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GC-MS Analysis of Phytosterol Content of Dried Mushrooms

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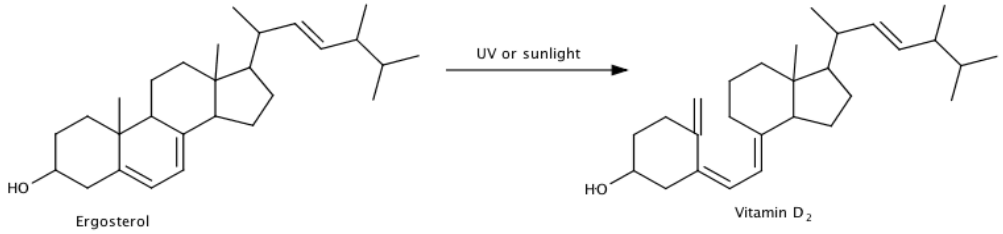
ABSTRACT:
The goal of this research was to determine the phytosterol content of dried, store-bought mushrooms and compare the results to that previously reported for fresh mushrooms. Ergosterol is particularly important due to its role as a light-activated precursor of vitamin D (vitamin D2). Dried mushrooms (*Pleurotus ostreatus* and *Morchella*) were Soxhlet extracted with petroleum ether and the extracts were concentrated and then saponified with 1-M NaOH in ethanol. Extraction again with petroleum ether was followed by drying with Na_2SO_4 and derivatization as TMS-ethers, which were analyzed by GC-MS. It was hypothesized that the mushroom drying process (e.g. sun drying vs. oven drying) could affect the phytosterol and vitamin D_2 content. Results suggest that the dried mushrooms analyzed have similar sterol content to that of fresh mushrooms. A sterol detected in both fresh and dried morel mushrooms was proposed to be 24-methylene cholesterol. 24-Methylene ergosta-5,22,24(24)-trienol was proposed to be the sterol detected in dried morel mushrooms. Ergosterol abundance relatively decreases in sundried morel mushrooms when compared to dried and fresh. This may be due to the conversion of ergosterol to vitamin D_2 via sunlight or UV rays.

INTRODUCTION

Mushrooms are a popular food item featured in the cuisines of almost every country and cultural group. This begs the question of what nutritional benefits come from eating them and how different species differ in nutrient content. One thing that makes mushrooms nutritionally valuable is that they contain phytosterols.[1] The body cannot make most phytosterols while edible plants and fungi produce a lot of them, so we must obtain these from our diets.[1,2] The most important phytosterol found in mushrooms, ergosterol (**1**), produces ergocalciferol upon photolysis. It is then rearranged (1) into vitamin D_2, a dietary form of vitamin D.

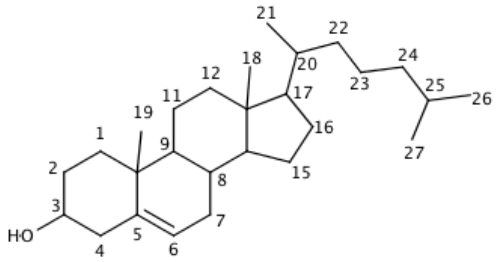


Ergosterol Vitamin D_2

(1)

UV treatment of ergosterol in *vitro* and yeast is used commercially. This produces vitamin D_2 for dietary supplements, pharmaceutical grade vitamin D preparations, and food fortification.[3] Recent research has looked at whether the amount of vitamin D_2 intake can be increased through ingestion of fresh mushrooms treated with UV-B light as opposed to untreated mushrooms.[3,4,5] This research did not specifically correlate ergosterol levels to vitamin D_2 levels but the authors recognized that in addition to vitamin D_2 there might be other vitamin D analogues present. For example, ergosta-5,7-dienol (**2**) was converted into vitamin D_4 in the UV-treated mushrooms.[3]

In another study[1], phytosterols were shown to be moderate cholesterol-lowering agents. In this study, rats were fed a cholesterol-based diet and a mixture of phytosterols decreased their dietary cholesterol concentration. Selective intestinal phytosterol absorption in the body occurs when the phytosterols enter the intestinal wall. It is thought that phytosterols, due to their structural similarity with cholesterol, help lower blood cholesterol because they compete with it for absorption into the intestines. [1–3]

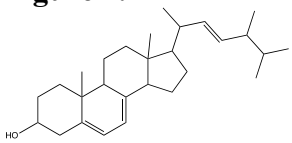


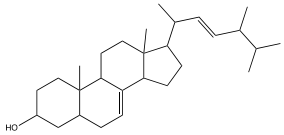
Cholesterol structure showing numbering used for sterol naming.

