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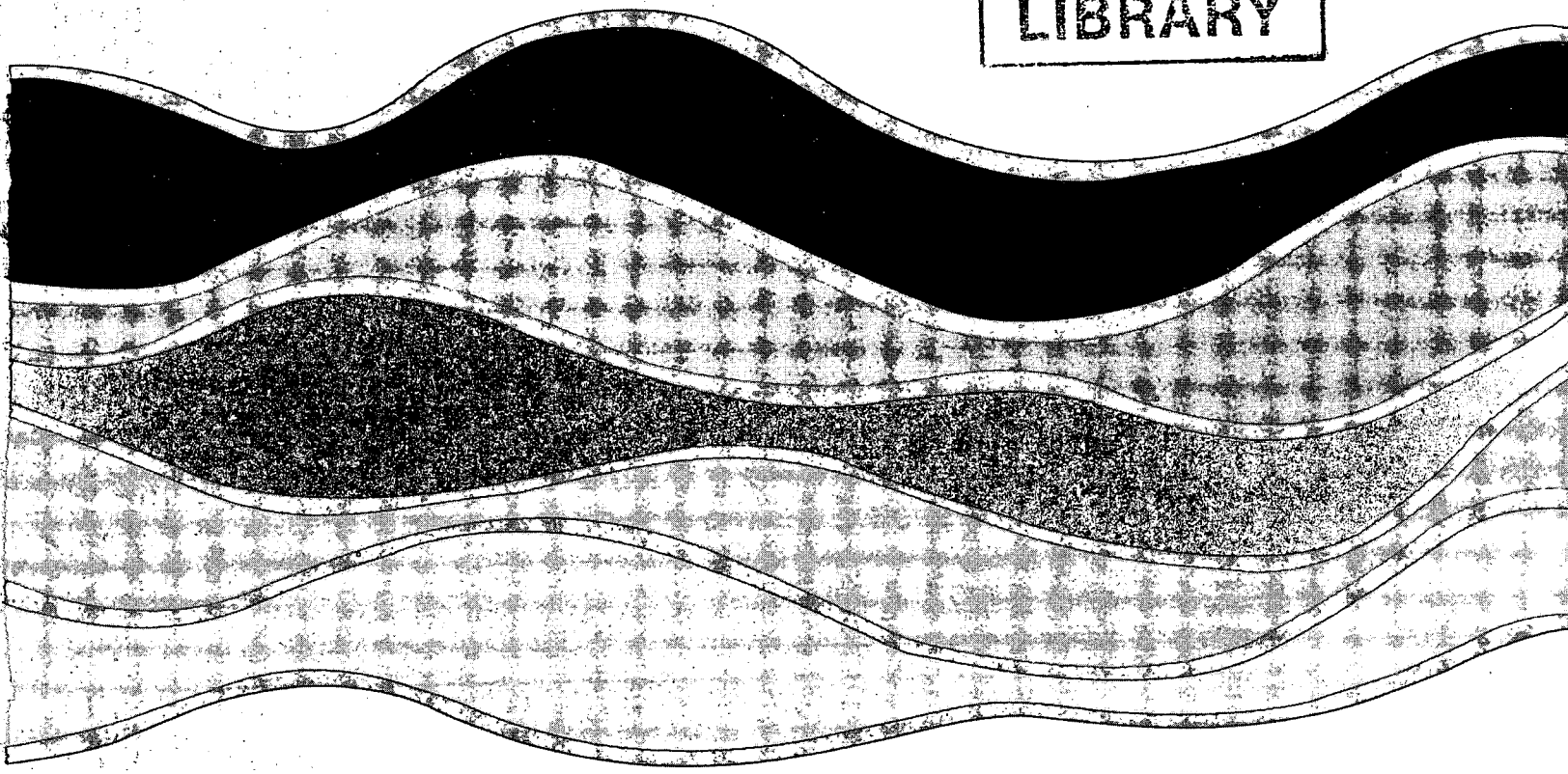

DERIVATIVES OF ESTROGENS FOR GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS

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Derivatives of estrogens for gas chromatography-mass spectrometry analysis

by

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MANAGEMENT PERSPECTIVE

The occurrence of estrogenic compounds in the environment has created concern lately since they have the potential to interfere with the reproductive systems of fish, wildlife, and humans. Among these endocrine disruptors, the most potent ones include the naturally occurring 17ß-estradiol and the synthetic 17α-ethynylestradiol which is used as an oral contraceptive for women. The presence of these estrogens at ng/L concentrations in sewage treatment plant effluents has been reported. This report describes new analytical methods for the detection of estrogens at trace levels.

SOMMAIRE À L'INTENTION DE LA DIRECTION

La présence de composés œstrogéniques dans l'environnement a suscité des inquiétudes dernièrement étant donné qu'ils peuvent perturber le système reproducteur des poissons, des animaux sauvages et des êtres humains. Parmi ces composés, les plus puissants comprennent le 17β-oestradiol d'origine naturelle et le composé de synthèse 17α-éthinyloestradiol utilisé comme contraceptif oral chez la femme. Une concentration de ces œstrogènes de l'ordre du ng/L a été mesurée dans les effluents des stations d'épuration des eaux d'égout. Le présent rapport décrit les nouvelles méthodes d'analyse pour le dépistage des œstrogènes à l'état de traces.

ABSTRACT

The syntheses of acetyl, pentafluoropropionyl, heptafluorobutyryl, and trimethylsilyl derivatives of estrogenic compounds such as diethylstilbestrol, 17ß-estradiol, estrone, estriol, and 17α-ethynylestradiol (EE_2) are described. These reactions were quantitative and the products were all stable at -20°C for weeks. Their separation on a HP-5 column was demonstrated. The GC/MS data indicated that, with the exception of EE_2, all hydroxy groups of these estrogens were derivatized. In the case of EE_2, the hydroxy group at the 17-position did not react. Intense molecular ions were observed, in many cases, for the pentafluoropropionyl and trimethylsilyl derivatives. Using these characteristic ions, detection of pg amounts of these estrogen derivatives could be achieved by GC/MS in selected ion monitoring mode.

Keywords: Estrogens, steroids, derivatives

RÉSUMÉ

La synthèse des dérivés acétyle, pentafluoropropionyle, heptafluorobutyryle et triméthylsilyle des composés œstrogéniques comme le diéthylstilbestrol, le 17β-œstradiol, l'œstrone, l'œstriol et le 17α-éthinyloestradiol (EE_2) est décrite dans le document. Il s'agissait de réactions quantitatives et les produits étaient tous stables à -20 °C pendant plusieurs semaines. Il est possible d'en faire la séparation sur une colonne HP-5. D'après les données de CG/SM, on peut obtenir des dérivés à partir de tous les groupes hydroxyle de ces œstrogènes, à l'exception du EE_2. Dans ce dernier cas, le groupe hydroxyle en position 17 ne réagissait pas. Dans bien des cas, les dérivés pentafluoropropionyle et triméthylsilyle présentaient des pics moléculaires intenses. À l'aide de ces ions caractéristiques, la chromatographie en phase gazeuse en mode de détection d'un seul ion permettrait de déceler des quantités de l'ordre du pg de ces dérivés œstrogéniques.

Mots-clés : oestrogènes, stéroïdes, dérivés.

INTRODUCTION

17ß-Estradiol (E_2), a naturally occurring steroid, is the principal estrogen responsible for the development and maintenance of the female reproductive system and secondary sex characteristics. In humans, E_2 is produced in the ovary, as well as in the testes and brain by the aromatization of testosterone. Clinically, E_2 is used in estrogen replacement therapy to treat, for example, symptoms associated with estrogen deficiency in post-menopausal women, and in the prevention of osteoporosis. Endogenous and orally administered E_2 is extensively metabolized to estrone (E_1) and estriol (E_3) in their conjugated and unconjugated forms.

