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PAIRED-ION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PARTIALLY BIODEGRADED LINEAR ALKYLBENZENESULPHONATE

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SUMMARY

The separation of mixtures of linear alkylbenzenesulphonate and *p*-sulphophenylcarboxylate salts was successfully achieved, without derivatisation, by paired-ion high-performance liquid chromatography. Partially biodegraded linear alkylbenzenesulphonate was analysed by the same method. Structural information on metastable intermediates formed was provided by stopped-flow ultraviolet spectra, comparison of retention behaviour with that of standards and analysis of collected fractions.

INTRODUCTION

Linear alkylbenzenesulphonate (LAS) is a surface active agent widely employed in detergent formulations. The commercial product is a mixture of secondary isomers of alkyl chain lengths of 9 to 13 carbon atoms. The biodegradation of LAS can be considered in two stages. Primary biodegradation is said to have occurred when the original molecule has its structure altered by bacterial action. Secondary biodegradation is the further conversion of the LAS molecule, ultimately to carbon dioxide, water, inorganic salts and products associated with the normal metabolic processes of bacteria.

Colorimetric analysis by the methylene blue method[1] detects LAS but not its metabolites, and may be employed to monitor primary biodegradation[2]. Analysis of partially degraded LAS mixtures by desulphonation gas–liquid chromatography (GLC)[2,3] has shown that the rate of initial bacterial attack is related inversely to the distance between the point of benzene ring attachment and the most remote end of the alkyl chain.

Swisher[4] has postulated that the first stage of the biodegradation of a LAS molecule occurs by ω-oxidation of the end of the alkyl chain furthest from the benzene ring. Next, the *p*-sulphophenylcarboxylate (SPC) salt so formed undergoes successive β-oxidation steps, each of which shortens the alkyl chain by two carbon atoms. At

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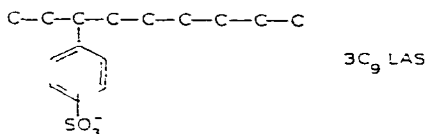
some point, further β-oxidation becomes more difficult than the previous steps, perhaps due to the proximity of the benzene ring. A SPC intermediate will then accumulate in solution until the bacteria "learn" to degrade it. Analysis of partially biodegraded LAS by desulphonation-GLC[5,6] has detected SPC salts as intermediates. The analytical method used is responsive to only some of the possible intermediates. Addition of a methylation step should expand this response, but gave no evidence for the presence of any other intremediates[7]. Further, but non-specific evidence for the formation of SPC intermediates was obtained from ultraviolet (UV) spectrometry[8] and by the use of ^{14}C ring-labelled LAS[9]. Recent developments have suggested that high-performance liquid chromatography (HPLC) would be an attractive technique for the analysis of such compounds and a preliminary study has been reported[10]. High-efficiency separations of sulphonates have been described employing a mobile phase containing cetyltrimethylammonium ($CTMA^+$) ions[11].

EXPERIMENTAL

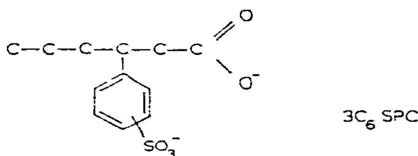
Chemicals

The pure isomers $3C_9$, $5C_{10}$, $3C_{12}$ and $2C_{14}$ LAS were a gift from the Unilever Research Laboratories, Port Sunlight, Great Britain.



$3C_9$ LAS

SPC salts are not available commercially (except dipotassium *p*-sulphobenzoate) but can be formed from the sulphonation and subsequent neutralisation of the corresponding phenylcarboxylic acid. Concentrated sulphuric acid (4 ml) and phenylcarboxylic acid (1 g) were reacted at 60° for 24 h. The mixture was carefully diluted with 20 ml distilled water, washed with 2 × 20 ml diethyl ether to remove unsulphonated material and neutralised to pH 7–9 by the addition of saturated aqueous sodium hydroxide solution. Three volumes of 2-propanol were added causing sodium sulphate to precipitate out. The remaining solution was filtered and the filtrate evaporated to dryness. The white solid so produced was purified by recrystallisation from methanol–diethyl ether mixtures. Yields of 30–60% were obtained for the synthesis of $2C_2$, $2C_3$, $2C_4$, $3C_4$, $4C_4$, $5C_5$, $2C_6$, $3C_6$ and $8C_8$ SPC disodium salts. The trisodium salt of 3-*p*-sulphophenylglutaric acid was prepared by the same method in a yield of 12%. The structures of the products were confirmed by nuclear magnetic resonance (NMR) spectroscopy employing deuterium oxide as the solvent. NMR spectra confirmed that monosulphonation had occurred in the *para* position and showed no evidence of impurities. Further confirmation of purity and structure was provided by microanalysis after drying at 130° for 2 h.



