-}COOH + H]^+$ fragment ions, with $n \geq 0$. These types of fragments were obviously absent in the spectrum from CA_8E_1C (Figure 3A). However, the neutral loss of $CH_2OH\text{-}COOH$, in addition to the H_2O loss, made evident that two carboxylic groups were present at the opposite ends of the molecule.

Figure 4 shows selected CID spectra of compounds producing chromatographic peaks at 3.5, 4.6, and 8.3 min. The common feature of the three related protonated molecules is that they lost two H_2O molecules by the CID process. This is evidence that they were species formed by oxidation of both main and side A-chains. Spectrum in Figure 4B displayed a signal at m/z 103 corresponding to the $[CH_2{=}CH\text{-}O\text{-}CH_2\text{-}COOH + H]^+$ fragment ion. Therefore, the MH^+ ion contained a third carboxylic group in the E-chain. From other additional evidences, it could be concluded that these spectra originated, respectively, from $C_2A_4E_3$, $C_2A_4E_2C$, and $C_2A_6E_4$, where C_2 indicates carboxylation of both main and side A-chains. By proceeding in the same way, structure assignments of all of the other intermediates originated by microbial degradation of $BA_{12}Es$ was accomplished.

Intermediate Quantitation. Analyte quantitation was accomplished by the internal standard quantification procedure. Alkyl branched AEs and AECs concentrations in the bioassay solution were assessed by assuming that the

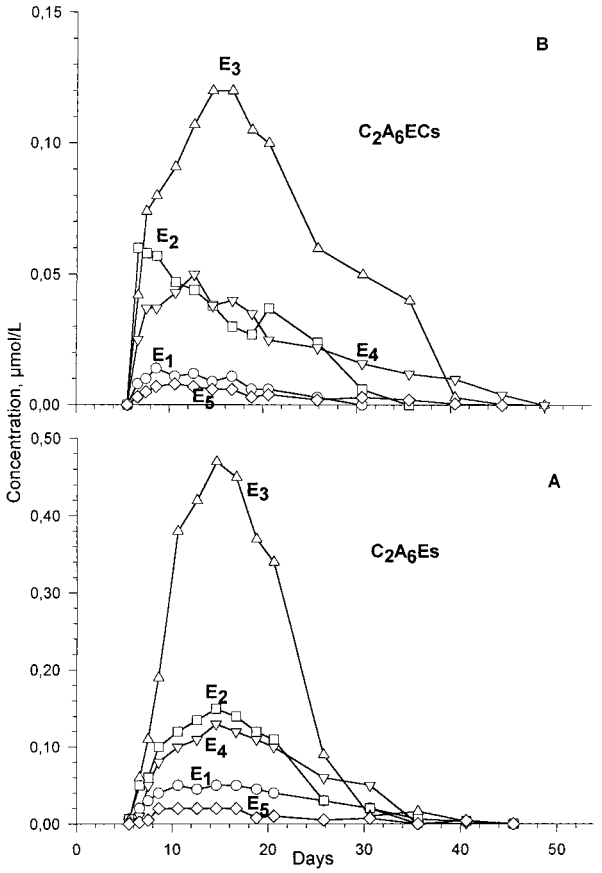

FIGURE 7. Concentration vs time plots of 2-butyl branched $A_{12}E$ metabolites arising from ω, β-oxidation process of both main and side alkyl chains plus carboxylation of the ethoxy chain. (○) $C_2A_6E_1$ or $C_2A_6E_1C$; (□) $C_2A_6E_2$ or $C_2A_6E_2C$; (△) $C_2A_6E_3$ or $C_2A_6E_3C$; (▽) $C_2A_6E_4$ or $C_2A_6E_4C$; (◇) $C_2A_6E_5$ or $C_2A_6E_5C$.