The synthetic estrogen, 17α-ethynylestradiol (EE_2), is a major ingredient in many oral contraceptive formulations that have been used since 1960. In contrast to E_2, EE_2 as well as diethylstilbestrol (DES), another synthetic yet non-steroidal estrogen, are metabolized rather slowly in the liver and other tissues, which result in their high intrinsic potency. The use of DES in humans, which at one time was prescribed to prevent miscarriage, has been banned because of severe side effects including increased incidence of reproductive tract carcinoma in the offspring. Chemical structures of the estrogens described in this work are depicted in Figure 1.

Recently, the endogenous and synthetic estrogens are suspected to play an important role as endocrine disruptors in the environment. For example, E_2 is several orders of magnitude more potent than nonylphenol, a weak environmental estrogen, to induce the proliferation of MCF_7 human breast tumor cells [1]. Even at ng/L levels, E_2 and EE_2 have been shown to promote the synthesis of the protein vitellogenin in male

rainbow trout [2,3]. Normally, this fish egg protein is only found in female fish. Although the information is scarce, the occurrence of these estrogens in municipal sewage treatment plant effluents at low and sub ng/L levels has been reported [4].

Apart from radioimmunoassay techniques [4], a number of HPLC methods using a reversed phase column and UV detection have been reported for the assay of E_1, E_2 and their derivatives [5-7]. Micellar electrokinetic chromatography and diode array detector have been used for the separation and detection of ten pharmaceutically important estrogens, including E_1, E_2, and E_3 [8]. For the GC determination of estrogens, formation of volatile and non-polar derivatives are usually required. For example, the formation of trimethylsilyl (TMS) derivatives for E_1, E_2, E_3, and DES [9-11], *tert*-butyldimethylsilyl derivatives of E_1, E_2, and E_3 [11], dimethylethylsilyl derivatives of EE_2 and other steroids used in oral contraceptive formulations [12], as well as the pentafluoropropionyl (PFP) derivative of testosterone acetate [13] has been demonstrated. In this report, we describe the formation of acetyl, PFP, heptafluorobutyryl (HFB), and TMS derivatives of E_1, E_2, E_3, EE_2, and DES as well as their GC/MS properties. These derivatives can be used for the gas chromatographic detection and confirmation of the estrogens in pharmaceutical formulations, in bodily fluids and excreta, and in environmental samples.

EXPERIMENTAL

Chemicals and reagents

The estrogens, acetic anhydride, pentafluoropropionic acid anhydride (PFPA), heptafluorobutyric acid anhydride (HFBA), bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), and pentafluorobenzyl bromide (PFBBr) were obtained from Sigma-Aldrich-Supelco Canada Ltd. (Oakville, ON, Canada). 3,5-(Bistrifluoromethyl)benzyl bromide was a product of Lancaster Synthesis Inc. (Windham, NH, USA). Solvents were distilled-in-glass grade provided by Burdick and Jackson (Muskegon, MI, USA).

Stock solutions of the estrogens, at 1000 µg/mL, were prepared in acetone. Mixtures of the estrogens, at 100, 10, and 1 µg/mL, were also prepared in acetone and stored at -20°C in the dark.

Derivatization procedures

(1) Acetic anhydride, PFPA, and HFBA reactions

In a centrifuge tube, the solvent of an estrogen mixture containing 20 µg of each estrogen was evaporated to dryness in a 40°C water bath using a gentle stream of nitrogen. Fifty µL of ethyl acetate and 50 µL of an anhydride were then added. The tube was then sealed with a Teflon-lined screw cap and the mixture was vortexed for 30 sec. The mixture was allowed to react for 20 min at room temperature (22°C) for the PFPA reaction, 60 min at 40°C for the HFBA reaction, and 60 min at 85°C for the acetic anhydride reaction. At the end of reaction, 3 mL of a 1% K_2CO_3 solution were added and the acylated products were extracted by vortexing with three 2-mL aliquots of petroleum

ether (30-60°C). After each extraction, the organic layer was passed through a 5 cm column of anhydrous sulfate prepared in a Pasteur pipet. The combined organic extract was evaporated and exchanged into 1 mL of iso-octane for GC-MS analysis.

(2) BSTFA-TMCS reactions

The above procedure was followed except that 100 µL of BSTFA-TMCS reagent was used instead of the anhydride and the reaction was carried out at 60°C for 60 min. At the end of the reaction, the mixture was evaporated just to dryness and the products were reconstituted into 1 mL of iso-octane for GC-MS analysis.

Gas chromatography-mass spectrometry of the derivatives

A Hewlett-Packard 5890A Series II gas chromatograph equipped with a split/splitless injector, and a Model 5792A Mass Selective Detector (MSD) was used. The analytical column was a Hewlett-Packard, 30 m x 0.25 mm ID x 0.25 µm thickness HP5-MS column. One µL splitless injection was made by a Model 7673 autosampler. The GC oven temperature program was 70°C, initial temperature, with a 1-min hold, increased to 180°C at 30°C/min, then increased to 290°C at 5°C/min and a 3.33-min hold at the final temperature. Injection port and detector interface temperatures were 250 and 280°C, respectively. Carrier gas (helium) linear velocity was held constant at 38.4 cm/sec. The electron energy and electron multiplier voltage were 70eV and 400 V above the autotune value, respectively. Full scan mass spectral data were collected from m/z 50 to 700.

RESULTS AND DISCUSSION

Formation of estrogen derivatives

Several commonly used reagents, namely, BSTFA with TMS, acetic anhydride, and PFPA, were evaluated for the trimethylsilylation, acetylation, and pentafluoroacylation, respectively, of the estrogenic compounds of interest. Trimethylsilylation and acetylation of the estrogens were completed in 30 min at 65 and 85°C, respectively. Pentafluoroacylation of the estrogens was completed in 20 min at room temperature. Reactions with PFPA at higher temperatures such as 50, 65 and 85°C were not recommended as the derivative of EE_2 degraded at such temperatures. All derivatives were stable at -20°C in the dark for at least two months.

As indicated by full scan GC/MS data (see later discussion), these reagents reacted readily with both hydroxy groups of DES and formed the TMS, acetyl, and PFP derivatives. In each case, two peaks, in an approximately 1:3 ratio, were observed for this nonsteroidal estrogen since DES exists as a mixture of *cis* and *trans* isomers. The smaller peak with a shorter retention time was assigned to the *cis* isomer. The hydroxy groups of the naturally occurring, endogeneous estrogens, i.e. 3-OH of E_1, 3- and 17-OH of E_2, and 3-, 16-, and 17-OH of E_3, also reacted readily with BSTFA, acetic anhydride, and PFPA to form the respective mono-, di-, and tri- substituted derivatives. Under the reaction conditions used, partially derivatized E_2 and E_3 (i.e. a mono-substituted E_2 and either a mono- or di-substituted E_3) were not observed. The absence of such intermediates

enables straight forward quantification of the estrogens. In contrast, only a mono-substituted derivative for the synthetic estrogen, EE_2, was formed with each of the reagents. Since mestranol, the 3-methyl ether derivative of EE_2, did not react with any one of these reagents even after prolonged reactions, it suggested that the hydroxy group at the 17 position was protected from being derivatized by the presence of the ethynyl group at the same location. It should be noted that, by virtue of GC/MS results, the formation of dimethylethylsilyl ether derivatives with the 3- *and* 17-OH for EE_2 as well as the 17-OH of mestranol has been documented [12].