$3C_6$ SPC

Laboratory biodegradation of LAS

River water was used both as the source of organisms and as the biodegradation medium. LAS was added to ten litres of filtered (Whatman No. 1) water collected from the River Avon at Keynsham, Great Britain. Solutions were stored at room temperature, away from direct sunlight and analysed at appropriate intervals.

Primary biodegradation was monitored by the methylene blue method[1]. Biodegradation of the benzene ring in LAS and its metabolites was monitored by UV absorbance measurement at 223 nm[12]. The UV spectra of LAS and SPC salts dissolved in water are similar, showing absorbance maxima at 224 and 222 nm, respectively. River water also absorbs UV light in the region of interest and so was employed in the reference cell of the double beam instrument. The UV spectrum at wavelengths above 203 nm could be observed using 2-mm path length cells. Measurements at 223 nm were corrected for errors due to turbidity and dirty cell faces, by subtracting the absorbance value at 280 nm. UV spectra observed during LAS biodegradation were similar in appearance to those of LAS and SPC salts.

Samples (1.5 l) were concentrated for analysis (by HPLC) by acidification with sulphuric acid to pH 2, followed by passage through a column containing 20 ml of XAD-4, at a flow-rate of 7 ml/min. Compounds retained by the resin were eluted with 3 × 25 ml portions of methanol and the combined eluates evaporated to dryness. The flask was washed out with 2 × 1 ml distilled water and the washings were transferred quantitatively to a 2-ml volumetric flask.

Paired-ion HPLC

The liquid chromatograph consisted of two high-pressure, reciprocating piston pumps (Waters Assoc. M 6000), a septum injector and a variable wavelength, double beam UV detector (Varian 635). Reversed-phase packing material, mean particle size 5 μm, was prepared by the reaction of LiChrosorb Si60 silica with octadecyltrichlorosilane[13]. Microanalysis of the dried product revealed an organic loading of 9.92% (w/w). Columns were prepared by slurry packing the phase (at 8000 p.s.i.) into seamless stainless-steel tubing (250 × 4.6 mm). The mobile phase was a solution of $(CTMA^+)_2SO_4^{2-}$ in methanol (AnalaR grade, distilled)–distilled water. It was prepared by the passage of a 2% (w/v) solution of $CTMA^+Br^-$ in methanol–water (1:1) through a column containing 25 g of the anion-exchange resin AG1-X8 (OH^-). The eluate was diluted and neutralised to the desired pH with concentrated sulphuric acid prior to use.

RESULTS AND DISCUSSION

HPLC of LAS mixtures

Liquid chromatography of LAS and SPC mixtures was attempted employing a bonded C_{18} column packing with a mobile phase consisting of methanol and water only, as has been reported[10]. However, the peaks observed were broad and highly asymmetric, column efficiency calculated from them being only 100–200 theoretical plates. Addition of a small amount of $(CTMA^+)_2SO_4^{2-}$ increased retention times and calculated column efficiency rose to approximately 3000 theoretical plates. The same column was employed for the chromatography of a non-ionic compound (*p,p*-DDT) with a mobile phase (90% methanol, 10% water) containing no $CTMA^+$,

the same flow-rate being used. A similar column efficiency was calculated (2800 theoretical plates).

Fig. 1 a shows the trace obtained from the analysis of a LAS mixture, namely "sodium dodecylbenzene sulphonate, technical", supplied by Koch-Light (Colnbrook, Great Britain). Peak identifications were made by co-injection with pure LAS compounds and confirmed by analysis of the LAS mixture by desulphonation followed by GLC of the resultant alkylbenzenes on 15-m OV-1 support coated open-tubular column.