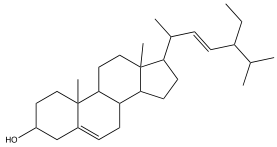
This competition is more effective with an increase in the alkyl substituent at C_{24}, which is true of all phytosterols commonly present in mushrooms. Phytosterols often differ in their side chain configuration but the side chain always is attached to C_{17}.[1,2]

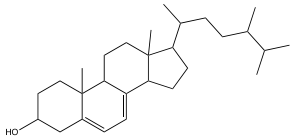
Phytosterols previously identified in mushrooms include: ergosterol (**1**), ergosta-5,7-dienol (**2**), ergosta-7-enol (**3**), ergosta-7,22-dienol (**4**), brassicasterol (**5**), campesterol (**6**), stigmasterol (**7**), sitosterol (**8**), and stigmastanol (**9**) (Figure 1).[3–5] All of these have the same carbon skeleton as cholesterol but with an ethyl or methyl group at C_{24}. The main variation between them is the number and locations of carbon-carbon double bonds.[1–3]

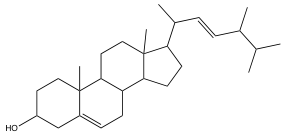
Figure 1. Structures of the different sterols found in various fresh mushrooms.

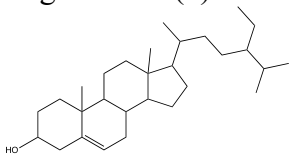

Ergosterol (**1**)

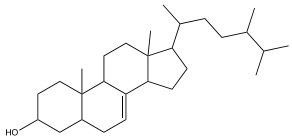

Ergosta-7,22-dienol (**4**)

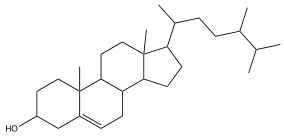

Stigmasterol (**7**)

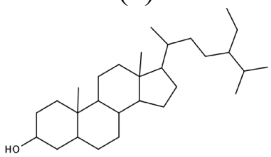

Ergosta-5,7-dienol (**2**)


Brassicasterol (**5**)


Sitosterol (**8**)


Ergosta-7-enol (**3**)


Campesterol (**6**)


Stigmastanol (**9**)

A great deal is known about the sterol content in edible fresh mushrooms but it is unknown if store-bought, dried mushrooms have similar sterol content or how the drying process affects sterol content. Processes used for drying include sun exposure, baking in an oven[6], using a food dehydrator, or blow drying with a fan.[7] A recent paper on fresh mushrooms commonly consumed in the US included detailed results for morel and oyster mushrooms but indicated one sterol detected in morels as "unknown".[3] For this research, we wanted to determine if dried morel and oyster mushrooms had similar sterol content to that of fresh. We also wanted to identify the unknown sterol reported for morel mushrooms. It was hypothesized that sterol content would vary in fresh and dried mushrooms and ergosterol (**1**) would be less abundant in sundried morel mushrooms due to the conversion of this sterol to vitamin D_2 through sunlight.

RESULTS AND DISCUSSION

Compounds **1** and **5-9** were bought, TMS derivatized with trimethylsilyl imidazole (TSIM), and used as standards. GC-MS was carried out using the same conditions as the literature reference. All compounds eluted in the same order as reported in fresh mushrooms[3], but the reported retention times were all around one minute longer then the retention times found in dried mushrooms (Table 1). Compounds **7-9** were not detected in dried mushrooms or reported in fresh mushrooms.

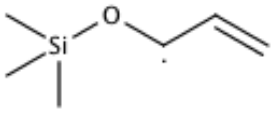
Table 1. Compounds Detected.

Mushroom	Retention Time for each Compound (min)										
	11[f]	**12**[f]	**5**	**13**[f]	**1**	**10**	**4**	**6**	**14**[f]	**2**	**3**
Fresh Oyster[a]	ND	ND	26.6	ND	28.6	ND	29.6	ND	ND	32.1	33.1
Dried Oyster[b]	25.8	26.2	ND	ND	27.8	ND	28.5	ND	ND	30.6	31.5
Fresh Morel (F1)[a,e]	ND	ND	26.6	ND	28.6	29.4	ND	29.6	ND	31.7	32.8
Fresh Morel (E2)[a,e]	ND	ND	26.6	ND	28.6	29.4	ND	29.6	ND	32.1	32.8
Sundried Morel[b,c]	ND	ND	25.7	27.2	27.7	28.4	ND	28.6	31.3	ND	ND
Dried Morel[b,d]	ND	ND	25.8	27.2	27.7	28.3	ND	28.6	31.3	ND	ND

[a] Reference 3. [b] This work. [c] Sundried drying process. [d] Unknown drying process. [e] Mushrooms obtained from two different suppliers. [f] Compounds only detected in dried mushrooms.

The mass spectra of all sterols show a loss of a methyl radical and TMSOH to form peaks at M-15 and M-90, respectively. Loss of a methyl radical occurs after the loss of TMSOH to give the peaks seen at M-105. Another common peak at *m/z* = 69 is a stable five carbon allyl carbocation (Table 2).

