Technical

The Analysis of Fatty Alcohols and Acids As *p*-(Methylthio)-benzoate Esters

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ABSTRACT

The *p*-(methylthio)-benzoate ester of fatty alcohols is investigated. This derivative shows either a molecular ion or M-168 m/e peak in its mass spectrum and mixtures may be qualitatively analyzed directly by mass spectrometry. The compounds absorb strongly in the ultraviolet, allowing quantitative analysis by high performance liquid chromatography to 200 pmol.

The quantitative and qualitative analyses of highly lipophilic alcohols in biological matrices at micro to ultramicro levels are difficult. In general, such analyses require prior derivatization of the alcohol components to enhance detectability limits, to improve chromatographic separation characteristics or to aid in structural identification of the alcohols present. A derivative which allows trace quantitation and also identification of these compounds by a technique as specific as mass spectrometry (MS) becomes increasingly important as the need increases to obtain maximal information on minimal sample. Analyses of these compounds by gas chromatography (GC), MS or liquid chromatography have been limited, especially at trace levels when sample size is limited, because the compounds often are not especially volatile, frequently exhibit weak molecular ions, and generally are not chromaphoric. Trimethylsilyl ether derivatives often are used in MS analysis of such alcohols. These derivatives exhibit weak molecular ions but large M-CH_3 peaks (1,2). Chemical ionization MS has been used to obtain mass spectral data on fatty acids directly and could be used with alcohols; however, the intensity of the quasi-molecular ions vary with sample introduction temperature and, therefore, the technique is unsuitable for quantitation (3). We report here the use of *p*-methylthiobenzoates as derivatives which allow quantitation at trace levels by high performance liquid chromatography (HPLC) of highly lipophilic alcohols and of molecules which can be readily converted to such alcohols. These derivatives absorb intensely in the ultraviolet (UV) region of the spectrum, allowing excellent sensitivity on HPLC with a UV-detector. They have an intense molecular ion which aids in mass spectral identification. Though the derivatives do not fluoresce strongly enough to increase the sensitivity of the HPLC analysis through use of a fluorescence detector, their characteristic fluorescence can be detected at the μg level, which can be an advantage in their separation and analysis by thin layer chromatography (TLC);

EXPERIMENTAL

Apparatus

Mass spectrometry. Mass spectra were recorded on a Hitachi Perkin-Elmer Model RMU-6E mass spectrometer. Mixture mass spectra were also recorded on a Finnigan Series 1015C gas chromatograph-mass spectrometer using the direct probe for sample introduction.

Liquid chromatography. Chromatograms were run on a Waters Associates' ALC-202 liquid chromatograph with a 254-nm UV detector. A 0.25 in. × 30 cm μ-Bondapak/C_{18} column was used, purchased from Waters Associates. The mobile phase was either acetonitrile or 5% water in acetronitrile. Peak areas were taken as the product of the peak height and the width at half-height.

Other instrumentation. UV spectra were obtained on a Perkin-Elmer Model 202 UV-visible spectrophotometer. Infrared (IR) spectra were taken with a Perkin-Elmer Model 621 IR spectrophotometer, and nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-360 spectrometer. Melting points were determined using a Mel-Temp apparatus and are corrected. Elemental analyses were performed on a Perkin-Elmer Model 240 elemental analyzer.

Materials

Straight-chain alcohols were prepared by lithium aluminum hydride (LAH) reduction of either the fatty acid methyl ester (PolyScience Corp.) or the fatty acid (Applied Science). Cholesterol was purchased from Matheson, Coleman, and Bell; 2,7-dimethyl-3,5-ocatadiyn-2,7-diol was from Farchan Research Laboratories; borneol, menthol, *cis*-1,2-cyclododecanediol and *p*-(methylthio-benzoic acid were from Aldrich Chemical Company.

p-*(Methylthio)-benzoyl chloride*. *p*-(Methylthio)-benzoic acid (1.68 g, [10 mmol] recrystallized from water and sublimed) was refluxed for 4 hr in 1.80 g (15 mmol) of thionyl chloride. The excess thionyl chloride was evaporated under nitrogen and the residue was sublimed (bath temperature 90 C) at 1 torr, mp 43-44, lit. (4), 40-44 C.