The reactions of estrogens and HFBA at room temperature as well as at 40, 50 and 60°C for periods from 30 min to 18 hr have also been attempted. The results indicated that these estrogens reacted more slowly with HFBA than with PFPA. Longer reaction times or higher reaction temperatures produced better yields for the DES, E_1, E_2, and E_3 derivatives. Particularly, the derivatization of DES at room temperature was very slow and incomplete, as mono-substituted products were found. Reactions at 60°C, however, yielded three di-substituted products for DES in a 1:1:2 ratio. In contrast, the best results for EE_2 were obtained at 40°C or lower for short reaction times such as 60 min or less. Under more drastic conditions, lower yields together with the presence of several unidentified peaks, presumably degradation products, were observed for the EE_2-HFB derivatives and mestranol. Although the HFBA reaction was deemed unsuitable for the present work since a derivatization condition could not be optimized for all estrogens, it could be useful for the GC analysis of selected estrogens.

Attempts to prepare ether derivatives of the estrogens with alkylating agents such as pentafluorobenzyl bromide and 3,5-bis(trifluoromethyl)benzyl bromide in the presence of pyridine as a catalyst resulted in little or no success.

GC and mass spectral properties of estrogen derivatives

Gas chromatograms of the PFP, HFB, TMS, and acetyl derivatives of the estrogens are shown in Figures 2A, 2B, 3A, and 3B, respectively. With the exception of the HFB derivatives of E_3 and E_1 (Figure 2B), all compounds were well separated by the HP-5 column under the GC conditions used.

Mass spectra of the PFP, HFB, acetyl, and TMS derivatives for the estrogens are shown in Figures 4 through 7, respectively. The characteristic ions and their relative abundance for each derivative are tabulated in Table 1.

All PFP derivatives displayed ions at m/z 69 (CF_3^+) and 119 ($C_2F_5^+$) which are characteristic of the pentafluoropropionyl group (Figure 4). Molecular ions of varying intensities were observed for the estrogen derivatives. In the cases of DES, E_1 and E_2, the molecular ions were either the base peak or very strong. In contrast, the molecular ions for E_3 and EE_2 were relatively weak. Note that the E_3-PFP derivative has a molecular mass of 726. The presence of this molecular ion was later confirmed on a Hewlett-Packard Mass Engine as it is beyond the upper mass range of our Mass Selective Detector. The molecular weight of the EE_2-PFP derivative, as in the cases of the other three types of derivatives, indicated that only one of the two hydroxy groups was derivatized. Since the *cis* and *trans* isomers of the DES-PFP derivative, as well as all

other derivatives of this compound, exhibited near identical mass spectra, only the mass spectrum of the *trans* isomer was shown in Figures 4 through 7. Another ion, m/z 359, was also prominent for all steroidal estrogens. This ion likely resulted from the loss of carbons at the 15, 16, and 17 positions of the 5-member ring (ring D), their substituents and a hydrogen from the molecular ion of each steroid. Using the E_2 derivative as an example, the ion m/z 359 or $(M-205)^+$, arised from the loss of C_3H_5O, C_2F_5CO, and H moieties from M^+. For EE_2, the ion of m/z 359 or $(M-83)^+$, was derived by the loss of C_3H_5O, the ethynyl moiety, and a hydrogen from M^+. This fragmentation pattern also provided direct evidence for the fact that the hydroxy group at the 17-position of EE_2 was not derivatized.

Ions at m/z 69 (CF_3^+) and 169 ($C_3F_7^+$), characteristic of the heptafluorobutyryl group, were present in all HFB derivatives of the estrogens (Figure 5). With the exception of E_3-HFB, the molecular ions of all derivatives were observed. In the case of E_1-HFB, the molecular ion (m/z 466) was also the base peak. Intense ions at m/z 409 and 356 were observed for the derivatives of all steroidal estrogens. Note that these ions were 50 mass units higher than the two mentioned above for the PFP derivatives due to the extra CF_2 moiety in the HFB derivatives.

The mass spectra of the TMS derivatives were characterized by the presence of readily observable molecular ions (Figure 6). In fact, these ions were the base peaks in the cases of DES and E_1, and were very strong for E_2 and EE_2. In addition, the ion m/z 73, attributed to $(CH_3)_3Si^+$, was also observed in high intensity for all TMS derivatives. Another major characteristic ion at m/z 285 was observed for the derivatives of E_2 and EE_2. For the E_2 derivative, the ion of m/z 285 or $(M-73-58)^+$ could also be

interpreted by the fragmentation pattern arised from the loss of TMS, C_3H_5O and hydrogen (from ring D) moieties in a way similar to the E_2-PFP derivative. In the case of the EE_2-TMS derivative, mass 285 corresponded to $(M-83)^+$ or the loss of C_3H_5O, C_2H (the ethynyl group) and H from the molecular ion. Again, this fragmentation pattern indicated that the hydroxy group on C_{17} of EE_2 was not silylated. In contrast, the ion at m/z 285 was weak for the TMS derivatives of E_1 and E_3.

Except for DES, the molecular ions of the acetyl derivatives of the estrogens were weak (Figure 7). The major fragmentation route of these derivatives involved the loss of a $COCH_2$ moiety. This was evidenced by the presence of the $(M-42)^+$ or $(M-COCH_2)^+$ ion which was the base peak for E_1, E_2, and E_3 and was very intense for DES and EE_2 derivatives. The base peak of DES, m/z 268, was attributed to the $(M-42-42)^+$ fragment. In the case of the EE_2 derivative, the base peak at m/z 213, or the $(M-42-83)^+$ fragment, was again due to the loss of $COCH_2$ at the 3-position followed by cleavage of ring D and loss of its substituents as explained above.

Selected ion monitoring of the PFP and TMS derivatives

The TMS and PFP derivatives of estrogens were further investigated for low level quantitative work using selected ion monitoring technique. These derivatives were chosen because of the presence of intense molecular or characteristic ions (Table 1) that could be used in the trace analysis of estrogens in complex matrices such as environmental samples. In addition, the electron capturing properties of the PFP

derivatives permitted the use of an ECD and potentialy the mass spectrometer operating in negative ion chemical ionization mode as alternate detectors.

The ions used for the quantitation and confirmation of the PFP and TMS derivatives for estrogens are listed in Table 2. In the case of the PFP derivatives, the amount of estrogen required to produce a signal-to-noise ratio of 5:1 was ca. 5 pg for DES, 10 pg for E_1 and E_2, and 20 pg of E_3 and EE_2. The amount of a corresponding TMS derivative to generate a similar signal-to-noise ratio was about twice as much as the PFP derivative.

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Table 1. Relative abundance of molecular and major ions of estrogen derivatives.