Dried morel mushrooms from two different sources, one using an unspecified drying process and the other identified as "sundried", were analyzed. GC-MS analysis was formed after petroleum ether extraction, saponification, and TMS derivatization. In both morel mushroom samples, compounds **1**, **5**, and **6** were detected and gave identical mass spectra to standards prepared for this research. The base peaks were at *m/z* = 363, 69, and 129, respectively (Table 1 and 2). A peak with the same mass spectrum as the unknown reported for morels (M^+ = 470, base peak at *m/z* = 129) (**10**) was detected in both dried morel samples (Table 1 and 2).[3] A fragment seen in all of compounds **5, 6,** and **10** is the loss of 129 and these compounds all also exhibited an abundant *m/z* = 129 peak. The M-129 and *m/z* = 129 peaks are thought to be degradation of the Δ^5-mono alkene structure present in structures **5** and **6**, so this gives us a hint as to the structure of **10**.



Radical fragment of M-129. Also found as a carbocation at *m/z* = 129.

A slight difference was seen in ergosterol (**1**) where a loss of 131 predominates. M-129 is still observed by is small because of a subsequent loss of H_2 is facile due to the hexadiene ring being able to aromatize.

Compounds **1-4** were detected in dried oyster mushrooms in good agreement with results reported for fresh oyster mushrooms (Table 1).[3] Compounds **2-4**, with respective base peaks at *m/z* = 365, 255, and 255 (Tables 1 and 2), were identified by comparison to the GC-MS data reported for fresh mushrooms[3]. Full mass spectra were not given by this source, only selected peaks and abundances, and standards were not available for these compounds so the identifiers are less certain then they are for **1, 5,** and **6**. Ergosta-7,22-dienol (**4**) showed a peak at M-127, corresponding to cleavage of the side chain followed

by dehydrogenation. Due to its structural similarity to that of ergosterol (**1**), ergosta-5,7-dienol (**2**) shows the same fragment of that observed for ergosterol at M-131. The base peak of ergosta-5,7-dienol at *m/z* = 365 seen for dried oyster mushrooms did not match the base peak at *m/z* = 143 reported for fresh mushrooms[3]. However, the next highest peaks were reported[3] as *m/z* = 365 and 339 while the next highest peaks from our mass spectrum were *m/z* = 339 and 143 so the disciplinary is singly due to varying relative intensities of the three strongest peaks. All of the other significant fragments reported in the literature was present in our spectrum.

Table 2. Mass Spectra of Compounds Detected.

Compound	M^+ (m/z)	Other significant fragments (m/z)											
1[a]	468	453	378	**363**	337	253	211	131	73	69			
2[b]	470	455	380	**365**	339	211	145	143	131	129	73		
3[b]	472	457	382	367	345	**255**	229	213	207	143	131	75	73
4[b]	470	455	380	365	345	343	**255**	229	143	131	73	69	
5[a]	470	455	380	365	341	255	215	213	129	125	73	**69**	
6[a]	472	457	382	367	343	289	261	255	213	207	**129**	73	
10[b]	470	455	429	386	380	365	343	341	253	213	207	**129**	73
11[c]	466	451	376	361	325	**251**	249	209	207	73	69		
12[c]	468	453	378	**363**	337	253	211	207	73	69			
13[c]	468	453	378	**363**	337	253	251	211	143	131	73	69	
14[c]	470	455	429	386	365	343	341	281	255	253	**207**	75	73

[a] Spectrum matched peak for peak with standard prepared for this research. [b] No standard was prepared but the spectrum matched peak for peak with literature values.[3] [c] Compounds only detected in dried mushrooms. [d] All base peaks are bolded.

Compounds **2** and **3** (~33 min) are present in fresh morels[3] but in the dry morels, an unknown peak at the expected retention time (31.3 min) was detected (compound **14**, Table 1). This peak with M^+ at *m/z* = 470 was not consistent with the mass spectrum of compound **2** or **3**. Both dried morel samples also showed another unidentified peak with M^+ at *m/z* = 468 at 27.2 min (compound **13**, Table 2). A very small amount (~1%) of brassicasterol (**5**) (27 min) was reported in fresh oyster mushrooms, but our dried oyster mushrooms showed no detectable compound **5**. Instead, the peaks observed at 25.8 min with M^+ at *m/z* = 466 and 26.2 min with M^+ at *m/z* = 468 (compounds **11** and **12**, Table 2) remain as unidentified phytosterols. Compounds **7-9** were not detected in any of the mushroom samples.

Fresh oyster mushrooms have a high relative abundance of ergosterol (**1**) (84%) while in the dried mushrooms that we analyzed, ergosterol is still high (68%) but the relative abundances are dispersed more evenly with compounds **2-4**, which relatively increased in abundance (8%, 10%, and 5% respectively) (Table 3). Dried oyster mushrooms therefore are less abundant in ergosterol then reported in fresh oyster mushrooms[3] and more abundant in compounds **2-4**.

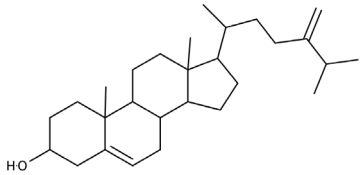
The two samples of dried morel mushrooms showed somewhat different sterol content, but the relative abundances of the known sterols are similar to that of fresh mushrooms.[3] Brassicasterol (**5**) had the same relative abundance in each mushroom sample (~52%) and was found to be the most abundant. However, ergosterol (**1**) was slightly less abundant and compounds **6** and **10** were relatively more abundant in sundried mushrooms than in the other dried morel mushrooms (20% and 25% respectively, Table 3).