p-*(methylthio)-benzoate (MeSBzO) esters: Pyridine method*. To 0.1 meq of alcohol were added 0.2 mmol of acid chloride and 3 mL dry pyridine. The mixture was heated at 50 C for 4 hr. After evaporation of the pyridine under nitrogen and addition of 5 mL water, the mixture was extracted 3 times with 3-mL portions of ethyl ether. The combined ether extracts were washed twice with 2-mL portions of saturated aq sodium carbonate. The ether phase was then dried by passing through a short column of anhyd sodium sulfate. The ether was evaporated under nitrogen and the residue was purified by TLC on Silica Gel G using benzene as the developing solvent (R_f values are in Table I). The product was eluted from the silica gel with ethyl acetate. The solid derivatives were recrystallized from absolute ethanol.

Sodium hydride method. To 0.15 mmol of pentane-washed

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TABLE I

Physical Properties of *p*-(Methylthio)-benzoate Esters

Derivative	% Yield (method)	mp (C)	Molecular ion (% Σ_{40}) 70 eV	Molecular ion (% Σ_{40}) 15 eV	ϵ293 (ethyl ether)	R_f (benzene)	IR (C = O) cm^{-1}
Tetradecyl MeSBzO	92 (pyr)	43.5- 44.5	7.5	67.1	18,500	0.73	1715
Hexadecyl MeSBzO	90 (pyr)	51 - 51.5	7.3	65.8	18,300	0.73	1720
Octadecyl MeSBzO	91 (pyr)	57 - 58	9.6	55.9	18,400	0.73	1720
Eicosyl MeSBzO	93 (pyr)	62.5- 63.5	21.0	48.3	18,300	0.73	1720
Docosyl MeSBzO	92 (pyr)	67 - 67.5	12.6	56.5	18,400	0.73	1720
Cholesteryl MeSBzO	100 (pyr)	189 -191.5	0.053[a]	0.14[a]	18,500	0.78	1710
Menthyl MeSBzO	63 (NaH)	–	2.7	21.8	18,400	0.74	1705
Bornyl MeSBzO	14 (NaH)	–	6.4	42.6	18,500	0.71	1710
cis-1,2-Cyclododecyl *bis*-MeSBzO	88 (NaH)	–	5.7	24.0	37,800	0.42	1715
2,7-Dimethyl-3,5-octadiyn-2,7-yl *bis*-MeSBzO	33 (NaH)	133 -133.5	0.84	6.0	38,500	0.53	1730

[a]M-168 is 13.3 and 72.2%, respectively.

sodium hydride were added 0.10 meq of alcohol and 0.125 mmol of *p*-(methylthio)-benzoyl chloride in 10 mL of dry dimethoxyethane. The mixture was heated at 50 C. Hydrogen evolution was monitored on a gas buret and when the reaction was complete, the excess sodium hydride was quenched with a drop of water. After evaporation of dimethoxyethane under nitrogen, 5 mL of water was added and the solution was extracted 3 times with 3-mL portions of ethyl ether. The combined ether extract was dried and purified as already described.

The NMR spectra of the esters' derivatives showed the following differences from the starting alcohols: addition of peaks at δ 7.85 (d, J = 8 Hz; *o*-CH), 7.18 (d, J = 8 Hz, *m*-CH), and 2.45 (s, S-CH_3) ppm; downfield shifts of the hydroxymethyl protons of 0.64 ppm for straight-chain esters to 1.50 ppm for the methyl or 1,2-cyclododecyl ester and of the protons on the α-carbons from 0.13 ppm for straight-chain esters to 0.25 ppm for the 2,7-dimethyl-3,5-octadiynyl diester, and loss of the OH resonance.

Anal. tetradecyl MeSBzO: calc. for $C_{22}H_{36}O_2S$: C 72.48; H 9.95. Found: C 72.79; H 9.70.

Hexadecyl MeSBzO: calc. for $C_{24}H_{40}O_2S$: M^+ m/e 392.27; M+1/M^+ 28.39% and M+2/M^+ 8.68%. Found: M^+ m/e 392.27; M+1/M^+ 28.22% and M+2/M^+ 8.76%.

