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Preparative liquid chromatographic separation of the oligomers of nonoxynol-9 and their characterization by ^{1}H-NMR and mass spectroscopy

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Abstract: Conditions are described for the preparative LC separation of a bulk nonoxynol-9 material into 16 components. ^{1}H-NMR analyses of these fractions provided evidence for all but the first of the components to be oligomers of nonylphenoxypolyethoxyethanol (nonoxynol) with *n*-values for $(OCH_2CH_2)_n$ ranging consecutively from 3 to 17, corresponding to the LC fractions 2–16. Mass spectral analysis of the separated LC fractions confirmed the oligomeric sizes deduced from ^{1}H-NMR spectral data, and provided EI fragmentation information for these oligomeric substances.

Keywords: *^{1}H-NMR (250 and 400 MHz); MS; nonoxynol-9; nonylphenoxypolyethoxyethanol.*

Introduction

In an earlier paper [1], a phase isocratic LC system was described for characterizing nonoxynol-9 spermicidal agents. These nonoxynol-9 (Fig. 1) non-ionic surfactant preparations were found to be complex mixtures comprising 16–17 components where the average number of $(OCH_2CH_2)_n$ ethyleneoxy repeating units per molecule was 9 [2]. Although nonoxynol-9 has been used for many years as a spermicide in vaginal contraceptive suppositories, foams, creams and jellies, as well as more recently for incorporation into vaginal contraceptive sponges and for coating (lubricated) condoms, only recently has the bulk material been evaluated for cytotoxicity and genotoxicity [3]. While the spermicidal activity of nonoxynol-9 is expressed by its bulk nature, and now that it can be separated by LC into its component oligomers, each of which could be characterized and differentiated by ^{1}H-NMR and mass spectrometry, there was interest in separating and collecting sufficient characterized material of each nonoxynol-9 component for evaluating individual spermicidal activity of the components versus activity of the bulk material. Recently, the use of preparative LC for the purification of ethoxylated anionic surfactants was reported [4], and conventional LC has been used to determine the ethoxylate oligomer distribution of both non-ionic and anionic surfactants [5, 6].

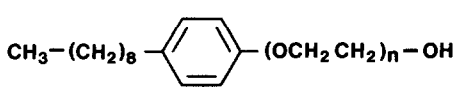

Figure 1
Structure of nonoxynol-9 oligomers.

Experimental

Preparative LC

The chromatograph consisted of a preparative LC (Varex VERSA Prep), a controller (Commodore PC 10-II), a UV detector (LDC/Milton Roy Spectro Monitor D at 280 nm), and an integrator (Spectra Physics SP4270).

For analytical scale separations, performed to monitor the composition of the preparative LC fractions, an analytical pump (Eldex AA-100-S), and a normal phase column (CSC, SSW, 25 × 0.46 cm, 5 μm) were used with a mobile phase of ethylacetate–methanol (50:50, v/v) at a flow rate of 1 ml min^{-1}.

Preparative scale separations were done on a preparative silica column (SUPELCOSIL PLC-Si, 15 μm, 25 cm × 21.2 mm), with

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Table 1
Gradient blends and time intervals for mobile phase of ethyl acetate and methanol during preparative scale separations of nonoxynol-9 oligomers

Run time (min)	% EtOAc	% MeOH
0–4	85	15
4–8	85–78	15–22
8–20	78–40	22–60
20–23	40	60
23–26	40–85	60–15
26–30	85	15

gradient solvent delivery (Table 1), and a flow rate of 25 ml min^{-1}. Fractions were collected manually according to UV peak response, collection for each peak being initiated and terminated at approximately 25% peak height to minimize oligomer mixing.

Up to 30 mg of bulk nonoxynol-9 (VLI Corporation, CA, Lot RM-1065-22) was applied to the preparative LC column per injection. The solvents employed were glass-distilled HPLC grade.

^{1}H-NMR spectroscopy

^{1}H-NMR spectra were determined at 250 MHz with a Bruker WM 250 spectrometer on specimens of the separated nonoxynol-9 components dissolved in acetone-d_6 containing 1% TMS (internal reference). Acetone-d_6 was found to be the solvent which resulted in the richest ^{1}H-NMR spectra of nonoxynol-9 samples [1]. More accurate analysis of the 250 MHz integration data was performed at a later date using the DISNMR87 program available in conjunction with a Bruker AM 400 instrument. Fractions 1 and 2 were also re-examined at this time at 400 MHz as $CDCl_3$ solutions.