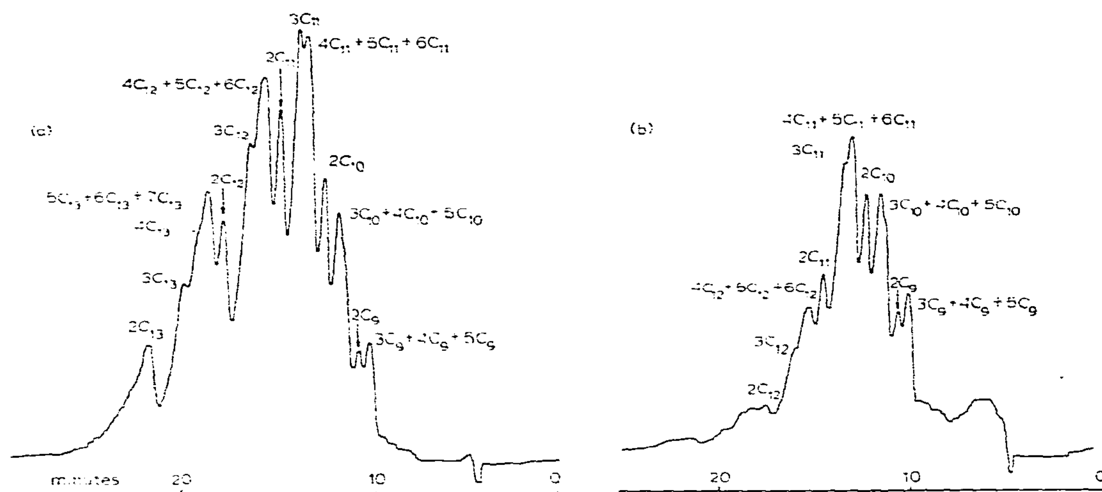


Fig. 1. Paired-ion HPLC of Koch-Light LAS (a) undegraded, (b) after partial biodegradation. Column: 250 × 4.6 mm, bonded C_{18} silica, d_p = 5 μm. Mobile phase: 13.7 · 10^{-3} M $(CTMA^+)_2SO_4^{2-}$ in 87.5% methanol, 12.5% water, pH 5.4. Flow-rate: 0.8 ml/min. UV detection at 224 nm, 0.5a.u.f.s.

Koch-Light LAS was added to river water at 60 μg/g, a sample concentrated on XAD-4 and analysed by paired-ion HPLC. The trace obtained showed the same mixture composition as in Fig. 1 a and a recovery of 96% was calculated. After 2.8 days, the values of the methylene blue and UV measurements had falled to 58 and 61%, respectively, of their original values. These figures do not indicate the formation of a significant amount of metastable, UV active, biodegradation intermediates. A further sample was concentrated on XAD-4 and analysed by paired-ion HPLC, producing the trace in Fig. 1b. Note that (a) the longer chain length LAS compounds were preferentially degraded and (b) for a given chain length, there was preferential degradation of the isomers with the benzene ring attached close to the end of the alkyl chain.

These findings were in agreement with previous results[2,3]. Methylene blue and UV measurements showed that no further biodegradation occured in the following 8 days. The most probable reason for the lack of bacterial activity was that the LAS concentration was too high.

HPLC of SPC mixtures

Under the conditions employed in Fig. 1, SPC salts were eluted close to the solvent front. In order to achieve separation of a mixture of SPC standards, the