Octadecyl MeSBzO: calc. for $C_{26}H_{44}O_2S$: M^+ m/e 420.31; M+1/M^+ 30.69% and M+2/M^+ 9.33%. Found: M^+ m/e 420.27; M+1/M^+ 30.79% and M+2/M^+ 9.39%.

Eicosyl MeSBzO: calc. for $C_{28}H_{48}O_2S$: M^+ m/e 448.34; M+1/M^+ 32.99% and M+2/M^+ 10.04%. Found: M^+ m/e 448.37; M+1/M^+ 32.95% and M+2/M^+ 10.01%.

Docosyl MeSBzO: calc. for $C_{30}H_{52}O_2S$: M^+ m/e 476.37; M+1/M^+ 35.30% and M+2/M^+ 10.79%. Found M^+ m/e 476.40; M+1/M^+ 35.27% and M+2/M^+ 10.83%.

Cholesteryl MeSBzO: calc. for $C_{35}H_{52}O_2S$: C 78.30: H 9.76. Found: C 78.32; H 9.82.

Menthyl MeSBzO: calc. for $C_{18}H_{26}O_2S$: C 70.54; H 8.55. Found: C 70.60; H 8.54.

Bornyl MeSBzO: calc. for $C_{18}H_{24}O_2S$: C 70.93: H 7.94. Found: C 71.11; H. 8.36.

cis-1,2-Cyclododecyl *bis*-MeSBzO: calc. for $C_{28}H_{36}O_4S_2$: M^+ m/e 500.21; M+1/M^+ 33.72% and M+2/M^+ 15.02%. Found: M^+ m/e 500.24; M+1/M^+ 33.72% and M+2/M^+ 15.10%.

2.7-Dimethyl-3,5-octadiyn-2,7-yl *bis*-*p*-(methylthio)-benzoate: calc. for $C_{26}H_{16}O_4S_2$: C 66.92; H 5.59. Found: C 66.98; H 5.75.

Total fatty acids (as alcohols) of coconut oil. One-half g of the liquid oil in several mL of dry diethyl ether was reduced with excess LAH. After 2 hr, the excess LAH was quenched with water. Dissolution of the aluminum salt was done by the addition of solid ammonium chloride followed by a drop of conc sulfuric acid. The resulting mixture was extracted with five 10-mL portions of redistilled petroleum ether (bp - 37 C). The combined petroleum ether phase was backwashed with several mL of water, dried over anhyd sodium sulfate and the solvent was evaporated under nitrogen. Twenty mg of the resulting fatty alcohol mixture was converted to the mixture of *p*-(methylthio)-benzoates by the pyridine method. A solution of the mixture of esters was analyzed by HPLC at a concentration of 100 ng of the mixture/μL of solvent.

RESULTS AND DISCUSSION

The mass spectra of the *p*-(methylthio)-benzoate esters studied show 3 prominent ions: the molecular ion and 2 fragment ions at m/e 168 and 151 (Fig. 1). Table I shows the contribution to the total ion current (Σ_{40}) by the molecular ion in the mass spectra of the compounds studied at ionization potentials of 70 and 15 eV, respectively. At 70 eV, the molecular ion is reasonable (3-21% Σ_{40}); at 15 eV, the molecular ion is considerably enhanced (22-67% Σ_{40}). The cholesteryl ester is an exception, exhibiting a small molecular ion and a base peak at m/e 368 (m-168) in both the 70 and 15 eV spectra. This fragment results from loss of the elements of *p*-(methylthio)-benzoic acid and is expected in view of the mass spectra of cholesteryl acetate (1) and its trimethylsilyl ether (4). In other components for which elimination of the acid is favored, such as 2,7-dimethyl-3,5-octadiyn-2,7-yl *bis* MeSBzO, the M-168 peak is also observed (m/e 298 [M-168] is 1.0% and 6.3% Σ_{40} at 70 and 15 eV, respectively).