Table 3. Relative Abundances for Detected Compounds.

Mushroom	Abundances (%)										
	1	**2**	**3**	**4**	**5**	**6**	**10**	**11**	**12**	**13**	**14**
Fresh Oyster[a]	83.9	1.1	2.4	2.3	0.8	ND	ND	ND	ND	ND	ND
Dried Oyster[b]	67.8	8.2	9.7	5.0	ND	ND	ND	5.2	4.2	ND	ND
Fresh Morel (F1)[a,e]	30.9	4.4	2.1	ND	44.1	2.8	15.8	ND	ND	ND	ND
Fresh Morel (E2)[a,e]	39.8	4.0	1.8	ND	42.1	6.7	5.7	ND	ND	ND	ND
Sundried Morel[b,c]	19.5	ND	ND	ND	51.5	8.4	10.5	ND	ND	13.8	2.0
Dried Morel[b,d]	24.5	ND	ND	ND	51.8	3.8	4.1	ND	ND	8.3	1.8

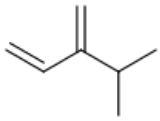
[a] Reference 3. [b] This work. [c] Sundried drying process. [d] Unknown drying process.
[e] Mushrooms obtained from two different suppliers.

One unidentified compound was reported in fresh morel mushrooms.[3] This compound with a retention time of 25.4 min and a M^+ at *m/z* = 470 was also detected in both of our dried morel mushroom samples. This peak had the same retention time as campesterol (**6**) but with a different molecular ion peak (Table 2). The mass spectrum did not show a *m/*z = 255 or *m/z* = 229 peak which would suggest a Δ^7-sterol. The peak found at *m/z* = 129 suggested a Δ^5-sterol, as claimed previously. The key peak that suggests a Δ^{24}-sterol is the peak at *m/z* = 386 ($M-C_6H_{12}$), which is not seen in any of the other mass spectra.[10] Therefore, we propose 24-methylene cholesterol (**10**) as the identity of this compound (Table 1). This structure makes sense due to it being a known intermediate in the biosynthesis of brassicasterol (**5**) in fungi.[12]



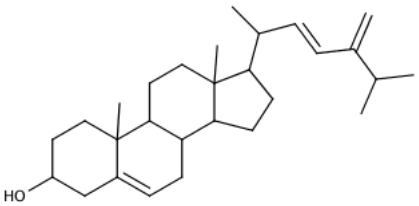
Unknown found in morels (**10**) = 24-methylene cholesterol.

Another unidentified compound (**13**) was detected in both dried morel mushrooms with a M^+ at *m/z* = 468. The retention time of 27.2 min (Table 1) is around the same retention time where brassicasterol (**5**) was reported in fresh morel mushrooms[3]. The peak found at *m/z* = 129 suggested a Δ^5-sterol. The peak at *m/z* = 372 (M-96) suggests the loss of 2-isopropyl butadiene.



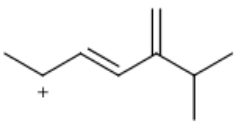
Fragment of M-96.

This fragment suggests a trienol and specifically a Δ^{22} and Δ^{24}-sterol. Compound **13** is proposed to be 24-methylene ergosta-5,22,24(24)-trienol, which is another known intermediate in the biosynthesis of brassicasterol in fungi.[12]

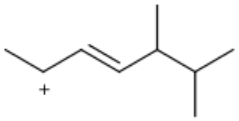


Unknown found in dried morels (**13**) = 24-methylene ergosta-5,22,24(24)-trienol.

Brassicasterol (**5**) shows a peak at *m/z* = 125, which is an allylic cleavage of the side chain to form the carbocation of C_9H_{17}. Similarly, compound **13** has a peak at *m/z* = 123. This illustrates the second double bond that is present in conjugation with the Δ^{22} in the side chain.



Carbocation at *m/z* = 123 in mass spectrum of compound **13**.



Carbocation at *m/z* = 125 in mass spectrum of compound **5**.

CONCLUSION

Fresh and dry mushrooms have similar sterol content except that there are unidentified compounds (**11-14**) detected in dry mushrooms. Also, brassicasterol (**5**), reported as a trace phytosterol in fresh oyster mushrooms[3], was not detected in the dried oyster mushrooms. In contrast to oyster mushrooms, the predominant sterol in morels is brassicasterol and this is true for both fresh (~40%) and dried (~50%) morels. Similarly, compounds **2** and **3** reported as present in small amounts (2-4%) in fresh morel mushrooms[3] were not detected in our dried morels. The unidentified sterol with a M^+ at *m/z* = 470 previously reported for fresh morels[3] was confirmed as present in dried morels and identified from the mass spectrum as 24-methylenecholesterol (**10**), which is a known intermediate in fungal biosynthesis of brassicasterol.