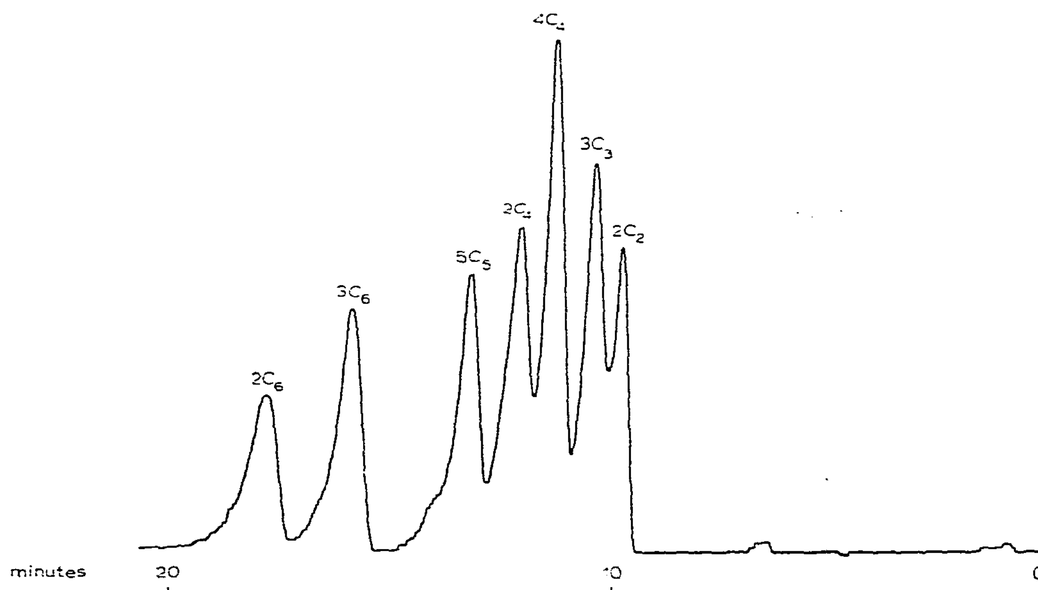


Fig. 2. Paired-ion HPLC of a mixture of SPC salts. Column: 250 × 4.6 mm, bonded C_{18} silica, $d_p = 5\,\mu m$. Mobile phase: $5.5 \cdot 10^{-3}$ *M* $(CTMA^+)_2SO_4^{2-}$ in 75% methanol, 25% water, pH 2.3. Flow-rate: 0.8 ml/min. UV detection at 222 nm, 0.5 a.u.f.s.

methanol content of the mobile phase was decreased from 87.5 to 75%. Fig. 2 depicts the separation employing a mobile phase, pH 2.3.

A 3-l volume of $CTMA^+$ OH^- in 75% methanol, 25% water was prepared (as previously described) and divided into 1-l portions. Concentrated sulphuric acid was added to produce solutions of $5.5 \cdot 10^{-3}$ *M* $(CTMA^+)_2SO^{2-}$ with pH values of 6.2, 4.6 and 2.2. Each solution was employed in turn as the mobile phase for the chromatography of a mixture of SPC salts. The effect of pH on retention behaviour is illustrated in Fig. 3. At pH 6.2, carboxylate groups are predominantly in the ionised form $R-CO_2^-$, which make ion pairs with $CTMA^+$. The decreased retention times observed at pH 4.6 are due to an increase in the concentration of the $R-CO_2H$ form, which do not make ion pairs with $CTMA^+$.

The figures in Table I demonstrate that aqueous solutions of SPC salts can be concentrated on XAD-4 resin. A solution (1 l) of 3 ppm SPC in distilled water was acidified with sulphuric acid to pH 2 and passed through a column of XAD-4 (20 ml). Retention by the resin was monitored by measurement of the UV absorbance (at 222 nm) of the eluate. Compounds adsorbed on the column were eluted with 3 × 25 ml methanol. The combined eluates were evaporated to dryness, dissolved in 2 ml water, and analysed by paired-ion HPLC. Retention by the resin of $2C_4SPC$, and hence its overall recovery, was greater at a column flow-rate of 7 ml/min than at 20 ml/min. SPC salts in neutral solution were poorly retained by XAD-4.

The biodegradation of a 10 μg/g solution of a LAS mixture supplied by the Soap and Detergent Association (SDA; New York, N.Y., U.S.A.) was studied. The composition of this mixture, as measured by desulphonation-GLC, is given in Table II. After 2 days, the methylene blue test and UV values had fallen to 19.0 and 89.3%, respectively, of the original figures, indicating that metastable intermediates con-