All of the monoesters studied absorbed strongly at 293 nm (ϵ = 28,000, Table I). The molar absorbtivity per chromophore is slightly enhanced in the diesters. The molar absorbtivity is comparable to that reported for closely allied 3,4-dinitrobenzoate esters (ϵ = 21,600 to 28,500 at 208 nm [5]) but the molar absorptivities for the *p*-(methylthio)-benzoate esters are more constant. Because of this constancy in the molar absorptivity of the monoesters (and only slight deviation in the diesters), the equivalent weight per chromophore of an unknown ester may be determined spectrophotometrically. This allows confirmation by UV spectroscopy of the molecular weight and the number of MeSBzO groups per molecule as determined by MS.

The constancy of the molar absorptivity of *p*-(methyl-

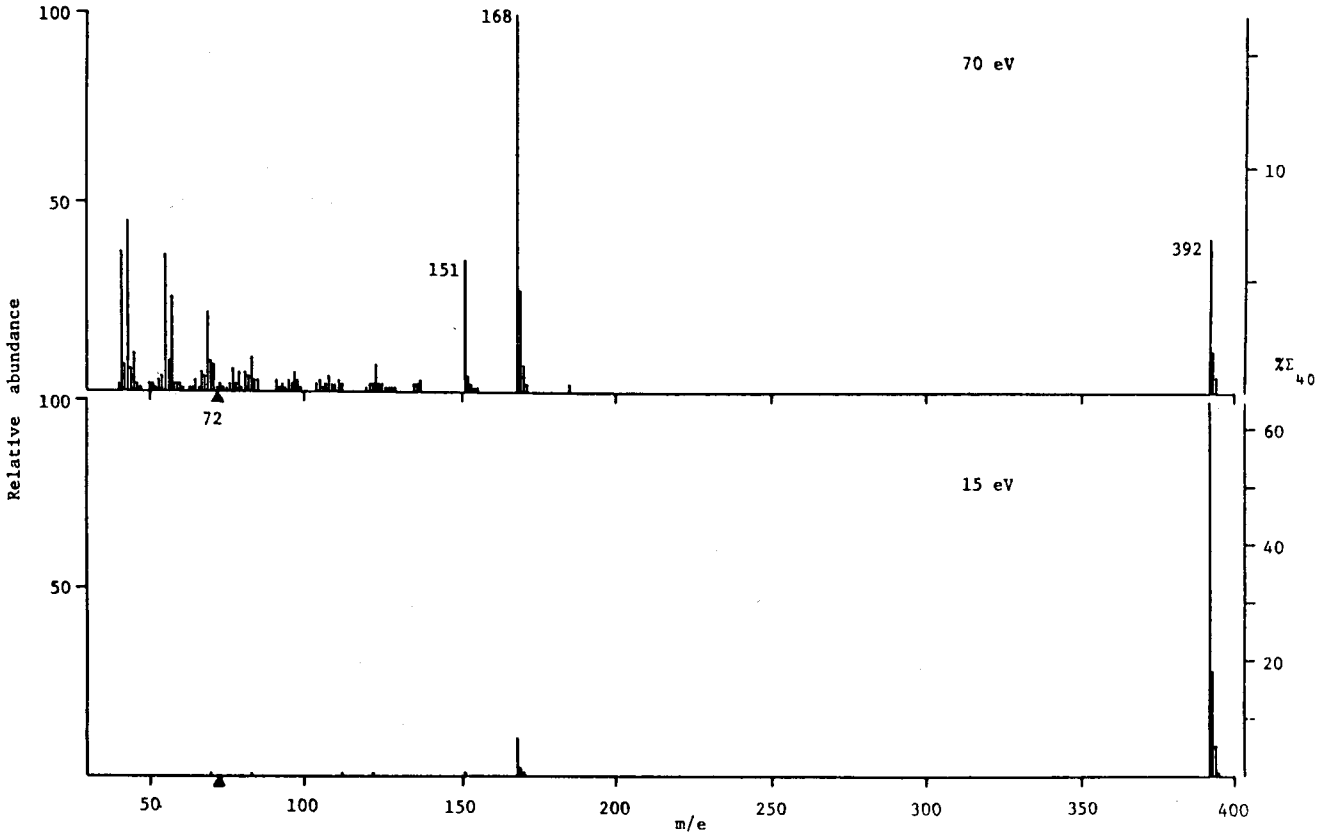

FIG. 1. Mass spectrum of the *p*-(methylthio)-benzoate of hexadecanoic acid at 70 and 15 eV.