The relative abundance of ergosterol appears to decrease after the drying process. In oyster mushrooms, ergosterol (**1**) decreased from 84% to 65% in the dried mushrooms. In fresh morel mushrooms[3], ergosterol decreased from 36% to 20%-25% abundant. This

may be to due the conversion of ergosterol into vitamin D_2 via sunlight. However, this needs further work to confirm.

EXPERIMENTAL

Dried oyster mushrooms from Festival Foods, sundried morel mushrooms from Northwest Wild Foods, and mushrooms dried by an unknown process from Morel Mushroom Store were ground with a mortar and pestle (~5 g dry mushroom sample) and placed in the porous thimble of the Soxhlet extractor. A sample of 2-3 g fresh mushroom was used in the literature procedure.[3] This was refluxed for 4-5 h at 60-80 ºC with 125 mL petroleum ether. The solvent was evaporated off at less than 80 °C under reduced pressure.[8]

Saponification was performed by adding 3 mL 1-M NaOH in ethanol and refluxing for 1 h at 60-80 ºC. The solution was extracted with 3 x 2.0 mL petroleum ether and 3 x 2.0 mL brine. The combined organic layers were dried with Na_2SO_4.[10] The solvent was then evaporated under reduced pressure.

The extracts were then derivatized with 0.20 mL trimethylsilylimidazole (TSIM) (Aldrich) in 0.80 mL pyridine (Aldrich).[11] This solution was refluxed for 1 h at 60-80 ºC. The extracts were then analyzed with GC-MS using the same parameters as reported previously for fresh mushrooms.[3] An Agilent 6890-5973 single-quadrupole GC-MS with a 5% diphenyl/95% dimethyl polysiloxane GC column (30 m x 0.25 mm x 0.25 μm) was used. The column inlet pressure was set at 117 kPa for a carrier gas flow of 0.9 mL/min. The oven temperature program was set at 250 °C, ramped at 0.5 °C/min to 265 °C, and held for 25 minutes. Injector, transfer line, and detector temperature were at 300 °C, and an 8.95-minute solvent delay was used. Mass spectrometer ionization energy was set at 70eV.[3]

Compound **1** (5 mg) was purchased from Sigma Aldrich and compounds **5-9** (1 mg) were purchased from ChromaDex. They were then derivatized with TSIM and analyzed with GC-MS using the same procedure to be used as standards. Relative amounts of phytosterols for fresh mushrooms from the literature reference were determined by triangulation of peaks in enlarged pictures of the published gas chromatographs.[3]

FUTURE WORK

We could determine the amount of vitamin D_2 in regularly dried mushrooms and compare that to the amount of vitamin D_2 after sun exposure by using HPLC with [^{3}H]-vitamin D_3 internal standard.[3] Yet to be identified peaks **11, 12,** and **14** could also be hypothesized and identified. Other future work could be done to prove identities proposed for compounds **10** and **13** by either synthesizing them or by purchasing and derivatizing them. Taking this research even farther would involve using internal standards to determine the absolute amounts of sterols and by analyzing different species of mushroom.