Estrogen	M^+	*Major ions*
PFP derivative		
DES*	560 (100)	531 (26), 291 (98), 253 (50), 119(70)
E_1	416 (100)	372 (82), 359 (52), 306 (37), 119(56)
E_2	564 (57)	401 (31), 359 (43), 306(41), 119(100)
E_3	726 (5)	563 (7), 399(22), 359 (9), 119 (100)
EE_2	442 (9)	359 (100), 306 (57), 279 (18), 119 (24)
HFB derivative		
DES	660 (36)	631 (8), 341 (100), 303 (47), 169 (41)
E_1	466 (100)	422 (93), 409 (60), 356 (48), 169 (40)
E_2	664 (24)	451 (32), 409 (48), 356 (52), 169 (100)
E_3	876 (2)	663 (4), 449 (28), 235 (49), 169 (100)
EE_2	492 (6)	409 (100), 356 (61), 329 (16), 169 (19)
Acetyl derivative		
DES	352 (39)	310 (58), 268 (100), 253 (9), 239 (40)
E_1	312 (9)	270 (100), 213 (10), 185 (19), 146 (16)
E_2	356 (6)	315 (22), 314 (100), 225 (8), 172 (14)
E_3	414 (7)	372 (100), 270 (8), 172 (13), 160 (26)
EE_2	338 (14)	296 (76), 255 (11), 213 (100), 160 (42)
TMS derivative		
DES	412 (100)	397 (14), 383 (16), 191 (13), 73 (65)
E_1	342 (100)	327 (11), 257 (38), 218 (29), 73 (41)
E_2	416 (94)	401 (8), 285 (72), 232 (21), 73 (100)
E_3	504 (19)	386 (12), 345 (20), 311 (18), 73 (100)
EE_2	368 (66)	353 (8), 301 (4), 285 (100), 73 (57)

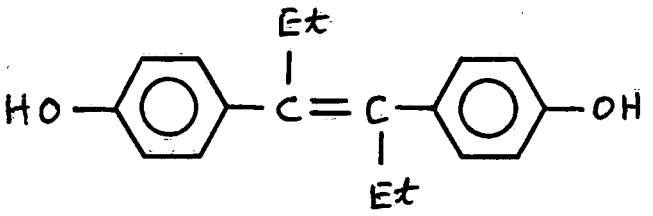
* Ions of the *trans*-DES derivative were listed. For each type of derivative, the *cis* and *trans* isomers of DES produced nearly identical mass spectra.

Table 2. Ions used for selected ion monitoring work for the PFP and TMS derivatives of estrogens.

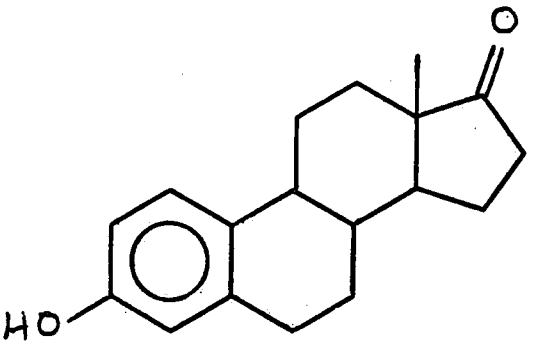
Estrogen	PFP derivative		TMS derivative	
	Quantitation	Confirmation	Quantitation	Confirmation
DES	560	291	412	383
E_1	416	372	342	257
E_2	564	401	416	285
E_3	399	563	504	345
EE_2	359	306	285	368

LIST OF FIGURES

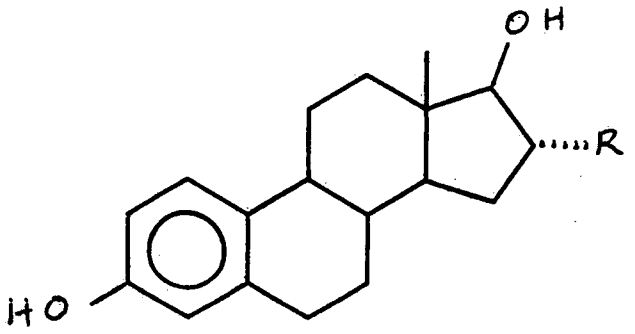
Figure 1



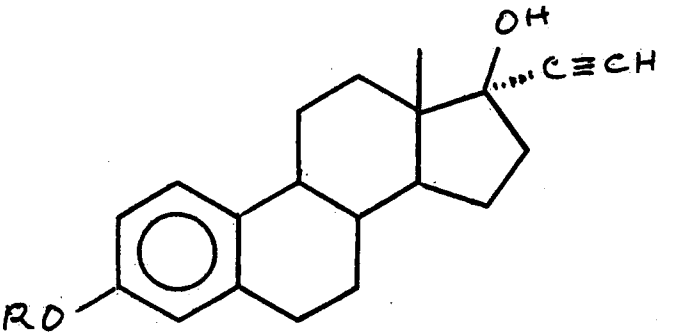
Diethylstilbestrol (DES)



Estrone (E_1)

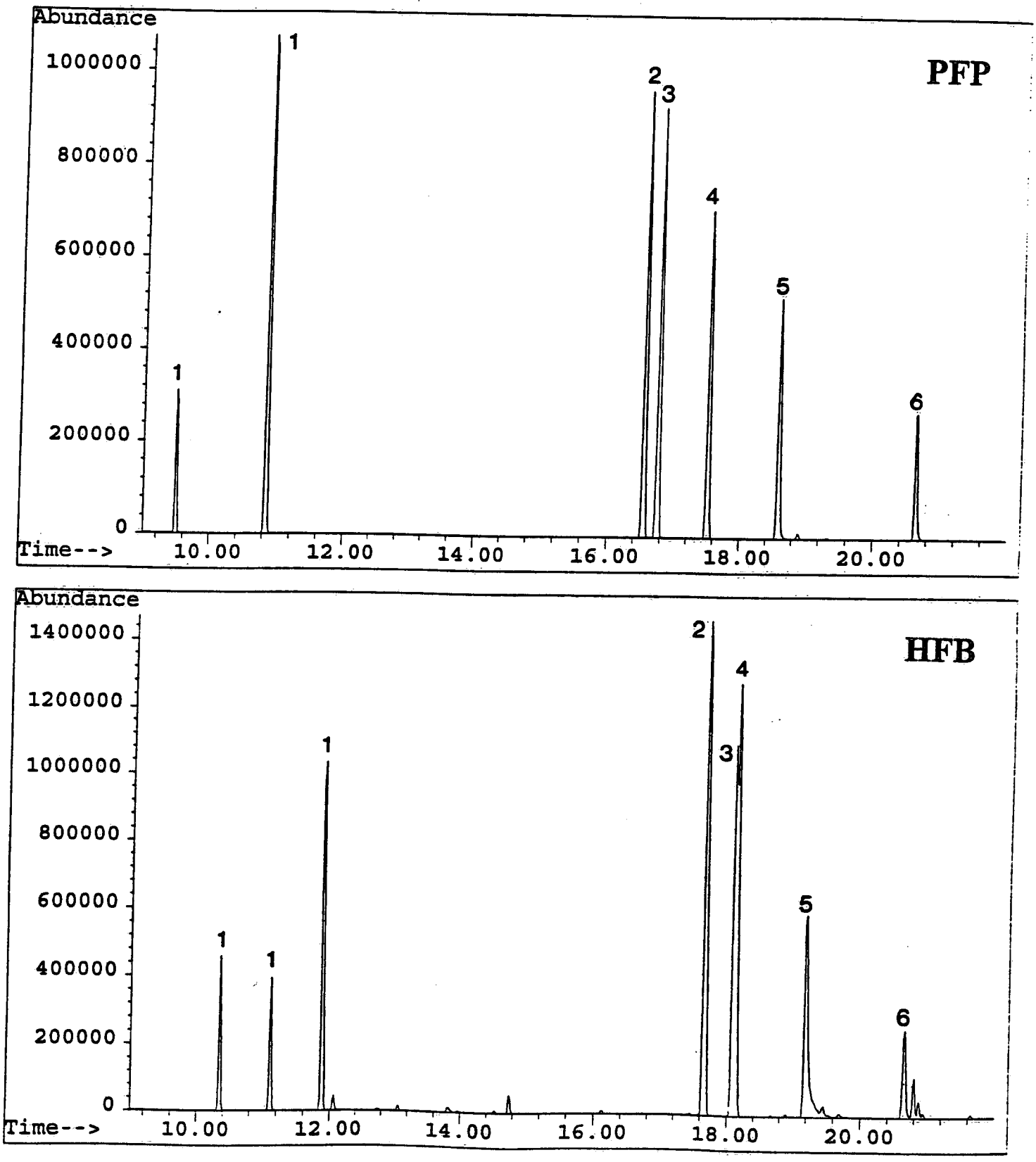


R=H, 17ß-Estradiol (E_2)
R=OH, Estriol (E_3)