thio)-benzoyl derivatives makes them ideal derivatives for quantitative analysis of appropriate mixtures by HPLC. A weighed mixture of the derivatized straight-chain alcohols (C_{14} to C_{22}) was analyzed by HPLC (Fig. 2). Each peak was identified by collection of the effluent, concentration of the solvent, and mass spectral analysis of the residue. The peak area ratios matched the corresponding molar ratios of the initial mixture (Table II). Weighed mixtures of long-chain fatty alcohols and of fatty acids reduced to the alcohols were esterified by the pyridine method. The mole ratios of the initial mixtures correspond well with the peak area ratios derived from the HPLC chromatogram (Table II). Thus, this derivative is useful for the quantitative analysis of alcohols and those compounds which can be converted quantitatively to alcohols.

Under the conditions used, the sensitivity of the detector to the tetradecyl MeSBzO ester was 0.003 AU/nmol at 0.02 AUFS. Two hundred pmol of the derivatives was easily detected. The sensitivity of this assay could be greatly enhanced at 280 nm, provided no loss of incident beam intensity were to occur, because this is closer to the maximal absorption band of the *p*-(methylthio)-benzoate.

To ensure the applicability of this procedure to the analysis of mixtures, the procedure was applied to LAH reduction products of the fatty acid present in coconut oil. Coconut oil consists of mixture of triglycerides. This procedure gives a fatty alcohol composition reflecting that of the original fatty acid composition of the oil. The triglycerides present in the oil were first converted into a mixture of fatty alcohols by direct reduction with LAH. Glycerol and short-chain, water-soluble alcohols are lost to the aqueous phase in the work-up. After esterification, the mixture was analyzed by HPLC (Fig. 3). The derivatives are well resolved and quantifiable under these conditions. The composition of a mixture of saturated fatty alcohols determined from this chromatogram is shown in Table III. The original fatty acid composition compares favorably to the fatty acid composition reported by other workers for coconut oil using different systems (6,7).

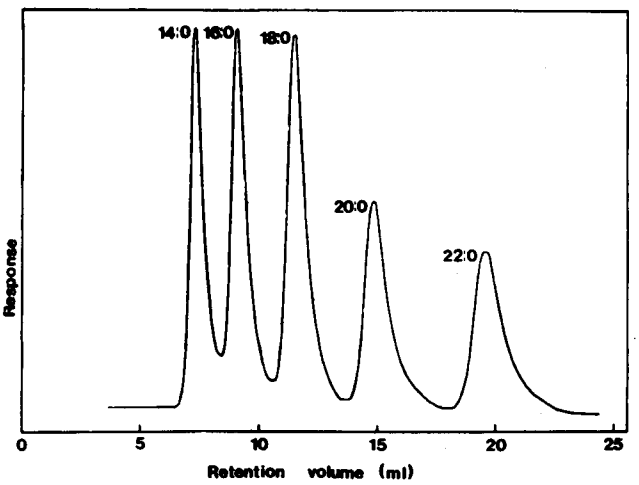

FIG. 2. High performance liquid chromatogram of a derivatized mixture of fatty acids.

Because of the strong molecular ion observed for these esters, particularly at 15 eV, the use of the mass spectrometer for quantitative analysis of a mixture was investigated. Table IV shows the variation in intensity of the respective molecular ion with temperature. Because of this variation, accurate quantitation would not be possible. The ratio

TABLE II

HPLC Quantitation of Standard Ester Mixtures Relative to C_{16}

Derivative	MeSBzO ester mix (derivative) Added	MeSBzO ester mix (derivative) Found	Alcohol mix (derivative) Added	Alcohol mix (derivative) Found	Fatty acid mix (derivative) Added	Fatty acid mix (derivative) Found
Tetradecyl MeSBzO	0.79	0.79	0.64	0.63	1.18	1.12
Hexadecyl MeSBzO	1.00	1.00	1.00	1.00	1.00	1.00
Octadecyl MeSBzO	1.22	1.20	0.29	0.27	0.74	0.77
Eicosyl MeSBzO	0.85	0.84	0.76	0.72	1.01	1.00
Docosyl MeSBzO	0.85	0.84	0.30	0.27	0.82	0.81