ACKNOWLEDGEMENTS

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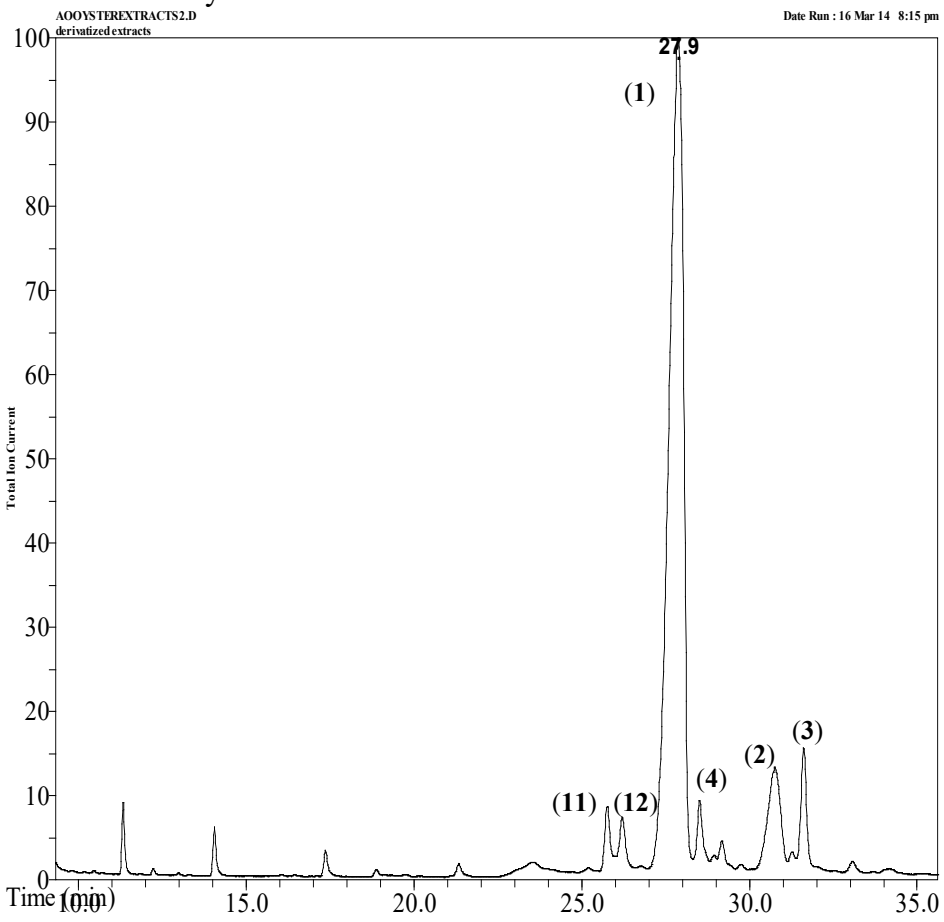
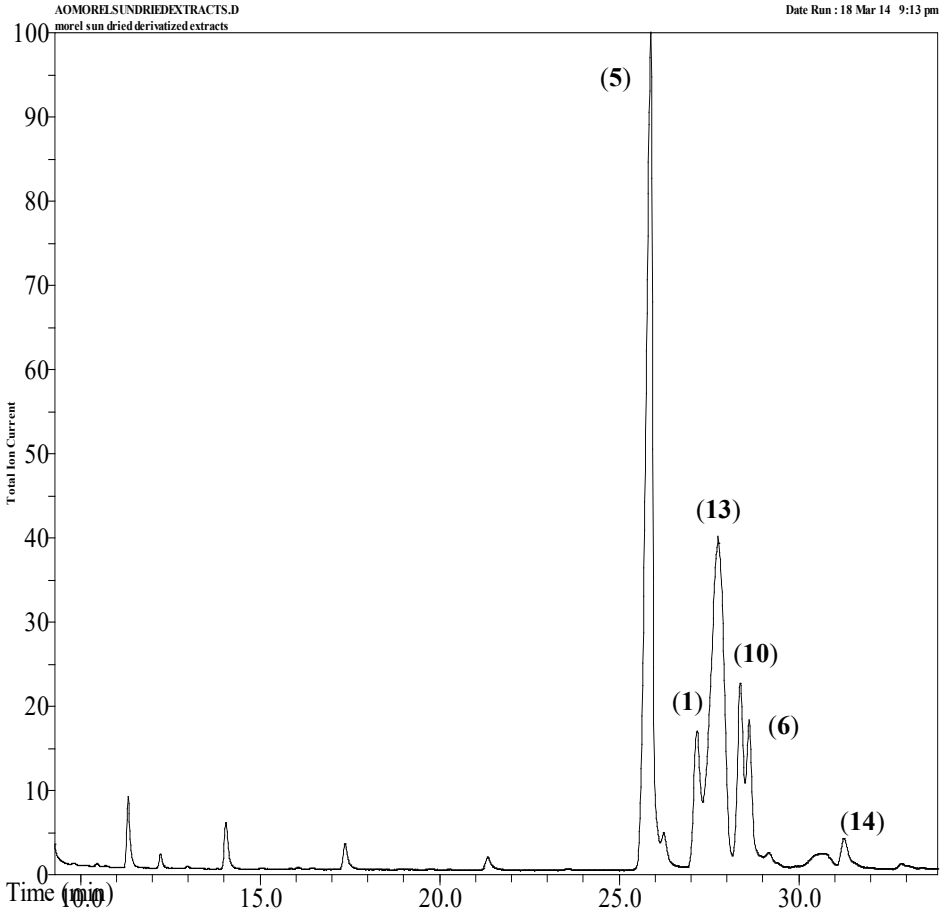
ATTACHMENTS

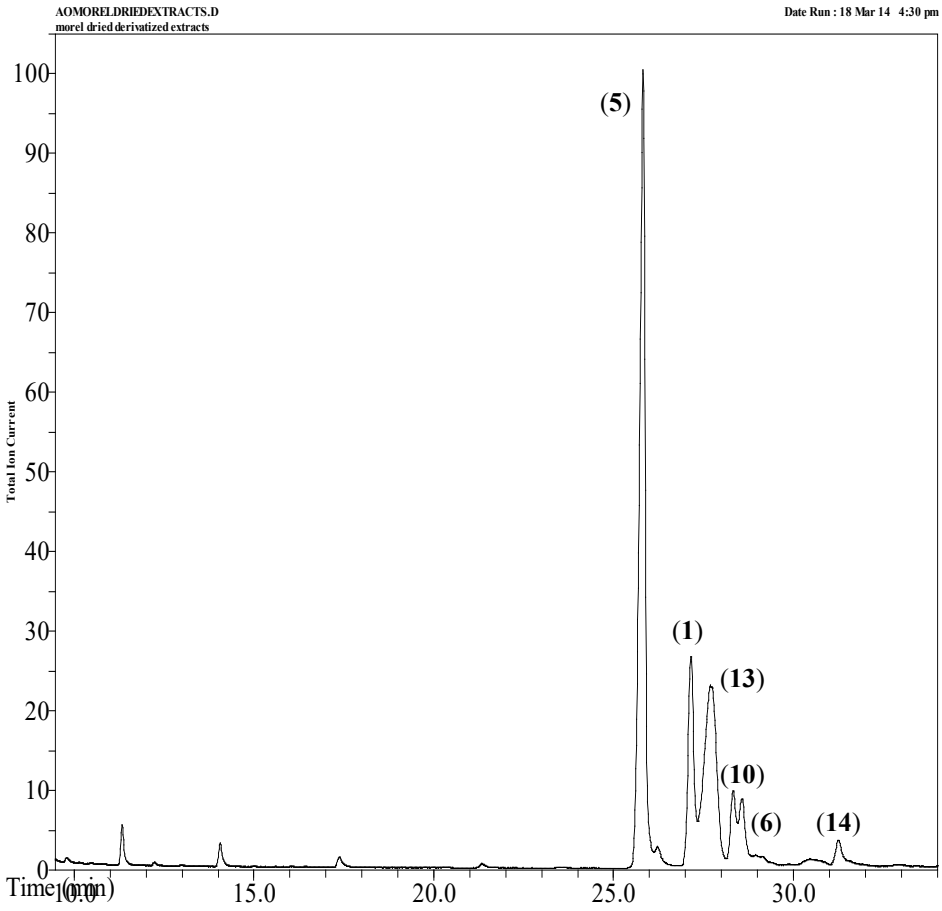
GC for Dried Oyster Mushrooms.