R=H, 17α-Ethynylestradiol (EE_2)
R=OCH_3, Mestranol

Figure 2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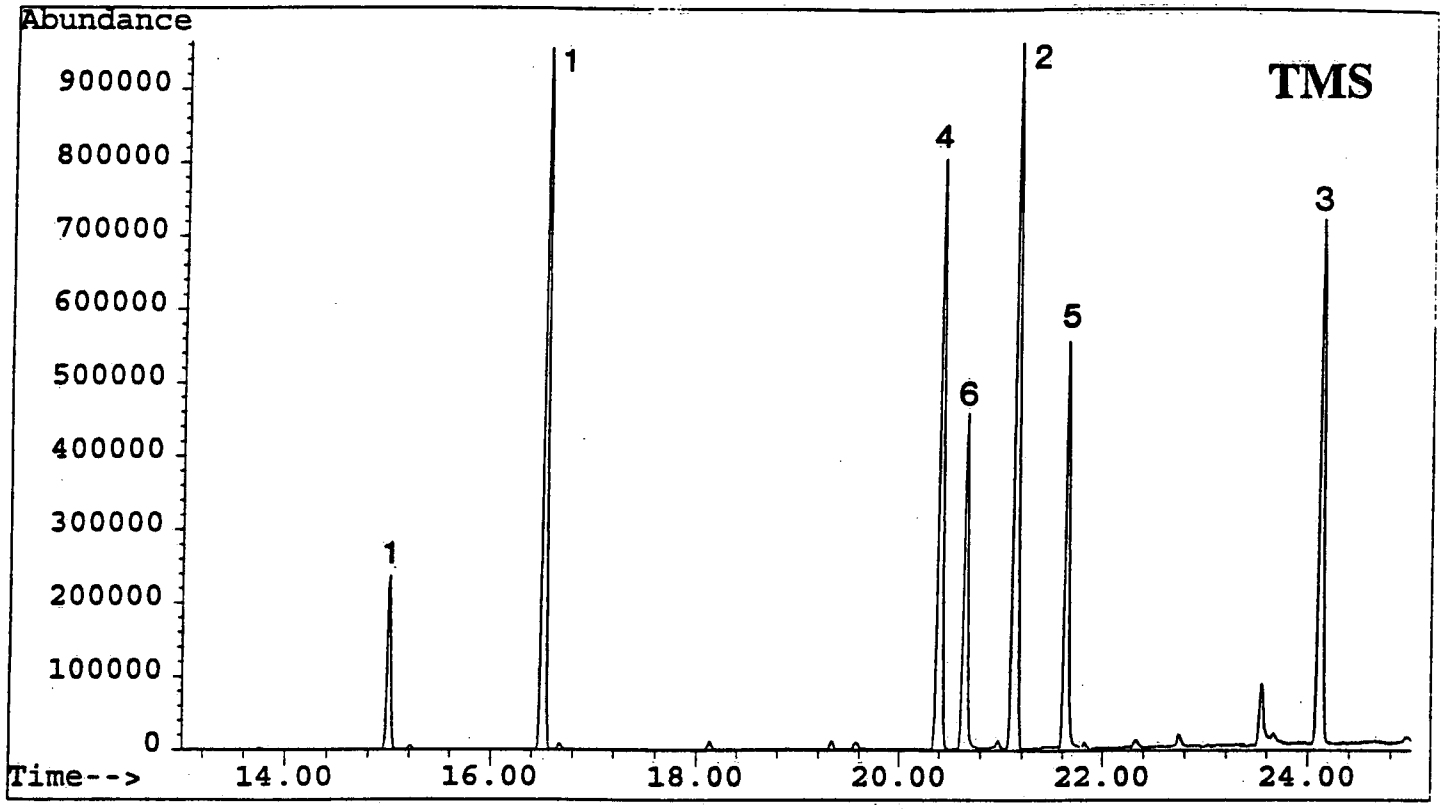
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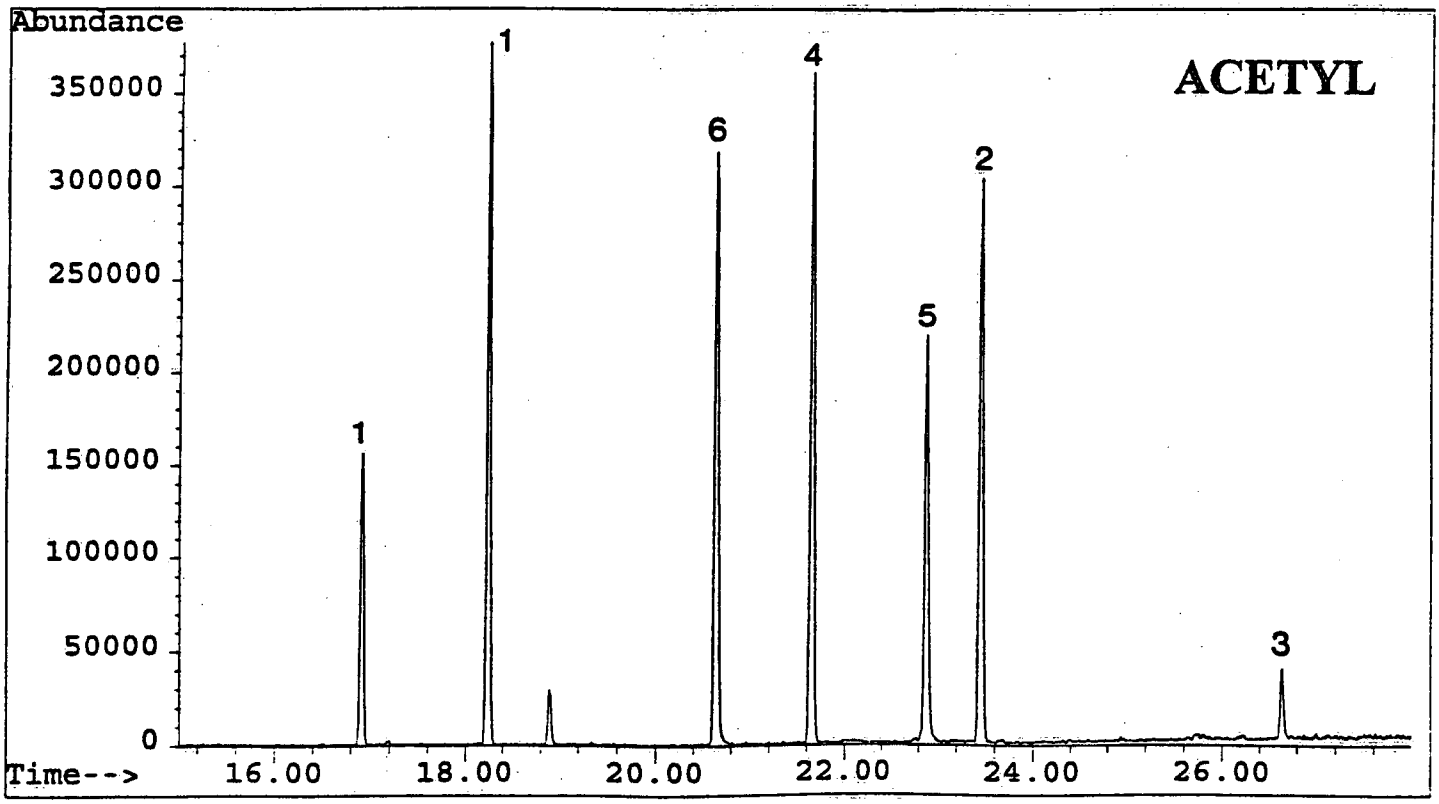
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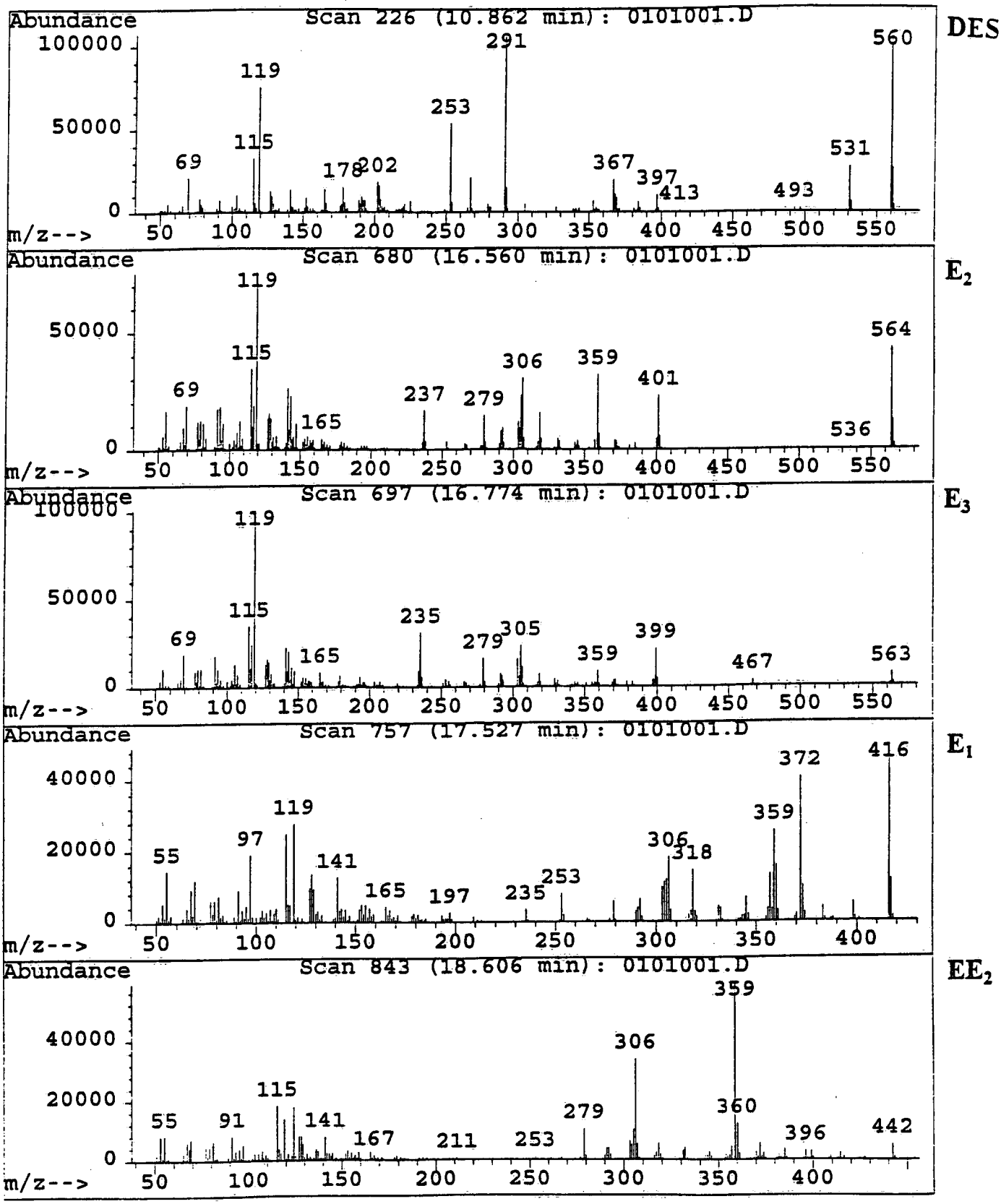
Figure 4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50 100 150 200 250 300 350 400 450 500 550
69 115 119 165 235 279 305 359 399 467 563
E3
Abundance
Scan 757 (17.527 min): 0101001.D
40000
20000
0
m/z-->
50 100 150 200 250 300 350 400
55 97 119 141 165 197 235 253 306 318 359 372 416
E1
Abundance
Scan 843 (18.606 min): 0101001.D
40000
20000
0
m/z-->
50 100 150 200 250 300 350 400
55 91 115 141 167 211 253 279 306 359 360 396 442
EE2