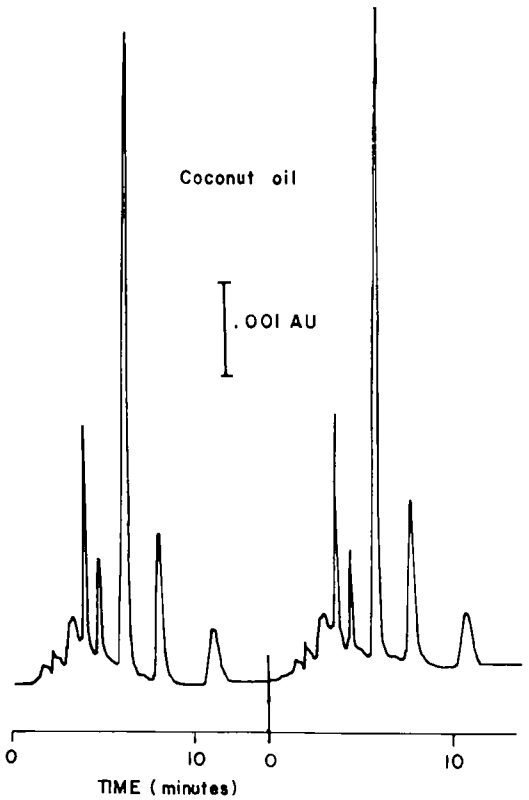

FIG. 3. High performance liquid chromatogram of derivatized coconut oil.

TABLE III

Composition of Coconut Fatty Acids by HPLC Analysis as *p*-(Methylthio)-benzoates

Acid	Relative %
Hexanoic (6:0)	3.7
Octanoic (8:0)	13
Decanoic (10:0)	8.6
Dodecanoic (12:0)	48
Tetradecanoic (14:0)	17
Hexadecanoic (16:0)	10

TABLE IV

Ion Intensity Variation of Coconut Oil Fatty Acids *p*-(Methylthio)-benzoates with Sample Inlet Temperature (Base Peak 168)

Parent acid	M^+	Inlet temperature (C) 100	120	180
18:0	420	–	5	13
18:1	418	–	5	8
16:0	392	–	50	10
14:0	364	4	99	9
12:0	336	65	100	20
10:0	308	29	2	6

of the dodecanoic to decanoic derivative, e.g., is about 2 at 100 C compared to 50 by the time 120 C is reached in the inlet. All components differing in molecular weight, however, were easily seen. A similar effect was noted by Murata et al. (3) in the chemical ionization MS of fatty acids for which direct quantitation was attempted.

The spectrum obtained on the unseparated components is sufficient to allow complete qualitative analysis of the fatty alcohols present (Fig. 4), demonstrating that a mixture of fatty alcohol *p*-(methylthio)-benzoate esters can be qualitatively analyzed by MS without prior separation into its individual components. This is possible because of the very intense molecular ions which are obtained in the 15 eV electron impact spectra. The only other important fragment which occurs is that due to the *p*-(methylthio)-benzoate ion which occurs at m/e 168. Thus, qualitative analysis of a mixture of these esters of long-chain fatty alcohols can be performed by MS. All fragments above m/e 168 represent molecular ions of the constituents of the mixture when the spectrum is obtained at 15 eV.

Additional information is obtained from the isotope cluster of molecular ions for an individual derivative. Because of the relatively large isotope ratio of ^{34}S to ^{32}S, about 4%, there is a relatively large $M+2^+$ peak. One can deduce from the ratios of $M+2/M^+$ the number of derivatizable sites in the molecule by comparing the actual ratio to that calculated for the molecular formula being tested.

The *p*-(methylthio)-benzoate esters appear to be of considerable utility in the analysis of fatty alcohols and alcohol-reducible compounds, especially in the analysis of limited size samples: the molecular weight may be determined both by MS due to the molecular ion intensity and in pure compounds by UV spectrophotometry because UV absorption is directly proportional to incorporated ester; qualitative analysis of mixtures may be done by MS; and finally, quantitative analyses by HPLC may be performed on mixtures with minimal intensity to 200 pmol.