Mass spectroscopy

Mass spectra of methanolic solutions (~1 μg μl^{-1}) of the separated nonoxynol-9 components were determined with a Finnigan 4610B mass spectrometer using the direct exposure probe (DEP) in the electron impact ionization (EI) mode. The previously cleaned DEP filament loop was dipped into a methanolic solution and allowed to dry leaving a residue of the specimen on the filament. Each sample was evaporated from the inserted DEP by applying a filament current of 0–1200 mA at 20 mA s^{-1}, approximately equivalent to 0–1200°C at a heating rate of 20°C s^{-1}. Mass spectra were determined for corresponding vapour emissions recorded as a reconstituted ion chromatogram (RIC). Fraction 9, showing M_W 660, constituted a practical limit for EI sensitivity for these substances.

Results and Discussion

Preparative LC

A typical preparative separation of 16 components of nonoxynol-9 eluting over a period of approximately 30 min is shown in Fig. 2. Subsequent analyses by LC of pooled fractions showed an 85–90% purity level from first-pass collections.

This level of purity was deemed adequate for spectroscopic characterization of the oligomers and for evaluation of intrinsic spermicidal activities in order to keep the total collection time (3–4 weeks) and solvent costs (50 l/week) within reasonable limits. This judgement was supported by the finding that the relative spermicidal activity of the separated oligomers was no different than that of the unseparated nonoxynol-9 material (H.S. Buttar, personal communication). Even with these compromises, only about 60 mg of components 2 and 14 were collected compared with an average of about 250 mg for each of other nonoxynol-9 components.

Previously, the ethylene–oxide content of ethoxylated alkylphenols has usually been based on iodometric or turbidimetric determinations, but as most technical surfactant products contain some poly(ethylene glycol), which iodometrically cannot be distinguished from other ethoxylated products and which influence the end-point in turbidimetric determinations, the degree of ethoxylation determined by these procedures is not always correct [5]. More recently, the assignment of ethoxylate number to oligomers has been made by comparison with standard reference materials and by the use of retention time–ethoxylate number relationships [6]. Alternatively peak areas are determined by electronic integration and compared with those of a standard solution containing an oligomer of known ethylene oxide units — the contributions of other peaks are calculated taking into account the respective relative molecular masses and assuming equal molar absorptivity [5]. The latter approach was verified by field-desorption mass spectrometry for molecular masses up to the low 600s [5]. ^{1}H-NMR spectral analysis has not been used either to

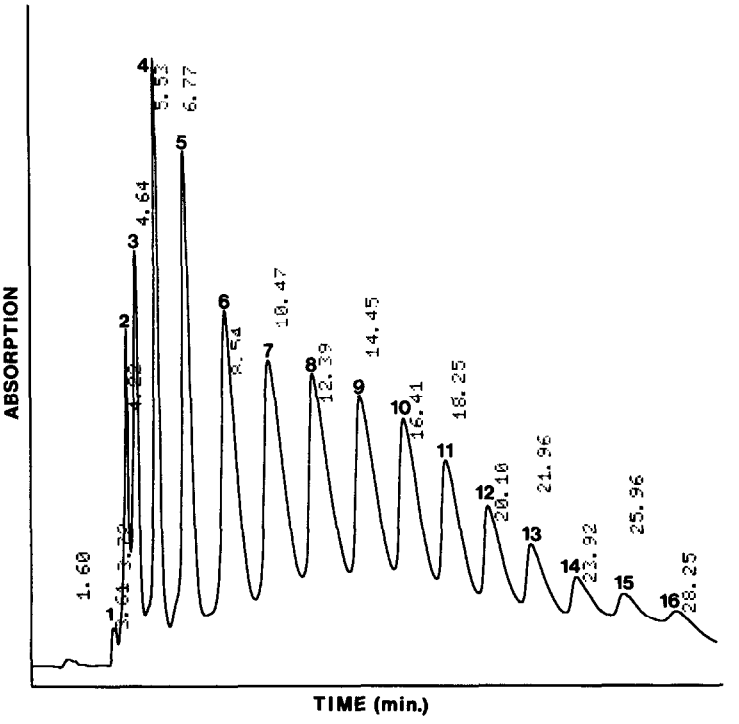

Figure 2
A typical preparative separation of 16 components from 30 mg of bulk nonoxynol-9.

provide confirmation of the chemical nature of oligomeric species present in ethoxylated alkylphenols or to afford insight into their molecular size from comparison of integration data. Likewise, attempts were made, in this study, to verify any ^{1}H-NMR findings by conventional mass spectrometric M_W determinations to the extent allowed by the sensitivity of the instrument and the nature of the substances examined.