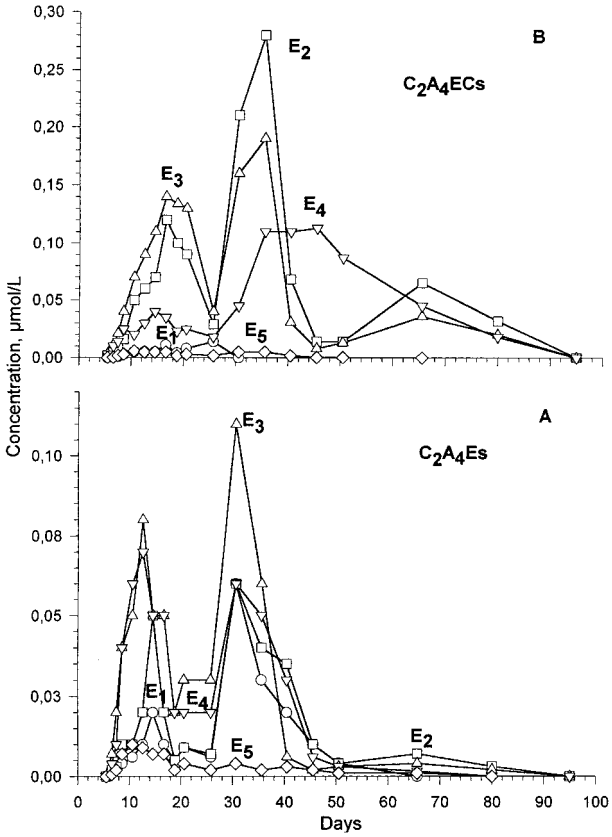

FIGURE 8. Concentration vs time plots of 2-butyl branched $A_{12}E$ metabolites arising first from precursors, whose time-concentration profiles are shown in Figure 7 via β-oxidation of the fatty acid chain, and then from other precursors, whose time-concentration profiles are shown in Figure 6 via ω, β-oxidation of the alkyl side chain. (○) $C_2A_4E_1$ or $C_2A_4E_1C$; (□) $C_2A_4E_2$ or $C_2A_4E_2C$; (△) $C_2A_4E_3$ or $C_2A_4E_3C$; (▽) $C_2A_4E_4$ or $C_2A_4E_4C$; (◇) $C_2A_4E_5$ or $C_2A_4E_5C$.

response of ES/MS detector is not dependent on different configurations of the alkyl chain. On this basis, calibration curves of $BA_{12}Es$ were constructed at six concentration levels by injecting known amounts of the commercially available $LA_{12}E$ standards from a solution containing the internal standard into the LC column. On the basis of a previous work (*10*), the molar response of $A_{12}E_7$ was assumed to be intermediate to those of $A_{12}E_6$ and $A_{12}E_8$. Also, molar responses of $A_{12}E$ species with $n_E > 8$ were considered to be equal to that of $A_{12}E_8$. Analogously, $BA_{12}ECs$ in the bioassay solution were quantified by suitably diluting $LA_{12}ECs$-containing reaction mixtures (see the Experimental Section) with an acidified water/methanol mixture and by adding a constant amount of the internal standard.

Because of the lack of any standard, quantitation of $BA_{12}E$ acidic intermediates, other than $BA_{12}ECs$, was rather laborious and less accurate than that of the species mentioned above. This procedure relied upon the assumption that, when setting the UV detector at 210 nm wavelength, the molar absorbances of compounds bearing, in addition to an E-chain, one or more carboxylic groups is dependent only upon the presence of the latter group(s). In fact, on injecting 10 μg of $A_{12}E_8$, no response was observed with the UV detector. The procedure followed to calculate molar response factors of $BA_{12}E$ acidic intermediates with the ES/MS detector is briefly described below. Firstly, molar responses with the UV detector of mono-, di-, and tricarboxylated compounds were estimated by injecting 0.1 μmol each of hexanoic, adipic, and 1,3,6-cyclohexane tricarboxylic acids and dividing peak areas by the number of moles injected. As calculated by us, the molar responses were 130, 180, and 330 arbitrary units/μmol, respectively. Secondly, extracts of the solution S (see the Experimental Section) containing unknown amounts of $BA_{12}E$ acidic intermediates were repeatedly injected into the LC column and analytes were detected with both UV and ES/MS detectors. When necessary, analyte coelutions were avoided by either varying the gradient elution program or the nature of the organic modifier of the LC mobile phase. Thirdly, the concentrations of the various mono-, di-, and tricarboxylated $BA_{12}E$ metabolites in the solution S were estimated by assigning to each class of acids the respective molar absorbance values reported above. Thereafter, molar responses of the compounds of interest relative to the internal standard and with the ES/MS detector were calculated. Finally, calibration graphs for the various acidic biointermediates were constructed by progressively diluting extracts of the solution S with an acidified water/methanol (80:20, v/v) mixture.