Figure 5

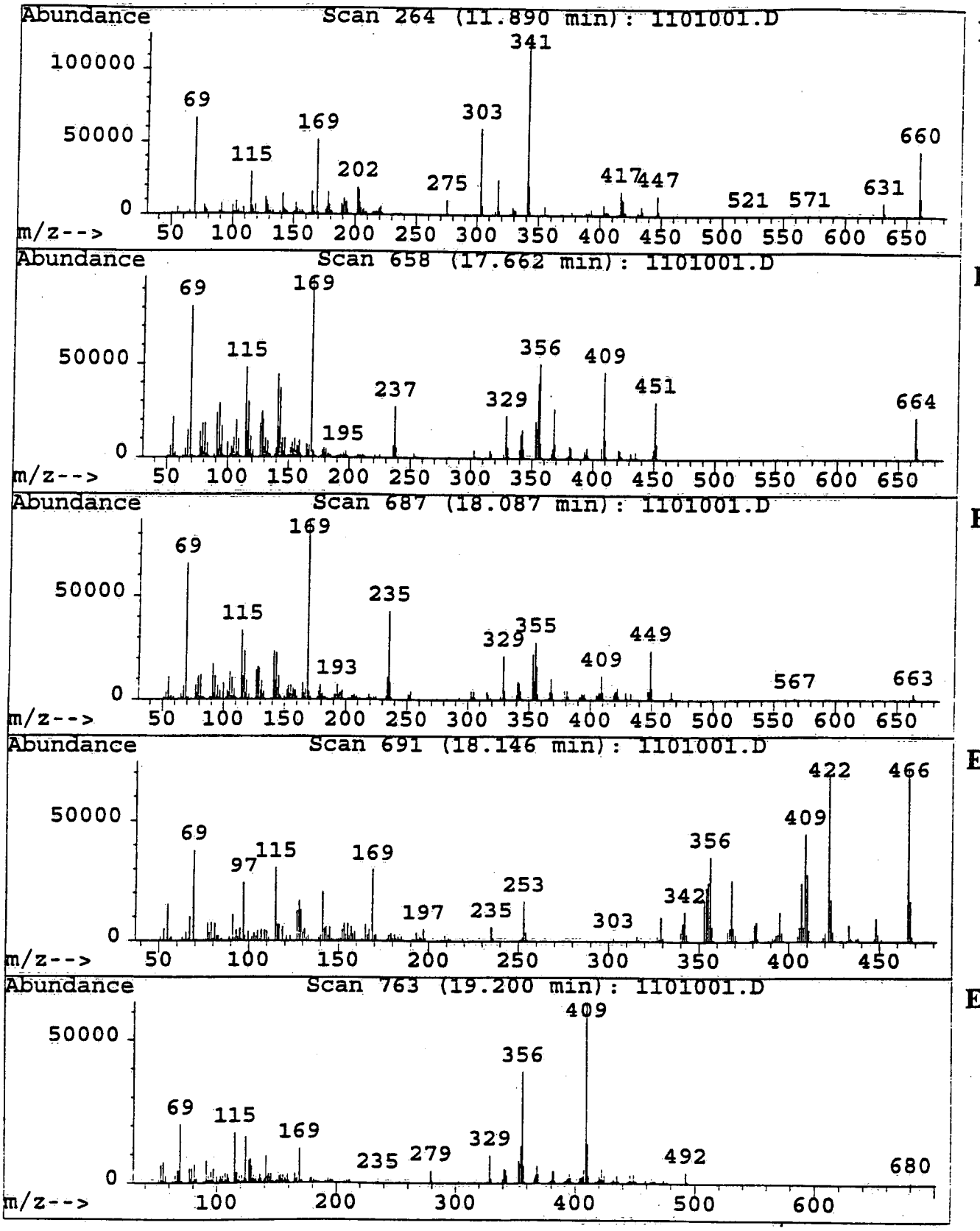

Abundance
Scan 264 (11.890 min): 1101001.D
DES
341
100000
69
303
169
50000
660
115
202
275
417
447
631
521
571
0
m/z-->
50 100 150 200 250 300 350 400 450 500 550 600 650
Abundance
Scan 658 (17.662 min): 1101001.D
E2
69
169
50000
115
356
409
237
329
451
664
195
0
m/z-->
50 100 150 200 250 300 350 400 450 500 550 600 650
Abundance
Scan 687 (18.087 min): 1101001.D
E3
169
69
50000
235
115
329
355
449
409
193
567
663