^{1}H-NMR spectral analysis

Figure 3 shows representative ^{1}H-NMR spectra, obtained from LC fractions 1, 2, 3, 5 and 8. Each of these spectra, with the exception of that for fraction 1, show bands of multiple resonances for the aromatic and $(OCH_2CH_2)_n$ protons over the ranges δ7.5–6.5 and δ4.4–3.2, respectively. The complex envelope of multiple resonances ranging from ~δ1.5–0.5 was due to the protons of the nonyl group attached to the phenyl ring; the intense band at ~δ2.2 arises from residual acetone-d_5 in the acetone-d_6 solvent. The two methylene muliplets appearing consistently at δ4.1 and 3.9 in a constant ratio (1:1) with the aromatic proton resonances, most likely arose from the two terminal OCH_2 groups, i.e. the phenyl OCH_2 group and the alcoholic CH_2OH group. These spectra show progressive diminishment of relative intensity for the aromatic and nonyl protons with increasing intensity for the $(OCH_2CH_2)_n$ portion of the spectra with increasing LC fraction number as expected for increasing value of n in the separated oligomers. The acetone-d_6 ^{1}H-NMR spectrum for fraction 1 (Fig. 3), virtually devoid of aromatic component, shows peaks at ~δ3.8 and 3.0 [but not present in the 400 MHz $CDCl_3$ spectrum of fraction 1 (Fig. 4)], which would appear to be due to an impurity or by-product of the nonoxynol-9 preparation and not one of its oligomers. The ^{1}H-NMR spectrum for fraction 2 (Fig. 3) shows some inclusion of the spurious material of fraction 1 (singlet resonance at ~δ3.8). Upon evaporation of the acetone-d_6 solution of fractions 1 and 2 for uptake of these residues in $CDCl_3$, the spurious component(s) were lost as shown by their 400 MHz spectra (Fig. 4). The residual material of fraction 1 (Fig. 4) showed ^{1}H-NMR characteristics of a substance containing a saturated long chain hydrocarbon. A direct exposure probe (DEP) mass spectrum of this material also gave a typical hydrocarbon profile.