Analytical recoveries of those biointermediates for which standards were available, namely AEs and AECs, were assessed by a conventional procedure. A water sample having the same composition in organics and inorganic salts as the biodegradation test solution was spiked with standard solutions of the compounds cited above and analyzed in triplicate. Mean recovery of all the species mentioned above were better than 95%.

Because of the lack of standards of acidic products, other than AECs, formed by $BA_{12}E$ degradation, a different procedure had to be followed to estimate their recovery. Briefly, analytes were extracted in triplicate from 100 mL of the solution S. Analyte recovery was evaluated by monitoring acidic biointermediates in both neutral and acidic extracts

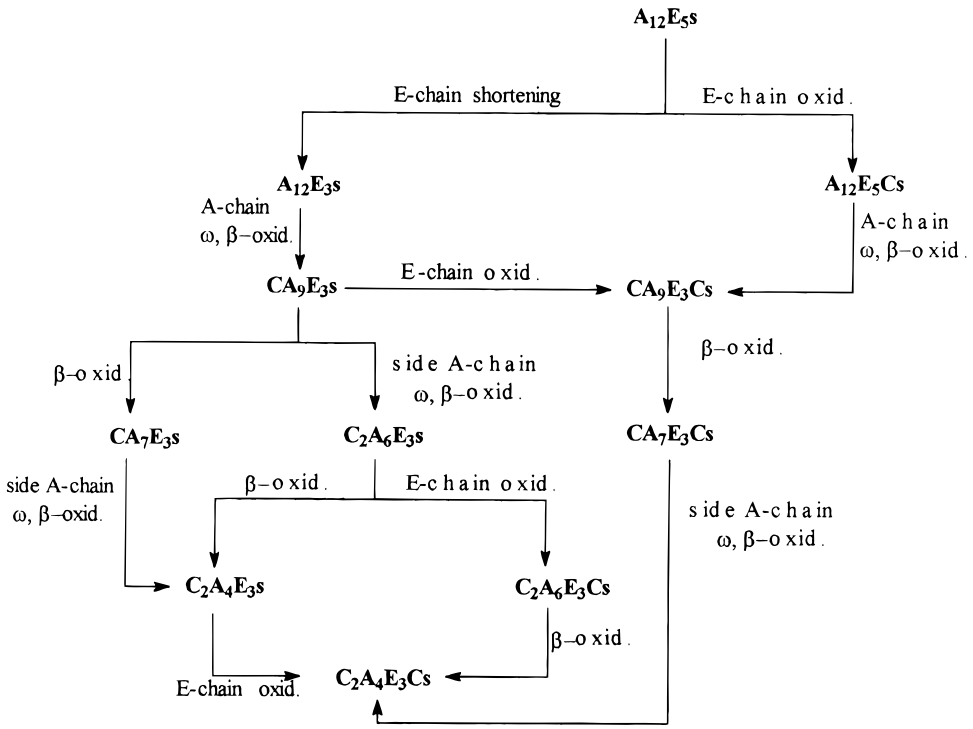

FIGURE 9. Proposed biodegradation pathway for 4-butyl branched $A_{12}Es$. A is for an alkyl group; the number following the letter E denotes approximately the average number of ethoxy units forming the ethoxy chain.

as well as by reanalyzing the aqueous effluent of the extraction cartridge. No significant analyte amounts were found in the neutral extract, while 3−7% of the amounts present in the acidic extracts were lost in the aqueous effluent.