0
m/z-->
50 100 150 200 250 300 350 400 450 500 550 600 650
Abundance
Scan 691 (18.146 min): 1101001.D
E1
422
466
50000
409
69
356
115
97
169
253
342
197
235
303
0
m/z-->
50 100 150 200 250 300 350 400 450
Abundance
Scan 763 (19.200 min): 1101001.D
EE2
409
50000
356
69
115
169
329
235
279
492
680
0
m/z-->
100 200 300 400 500 600

Figure 6

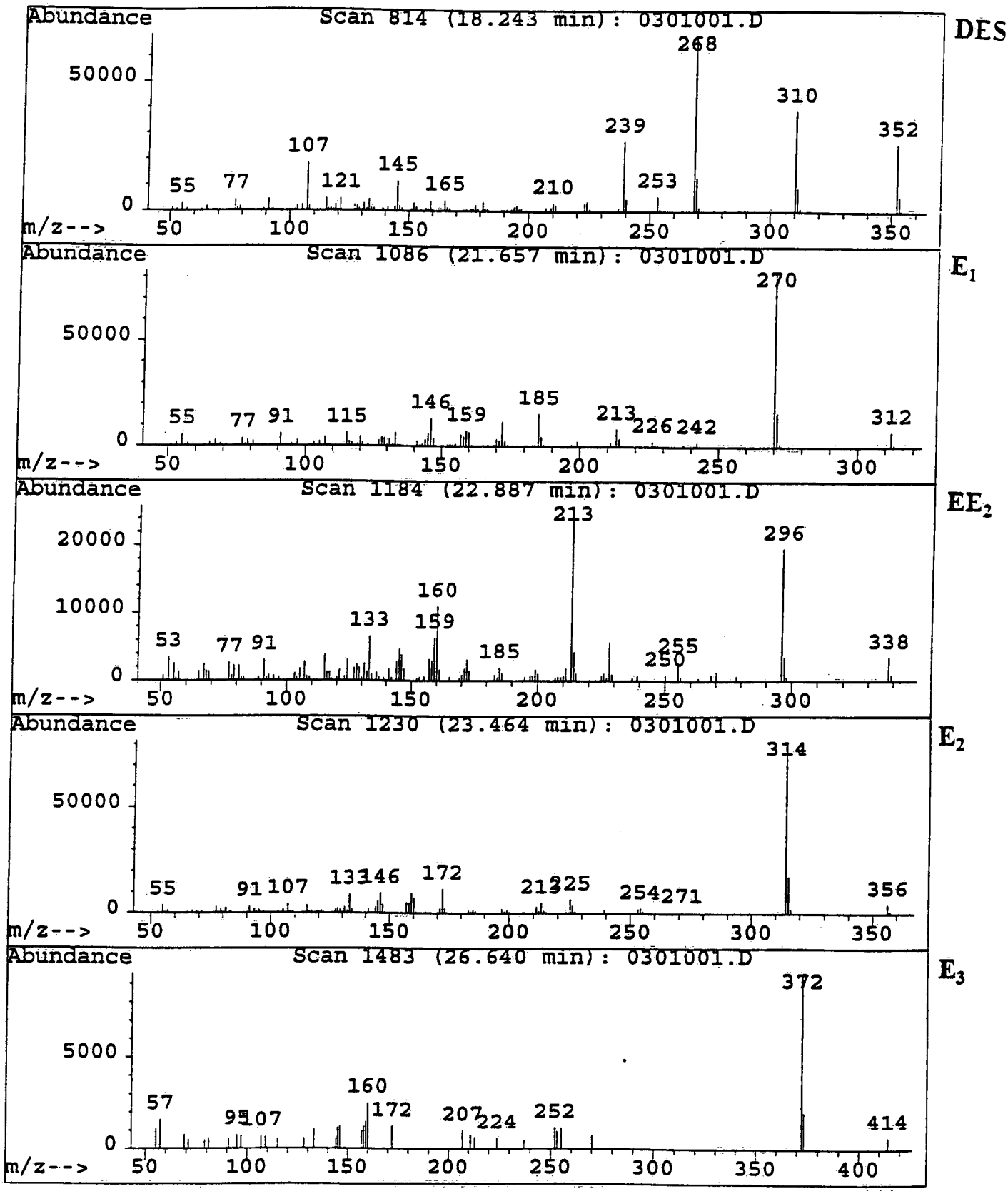
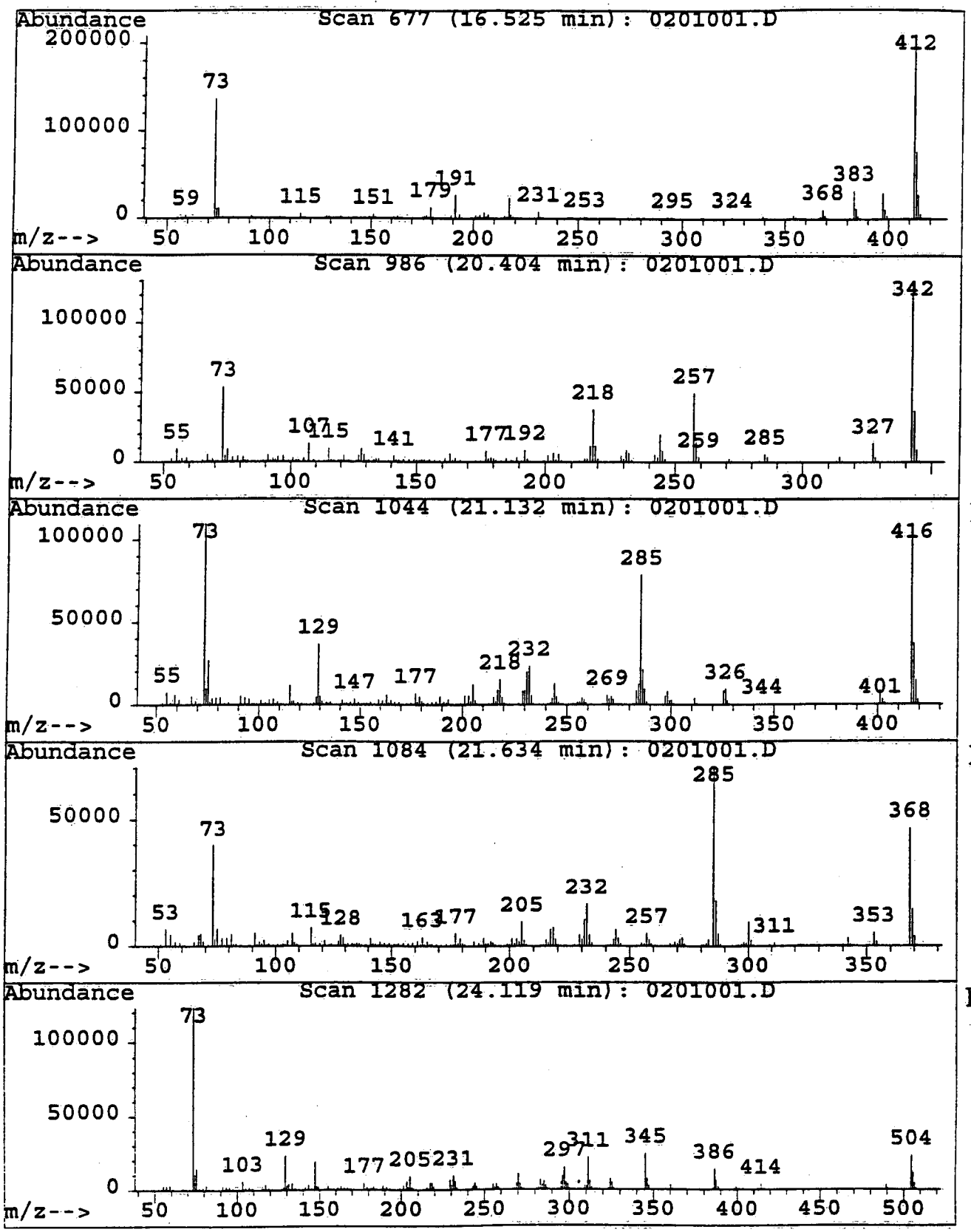
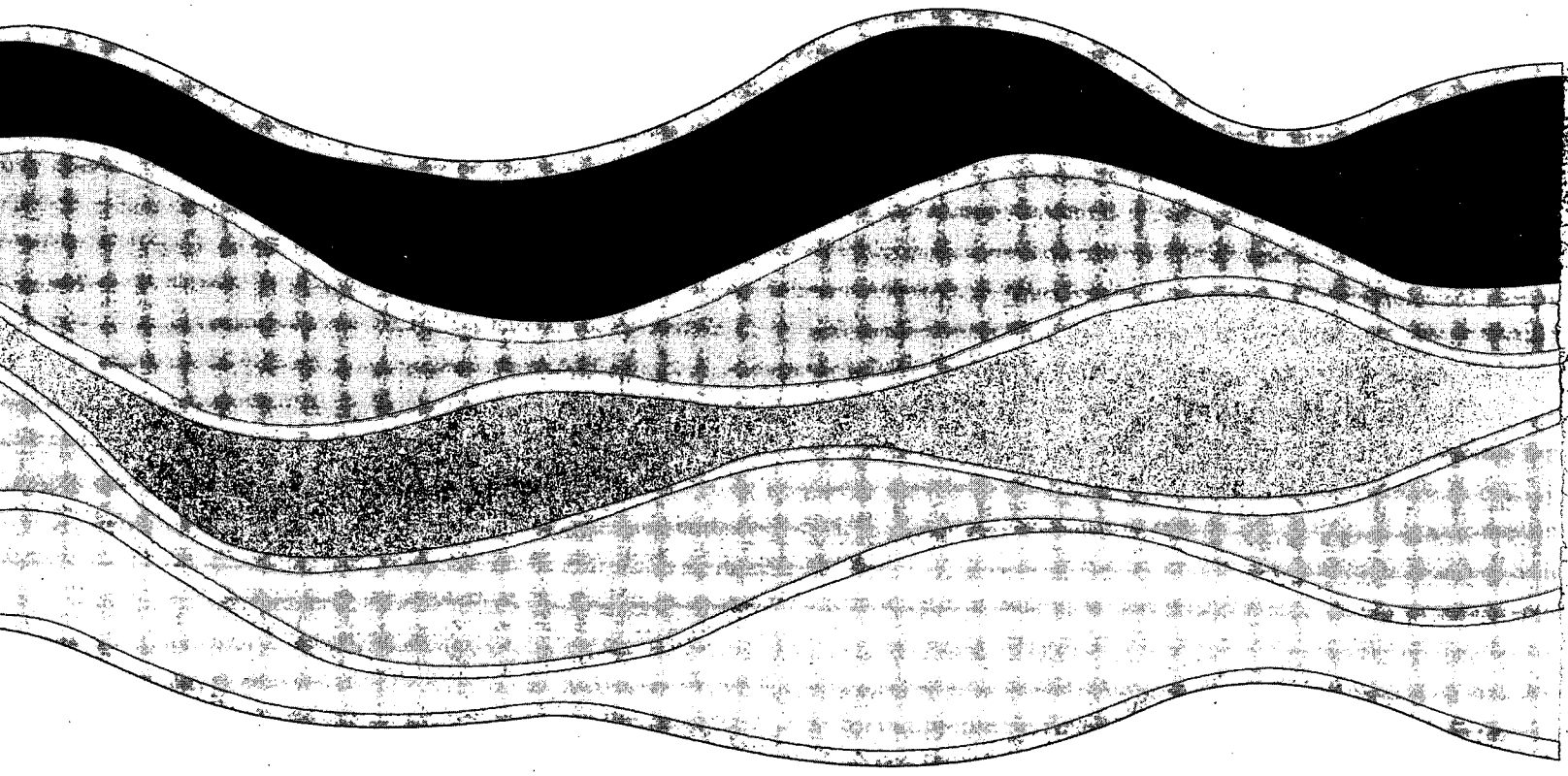