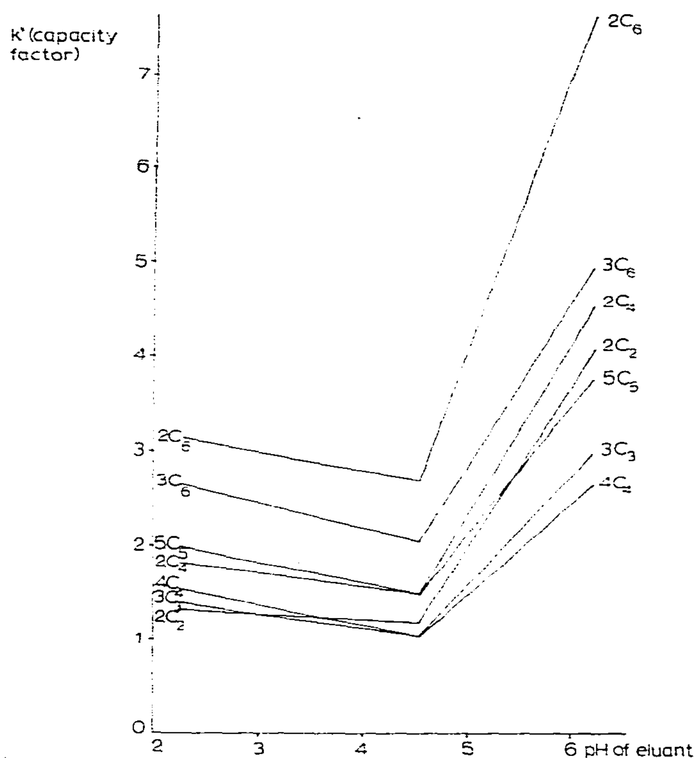


Fig. 3. Paired-ion HPLC of SPC salts, variation of pH of mobile phase. Column: 250 × 4.6 mm, bonded C_{18} silica ,d_p = 5 μm. Mobile phase: 5.5 · 10^{-3} M $(CTMA^+)_2SO_4^{2-}$ in 75% methanol, 25% water.

taining the benzene ring had been formed. A sample was concentrated on XAD-4 and analysed by paired ion HPLC under the conditions of Fig. 2. The trace obtained (Fig. 4) contained a number of peaks with similar retention times to SPC standards. Note that none of these peaks corresponded to undegraded LAS, which had a much

TABLE I

CONCENTRATION OF AQUEOUS SPC SOLUTIONS ON XAD-4

Experiment 1:3 ppm $2C_2$, $2C_3$, $2C_4$, $2C_6$ SPC. Flow-rate: 20 ml/min; experiment 2: 2 ppm $2C_4$, $3C_4$, $4C_4$ SPC. Flow-rate 7 ml/min.

Parameter	*Volume of solution passed (ml)*	*Experiment 1*	*Experiment 2*
Retention	100	87.9	
by resin (%)	500	61.8	
	950	54.2	94.6
Overall SPC recovery (%)		$2C_2$ 21.0	
		$2C_3$ 45.1	
		$2C_4$ 72.5	$2C_4$ 90.3
			$3C_4$ 93.2
			$4C_4$ 92.5
		$2C_6$ 92.7	

TABLE II

COMPOSITION OF SDA LAS

LAS isomer	*Percentage of total*	
$5C_{10}$	2.9	
$4C_{10}$	3.1	16.5
$3C_{10}$	3.4	
$2C_{10}$	7.1	
$6C_{11}$, $5C_{11}$	12.7	
$4C_{11}$	9.1	43.8
$3C_{11}$	9.2	
$2C_{11}$	12.8	
$6C_{12}$	7.2	
$5C_{12}$	5.8	
$4C_{12}$	5 3	35.5
$3C_{12}$	7.3	
$2C_{12}$	9.9	
$7C_{13}$, $6C_{13}$	0.9	
$5C_{13}$	0.5	4.2
$4C_{13}$	0.7	
$3C_{13}$	0.9	
$2C_{13}$	1.2	

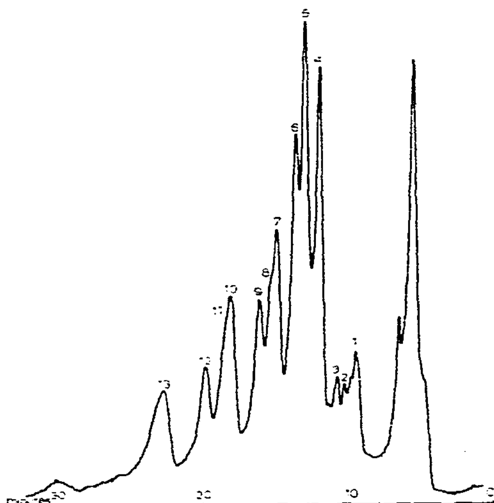


Fig. 4. Paired-ion HPLC of partially degraded SDA LAS. 5-μl injection of 2-day concentrate. Chromatography conditions as in Fig. 2.