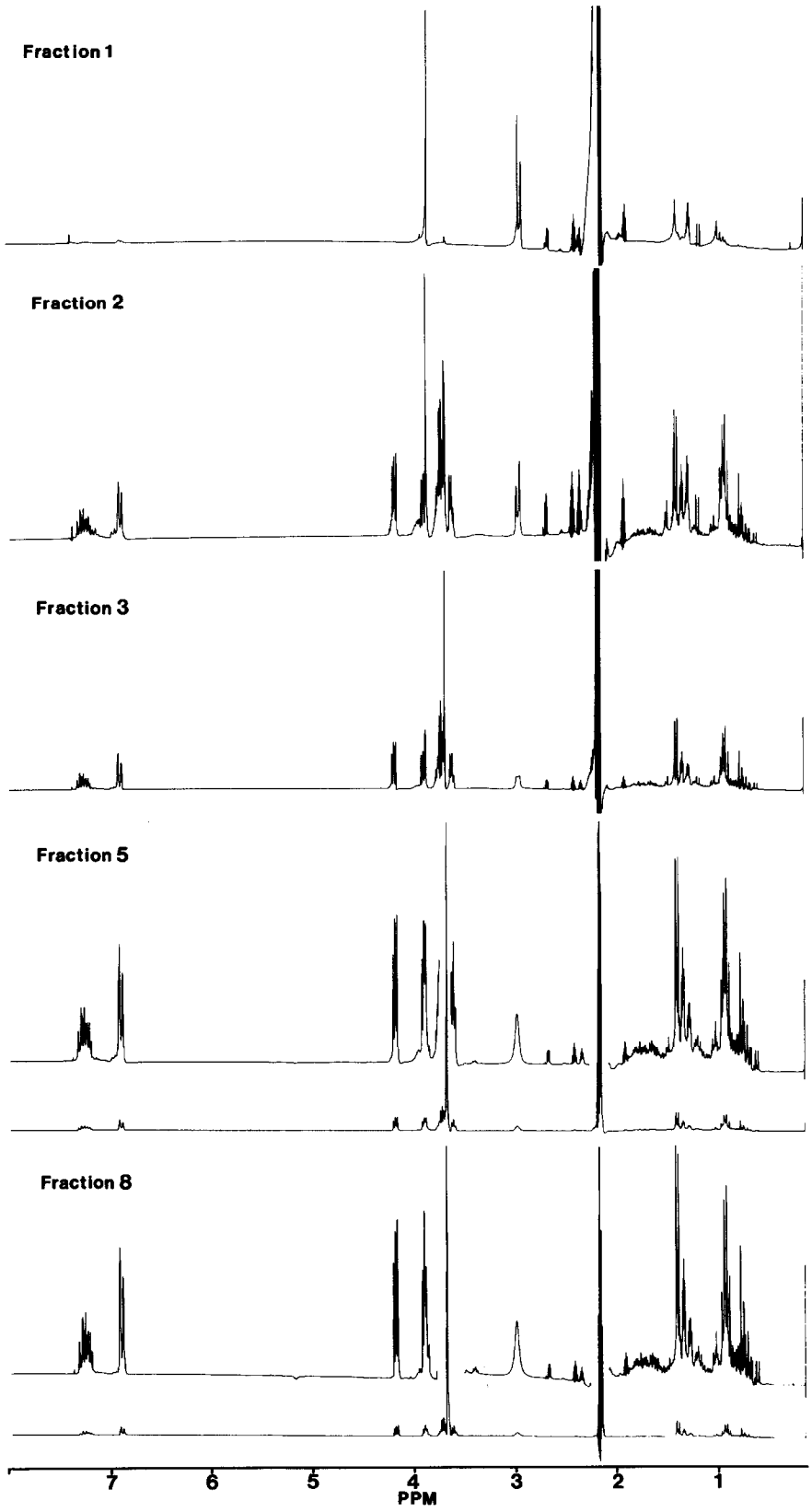

Figure 3
Selected ^{1}H-NMR spectra (250 MHz) of the indicated LC fractions of bulk nonoxynol-9 examined in acetone-d_6.