Abundance
Scan 814 (18.243 min): 0301001.D
DES
50000
0
m/z-->
50 100 150 200 250 300 350
55 77 107 121 145 165 210 239 253 268 310 352
Abundance
Scan 1086 (21.657 min): 0301001.D
E1
50000
0
m/z-->
50 100 150 200 250 300
55 77 91 115 146 159 185 213 226 242 270 312
Abundance
Scan 1184 (22.887 min): 0301001.D
EE2
20000
10000
0
m/z-->
50 100 150 200 250 300
53 77 91 133 159 160 185 213 250 255 296 338
Abundance
Scan 1230 (23.464 min): 0301001.D
E2
50000
0
m/z-->
50 100 150 200 250 300 350
55 91 107 131 146 172 213 225 254 271 314 356
Abundance
Scan 1483 (26.640 min): 0301001.D
E3
5000
0
m/z-->
50 100 150 200 250 300 350 400
57 95 107 160 172 207 224 252 372 414

Figure 7


Abundance
Scan 677 (16.525 min): 0201001.D
DES
200000
100000
0
73
412
59
115
151
179
191
231
253
295
324
368
383
m/z-->
50
100
150
200
250
300
350
400
Abundance
Scan 986 (20.404 min): 0201001.D
E1
100000
50000
0
342
73
257
218
55
107
115
141
177
192
259
285
327
m/z-->
50
100
150
200
250
300
Abundance
Scan 1044 (21.132 min): 0201001.D
E2
100000
50000
0
73
416
285
129
232
55
147
177
218
269
326
344
401
m/z-->
50
100
150
200
250
300
350
400
Abundance
Scan 1084 (21.634 min): 0201001.D
EE2
50000
0
285
368
73
232
53
115
128
163
177
205
257
311
353
m/z-->
50
100
150
200
250
300
350
Abundance
Scan 1282 (24.119 min): 0201001.D
E3
100000
50000
0
73
129
345
504
103
177
205
231
297
311
386
414
m/z-->
50
100
150
200
250
300
350
400
450
500



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