greater elution time under these conditions. Peak 3 and the peaks eluted close to the solvent front were present in the HPLC trace of river water (no LAS added) concentrated by the same method. Further analyses revealed changes in the peak pattern as biodegradation proceeded (Fig. 5). A methylene blue analysis performed after 3.2 days revealed that no intact LAS remained in solution.

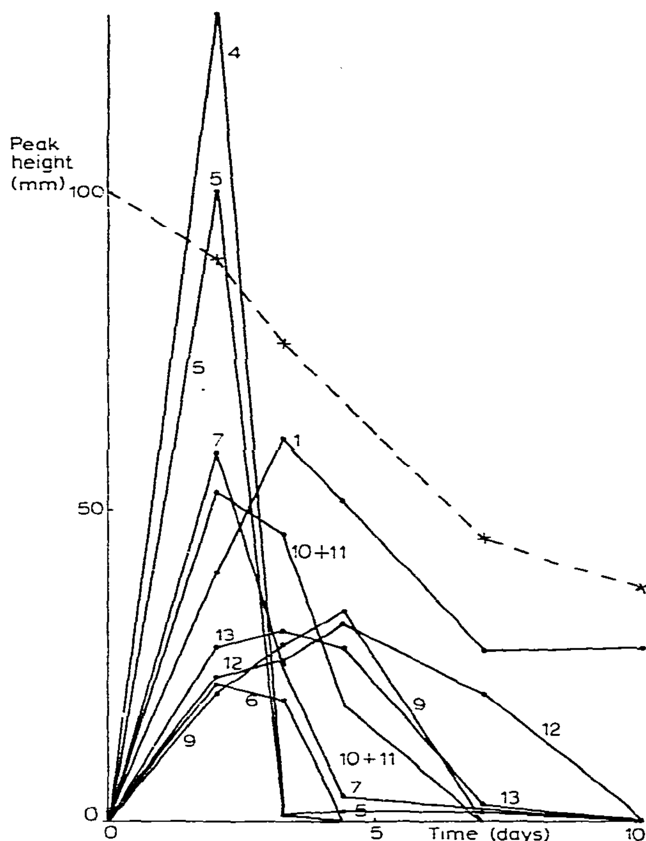


Fig. 5. Concentration profile of metastable intermediates formed during the biodegradation of SDA LAS. Peak height measured from 6-μl injection of concentrate, employing UV detection at 223 nm, 0.5 a.u.f.s. (*e.g.* Fig. 4). --------, 223–280 nm UV absorbance value (vertical axis represents % of $T = 0$).

A study of the biodegradation of a 15 μg/g solution of $3C_9$ LAS was performed (Fig. 6). Paired-ion HPLC analysis of concentrated, partially degraded $3C_9$ LAS employing the conditions of Fig. 2 revealed that four metastable intermediates, labelled A, B, C, D in order of increasing column retention, were formed. Intermediates B and C corresponded to 6 and 7 (observed during the biodegradation of SDA LAS), respectively.

Identification of LAS biodegradation intermediates

Stopped-flow UV spectra of all SPC standards showed a single absorbance peak at 222 nm. The stopped-flow spectra of all LAS biodegradation intermediates were similar to those of SPC standards, with the exception of the spectrum of intermediate 5 which contained a broad peak at 252 nm as well as a 222-nm peak. A study of the biodegradation of LAS by pure strains of bacteria[14] has shown that some species degrade LAS via metastable phenylcarboxylate and *p*-hydroxyphenylcarboxylate intermediates. The paired-ion HPLC of sodium phenylacetate and sodium *p*-hydroxyphenylacetate revealed that these compounds were eluted close to the solvent