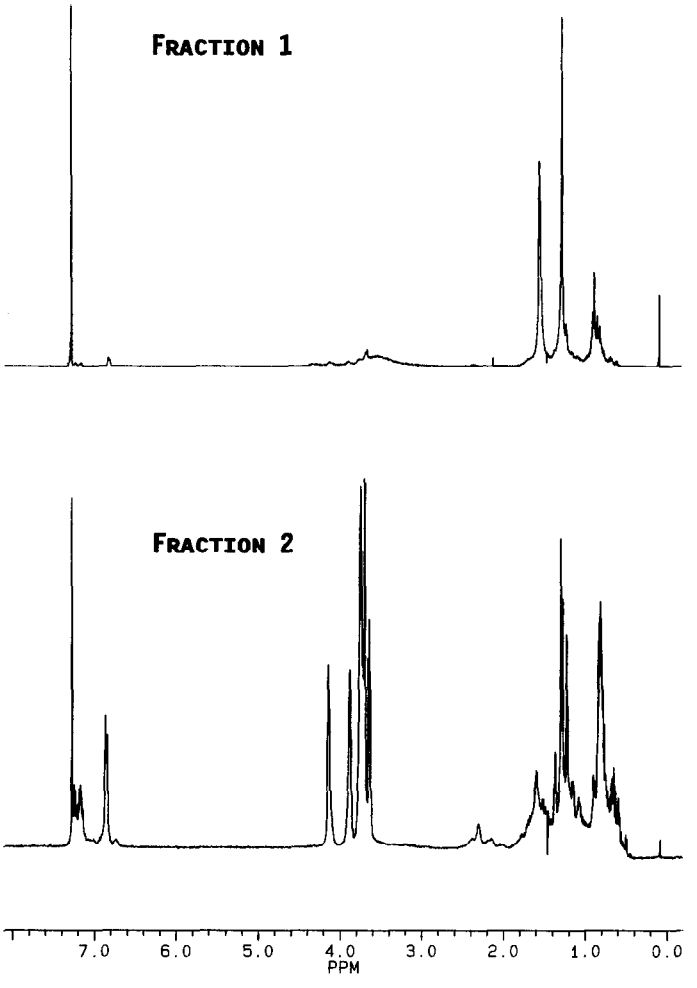

Figure 4
^{1}H-NMR spectra (400 MHz) of LC fractions 1 and 2 from bulk nonoxynol-9 examined in $CDCl_3$.

Integration of the ^{1}H-NMR spectra for nonoxynol-9 fractions provided insight into oligomer size when the ratios (R) of total integrals for aromatic and $(OCH_2CH_2)_n$ protons were determined. As can be seen from Table 2, the R values determined from integration data agree well with calculated R values for given n values for most of the separated oligomers. For values of $n > 10$, i.e. fractions 10–17, the good agreement between experimentally determined and calculated R values was only possible by use of the newer Bruker NMR program for analysis of integration data because of markedly diminishing intensity for the aromatic portion against overwhelming intensities for the $(OCH_2CH_2)_n$ band.

Mass spectral analysis

Typical DEP–EI mass spectra obtained from the HPLC fractions are shown in Fig. 5 for fractions 4 and 6. Molecular weights determined for the separated oligomers (Table 2) are in excellent agreement with the oligomeric sizes deduced from ^{1}H-NMR integration data.

Each of the mass spectra obtained was characterized by a moderately weak (~5% relative intensity) molecular ion ($M^{+\cdot}$) (e.g. 440 and 528 for fractions 4 and 6, respectively). In each of the spectra, the high mass range was characterized by initial M-15 fragmentation (weak CH_3 elimination) and M-29 (moderate CH_3 plus CH_2· elimination) fragmentation followed by four successive eliminations of CH_2 radicals or losses of $CH_2{=}CH_2$ (28 a.m.u.) to give a moderately strong (~50% R.I.) fragment ion, e.g. m/z = 355 and 443 in fractions 4 and 6, respectively. Thereafter, the fragmentation is very weak and uncharacteristic until the oligomers are virtually disintegrated producing a characteristic set of fragment

Table 2
Summary of ^{1}H-NMR integration data for determining size of nonoxynol-9 oligomers and mass spectroscopically determined molecular weights

Oligomer n-value	Calc. M_W	Number of protons Ar	$(OCH_2CH_2)_n$	Calc. R = Ar/$(OCH_2CH_2)_n$	R value found	HPLC fraction number	M_W by MS
3	352	4	12	0.33$\dot{3}$	0.34	2	352
4	396	4	16	0.250	0.26	3	396
5	440	4	20	0.200	0.21	4	440
6	484	4	24	0.16$\dot{6}$	0.17	5	484
7	528	4	28	0.143	0.15	6	528
8	572	4	32	0.125	0.14	7	572
9	616	4	36	0.11$\dot{1}$	0.12	8	616
10	660	4	40	0.100	0.12	9	660
11	704	4	44	0.091	0.09	10	—
12	748	4	48	0.08$\dot{3}$	0.09	11	—
13	792	4	52	0.077	0.08	12	—
14	836	4	56	0.071	0.08	13	—
15	880	4	60	0.067	*	14	—
16	924	4	64	0.063	0.08	15	—
17	968	4	68	0.059	0.06	16	—

*Not determined because sample was found to contain material that would not dissolve in acetone-d_6.