GC for Sundried Morel Mushrooms.

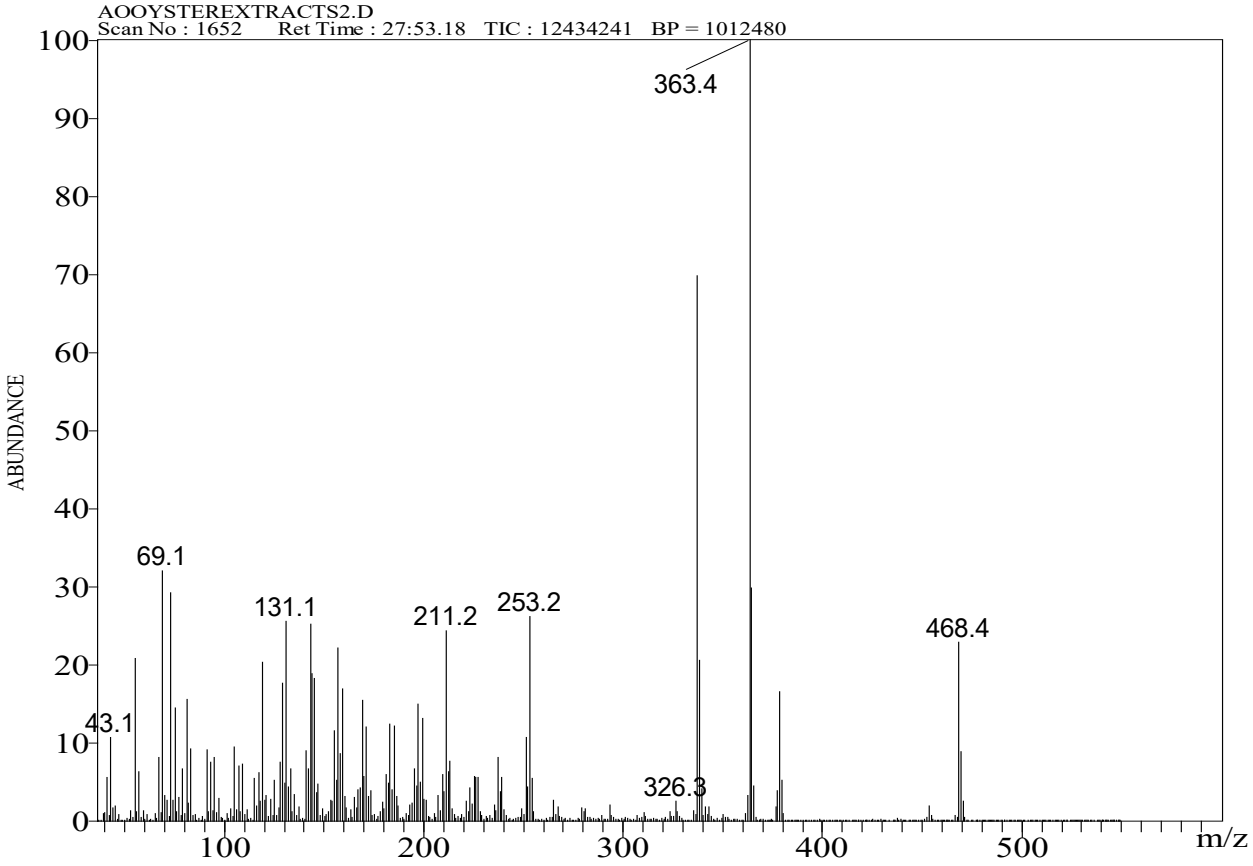


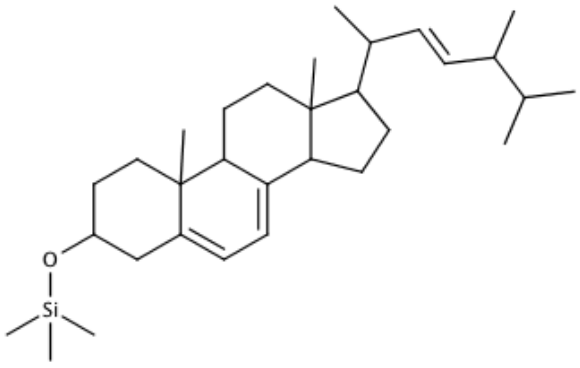
GC for Dried[a] Morel Mushrooms.