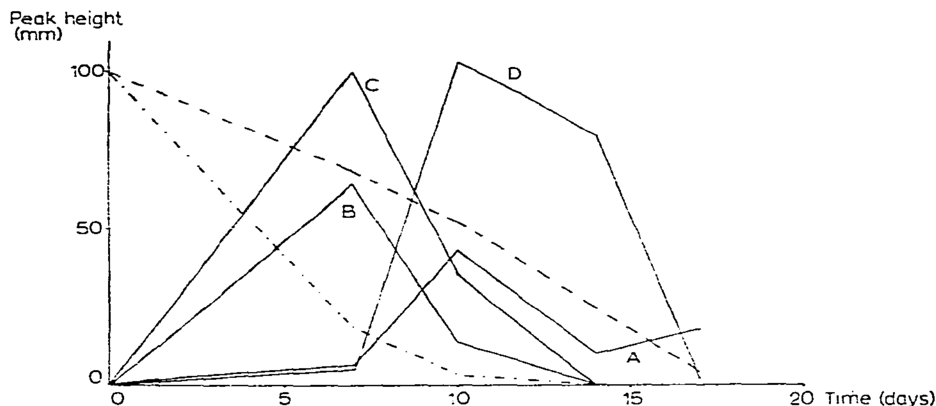


Fig. 6. Biodegradation of $3C_9$ LAS. Metastable intermediates A,B,C,D were observed as peaks in paired-ion HPLC (Fig. 2 conditions), 3-μl injection of concentrate. ---------, 223–280 nm UV absorbance value (vertical axis represents % of $T = 0$); -·-·-·-, methylene blue test value (vertical axis represents % of $T = 0$).

front ($k' = 0.39$ and 0.20, respectively) under the conditions of Fig. 2. The stopped-flow UV spectrum of sodium phenylacetate consisted of a peak at 212 nm and that of *p*-hydroxyphenylacetate, peaks at 224 and 276 nm. It has been suggested[10] that *p*-sulphophenyldicarboxylate salts may be formed as metastable intermediates during the biodegradation of LAS. Paired-ion HPLC of the trisodium salt of 3-*p*-sulphophenylglutaric acid revealed that it was weakly retained ($k' = 0.98$) under the conditions of Fig. 2. The stopped-flow UV spectrum consisted of a single absorbance peak at 222 nm.

The retention behaviour of the intermediates observed was compared with that of SPC standards employing mobile phases of various compositions and pH values. If the ion-pairing reagent was omitted from the mobile phase, whilst retaining the same percentage of methanol, the intermediates were eluted as a single broad peak close to the solvent front. The evidence suggested that all of the intermediates contained a functional group capable of forming an ion pair with $CTMA^+$ at pH 2.3 → 6.2, and so were probably sulphonates. Peaks 4, 9 and 10 were identified as $3C_4$, $3C_6$ and $2C_6$ SPC, respectively, by direct comparison with the retention behaviour of these standards. The retention behaviour of SPC salts was predicted from the HPLC of the range of SPC standards available. Structures were tentatively assigned to other intermediates on the basis of these estimates.

A mobile phase of $5.5 \cdot 10^{-3}$ *M* $(CTMA^+)_3PO_4^{3-}$ in methanol–water (3:1) pH 3.4 was employed for the paired-ion HPLC of partially degraded $3C_9$ LAS. Fractions containing separately the four intermediates A, B, C, D were collected, neutralised with sodium hydroxide solution, evaporated to dryness and examined by the Lassaignes sodium nitroprusside test for sulphur. A positive result was obtained in each case whereas the same test performed on a fraction collected where no peaks were being eluted gave a negative result.

Further analysis of fractions collected during paired-ion HPLC of partially degraded $3C_9$ LAS was attempted by Fourier transform NMR spectroscopy. Fractions containing micromole quantities of the four intermediates were collected,

neutralised with sodium hydroxide solution and passed through a column containing 20 ml of the cation-exchange resin AG-50 W (H^+) (to remove the ion-pairing reagent $CTMA^+$). The fractions were neutralised to pH 7 again, evaporated to dryness at 130° for 8 h and dissolved in 99.97% purity deuterium oxide. The spectra of all four intermediates contained two doublet signals in the τ 2–3 region, a feature characteristic of a benzene ring para substituted with two different groups. Otherwise the spectra were difficult to interpret, being noisy and complicated by some spurious signals.