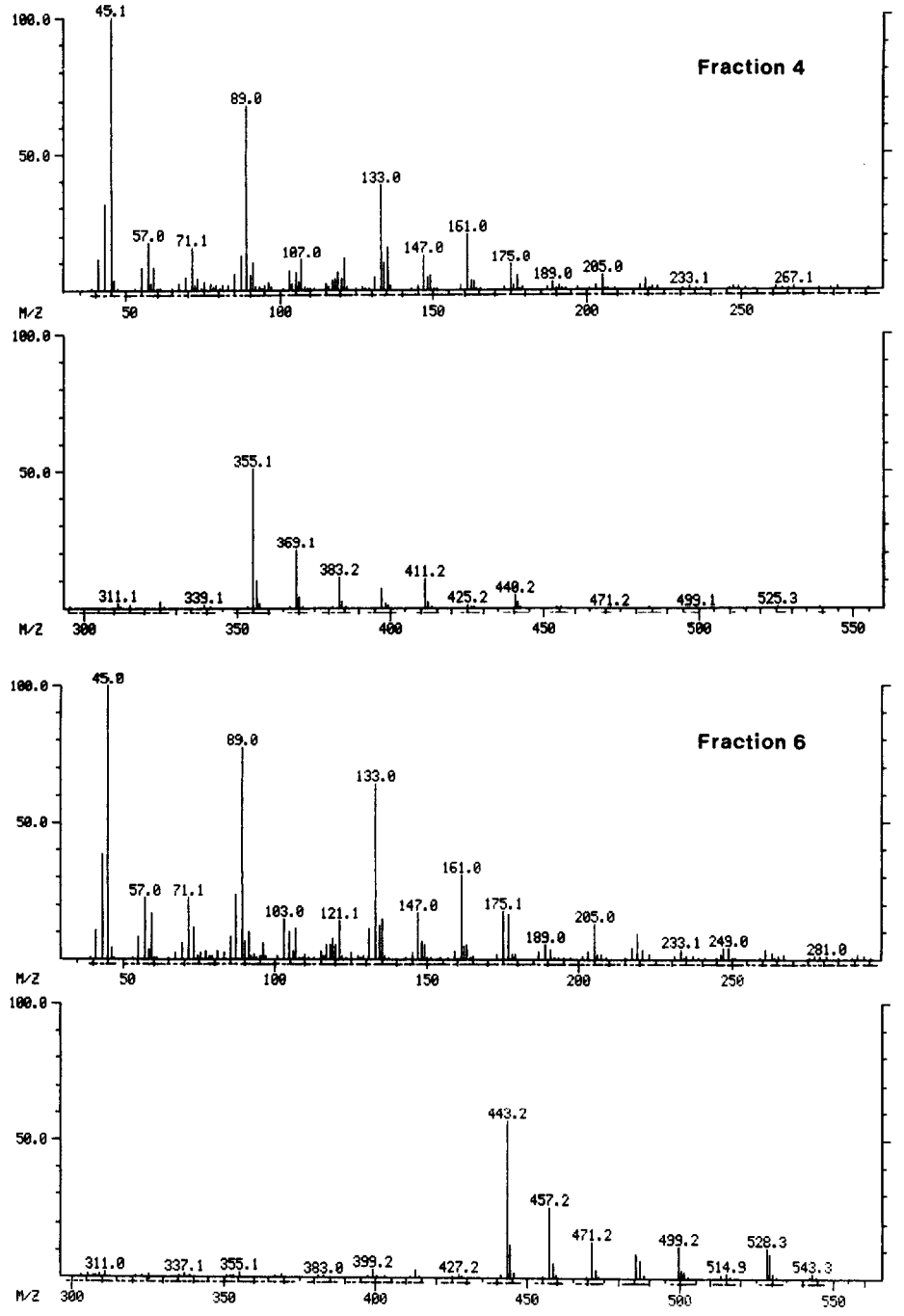

Figure 5
Representative EI mass spectra obtained from LC fractions 4 and 6 of the separated components comprising bulk nonoxynol-9.

ions in the lower mass range, e.g. m/z = 45, 89, 133, 161, 175 and 205 common to each of the oligomers.

References

[1] D.B. Black, B.A. Dawson and G.A. Neville, *J. Chromatogr.* **478(1)**, 244–249 (1989).
[2] *USP Pharmacopeial Forum*, p. 1027 (1986).
[3] H.S. Buttar, S.H.H. Swierenga and T.I. Matula, *Toxicol. Lett.* **31**, 65–73 (1986).
[4] P.K.G. Hodgson and N.J. Stewart, *J. Chromatogr.* **387**, 546–550 (1987).
[5] R.H. Schreuder and A. Martijn, *J. Chromatogr.* **435**, 73–82 (1988).
[6] R.E.A. Escott, S.J. Brinkworth and T.A. Steedman, *J. Chromatogr.* **282**, 655–661 (1983).

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