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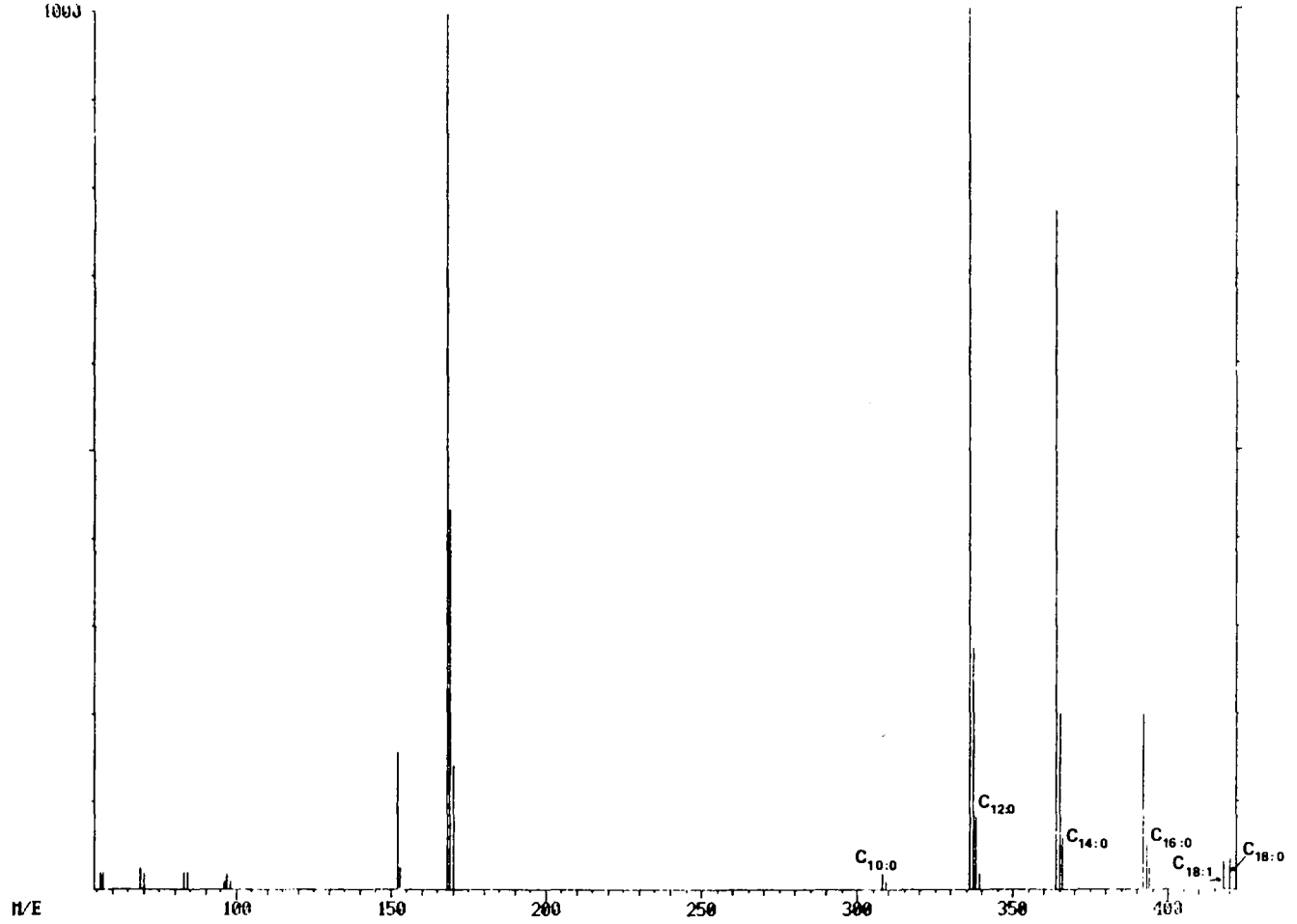

FIG. 4. Mass spectrum of the *p*-(methylthio)-benzoates derived from coconut oil.

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