Biodegradation Pathway of the 2-Butyl-Branched $A_{12}E$ Mixture. Microbial attack of the E-chain was the initial act of $BA_{12}E$ biotransformation. Two routes for the hydrophilic moiety degradation were acting simultaneously: nonoxidative shortening by cleavage of C_2 units and oxidation of the terminal alcoholic group to form AECs. For clarity of presentation, concentrations vs time plots of the most abundant forms of both AEs and AECs are shown, respectively, in Figures 5. These plots highlight that the former biodegradation mechanism largely prevailed over the latter one. Another outstanding aspect of the initial part of the $BA_{12}E$ biotransformation was that no long-chain PEG-type compounds were detected in the test liquor. This finding confirms that the central scission mechanism dominating the LAEs degradation process can be deactivated with branched AEs because of steric hindrance. In the final stage of the degradation process, attack at the central ether bridge after shortening of both A- and E-chains can be predicted. In this case, very polar species, such as ethylene glycol and glycolic acid, are released. Under our experimental conditions, however, these compounds could not be detected (*7*). After more than 4 days of incubation, bacterial attack on the main A-chain of $A_{12}ECs$ produced CA_9ECs by the ω, β-oxidation mechanism. The day after, the same mechanism produced CA_9Es from ethoxylate-shortened $A_{12}Es$. CA_9ECs were more persistent than CA_9Es. None of these species, however, accumulated in large amounts in the test liquor, as further shortening of the fatty acid chain by the β-oxidation mechanism converted them in few hours, respectively, to CA_7ECs and CA_7Es. Figure 6 shows concentration vs time plots for the most abundant forms of CA_7Es and CA_7ECs. Forms bearing only one ethoxy unit and one -O-CH_2-COOH unit, which were absent in the precursor species, were now present in CA_7Es and CA_7ECs, respectively. This indicated that shortening mechanisms of the two chains at the opposite sides of the ether bridge were simultaneously operating. CA_7-ECs were species much more persistent than CA_7Es. This could be explained by considering that the former species can originate also from E-chain oxidation of the latter species and assuming that the particular molecular configuration of CA_7ECs make them less prone to bacterial attack than CA_7Es.

Less abundant intermediates were formed by $BA_{12}E$ biotransformation. Time-concentration profiles of C_2A_6Es and C_2A_6ECs are visualized in Figure 7, while those of C_2A_4Es and C_2A_4ECs are shown in Figure 8. Although largely less favored than that leading to abundant production of CA_7Es and CA_7ECs, an alternative route for biodegradation of labile CA_9Es generated C_2A_6Es via the ω, β-oxidation mechanism of the side A-chain. Next, C_2A_6Es were in part converted to C_2A_6ECs by the E-chain oxidation process. Against our expectations, extensively degraded forms of $BA_{12}Es$, such as C_2A_4Es and C_2A_4ECs, were already present in the initial part of the biodegradation process, together with their supposed natural precursors, namely CA_7Es and CA_7ECs. This apparently anomalous situation could be accounted for by rapid degradation of C_2A_6Es and C_2A_6ECs via β-oxidation process of the main fatty acid chain leading, respectively, to formation of C_2A_4Es and C_2A_4ECs. After a rather long acclimation time, these very polar species originated also from ω, β-oxidative attack of the side A-chain of CA_7Es and CA_7ECs. This picture accounts for the appearance of second maxima in time-concentration profiles of C_2A_4Es and C_2A_4ECs, just following gradual disappearance of the above species. The appearance of third maxima for $C_2A_4E_2C$ and $C_2A_4E_3C$ concentrations can be explained by shortening of the E-chain of $C_2A_4E_4C$. As said above, this mechanism is active in any stage of the $BA_{12}E$ biotransformation. In fact, very small amounts of CA_7E_1C reappeared on day 50 after more than 20 days of latency.

Conclusions

This study has shown that LC-ES-MS is a powerful technique for unambiguous identification of intermediates which can

originate from BAE biotransformation. This allowed us to elucidate many aspects of the biodegradation pathway of a 2-butyl branched $A_{12}E$ surfactant mixture (Figure 9). Under analogous conditions, the primary degradation rate of this surfactant mixture was comparable to that of $LA_{14}E_8$, reported in a previous study (*3*). However, degradation products of the $BA_{12}E$ surfactant mixture here considered are much more recalcitrant to further degradation than PEGs originated from $LA_{14}E_8$ degradation (*3*). The resistance of BAE intermediates to microbial attack coupled with their mobilities deriving from their highly polar nature could make them of significant environmental interest. Commercial detergent products may contain BAE mixtures with both shorter and longer side A-chains than that of the surfactant considered in this study. In the near future, our attention will be addressed to elucidate the biodegradation pathways of these surfactant mixtures. Efforts will be also devoted to develop a sufficiently sensitive LC-ES-MS method for monitoring BAE degradation products in environmental waters.

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