The evidence indicated that all of the metastable intermediates observed during the biodegradation of LAS contained the structural unit

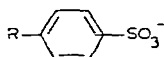


Table III shows structural assignments made, those in brackets being tentative.

TABLE III

STRUCTURES ASSIGNED TO INTERMEDIATES OBSERVED DURING THE BIODEGRADATION OF LAS

	Intermediate	*Structure*
SDA LAS	1	(*p*-Sulphophenyldicarboxylate)
	2	(*p*-Sulphophenyldicarboxylate)
	4	$3C_4$ SPC
	5	? not an SPC
	6	($3C_5$ SPC)
	7	($4C_6$ SPC)
	8	($2C_5$ SPC)
	9	$3C_6$ SPC
	10	$2C_6$ SPC
	11	($4C_7$ SPC)
	12	($3C_7$ SPC)
	13	($2C_7$ SPC)
$3C_9$ LAS	A	(*p*-Sulphophenyldicarboxylate)
	B	($3C_5$ SPC)
	C	($4C_6$ SPC)
	D	? not an SPC

CONCLUSION

The relatively new technique of paired-ion HPLC was shown to be capable of the analysis, without derivatisation, of complex LAS and SPC mixtures. Paired-ion HPLC was also well suited to the analysis of partially degraded LAS. Compounds which absorb UV light at 223 nm were detected (which includes all compounds containing a benzene ring). The method has advantages over desulphonation-GLC[5,6] which causes structural rearrangement and is not responsive to all possible intermediates. Hence paired-ion HPLC is likely to give a more accurate and complete analysis of metastable intermediates formed. Examination of a partially degraded LAS mixture revealed that at least thirteen metastable intermediates were formed.

Some of the intermediates corresponded to those observed by desulphonation-GLC as well as several that did not.

The ω,β-oxidation theory predicts that a single metastable intermediate will be formed from the biodegradation of $3C_9$ LAS, namely $3C_5$ SPC. Paired-ion HPLC analysis of partially degraded $3C_9$ LAS indicated that the true biodegradation scheme may be more complex. Four metastable intermediates were observed, one of which was probably $3C_5$ SPC. A method for the conclusive identification of all metastable LAS biodegradation intermediates is required to elucidate the situation. The characterisation of sulphonates by formation of the methyl ester derivative and subsequent examination by mass spectroscopy, has been reported[15]. Such a method could be applied to fractions collected during paired-ion HPLC.

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REFERENCES

1 J. Longwell and W. D. Maniece, *Analyst (London)*, 80 (1955) 167.
2 R. D. Swisher, *J. Water Poll. Contr. Fed.*, 35 (1963) 877.
3 R. L. Huddleston and R. C. Allred, *Dev. Ind. Microbiol.*, 4 (1963) 24.
4 R. D. Swisher, *Surfactant Biodegradation*, Marcel Dekker, New York, 1970.
5 R. D. Swisher, *J. Water Poll. Contr. Fed.*, 35 (1963) 1557.
6 H. Leidner, R. Gloor and K. Wuhrmann, *Tenside Detergents*, 13 (1976) 122.
7 R. D. Swisher, *Chem. Eng. Progr.*, 60 (1964) 41.
8 R. D. Swisher, *Japan Analyst*, 21 (1972) 130.
9 W. E. Gledhill, *Appl. Microbiol.*, 30 (1975) 922.
10 R. D. Swisher, W. E. Gledhill, R. A. Kimerle and T. A. Taulli, *7th Int. Cong. Surf. Act. Subst.*, Paper 117, Moscow, 1976.
11 J. H. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1976) 17.
12 R. D. Swisher, *Ind. Chim. Belge*, 32 (1967) 718.
13 B. B. Wheals, *J. Chromatogr.*, 122 (1976) 85.
14 R. B. Cain, A. J. Willats and J. A. Bird, *Proc. Int. Biodetesior. Symp., 2nd, 1971, Lunteren*, Applied Sci. Publ., Barking, 1972, pp. 136–144.
15 A. Heywood, A. Mathias and A. E. Williams, *Anal. Chem.*, 42 (1970) 1272.