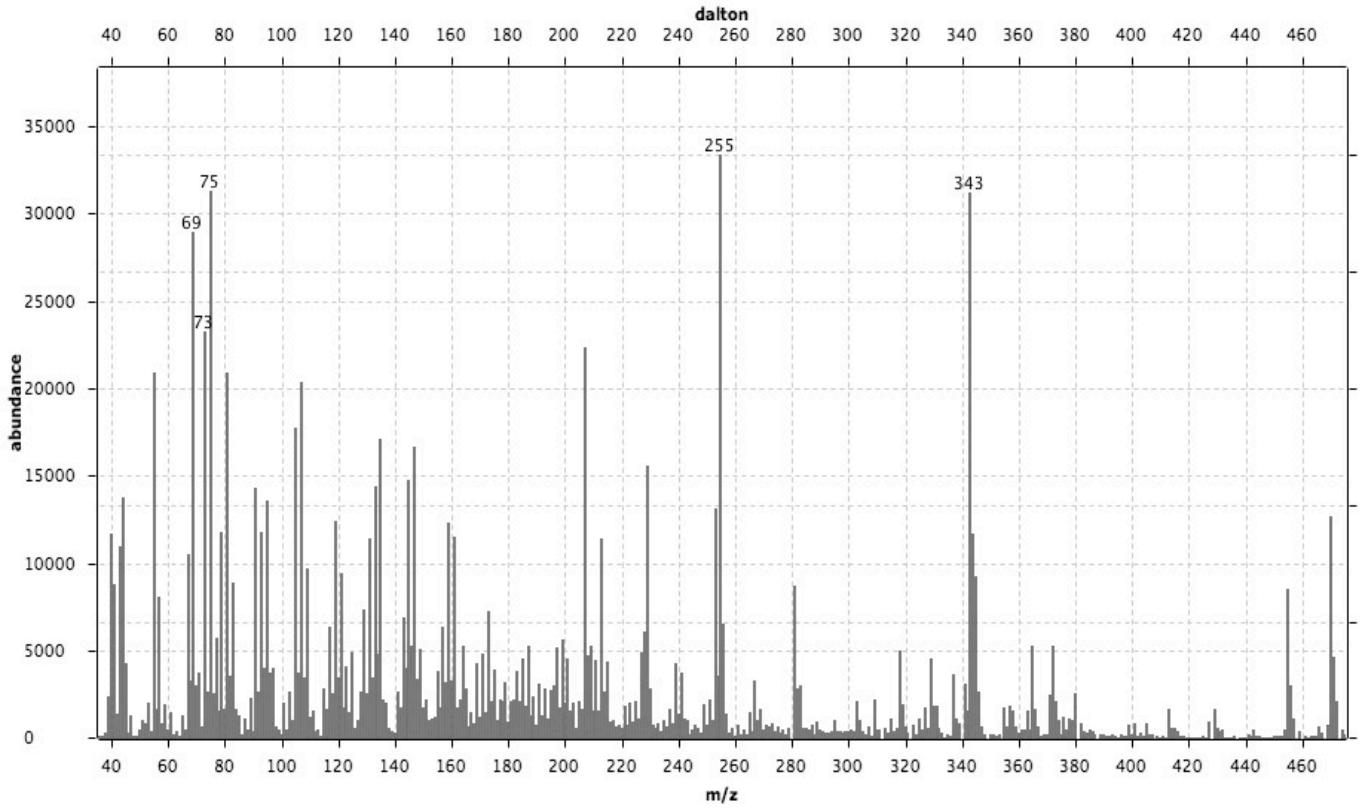
[a]Unknown drying process.

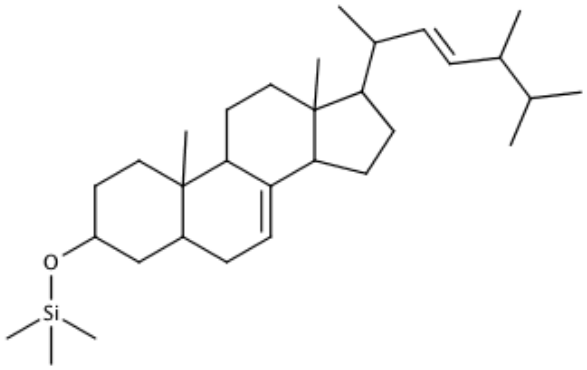
Mass Spectrum for Dried Oyster Mushrooms (**1**).



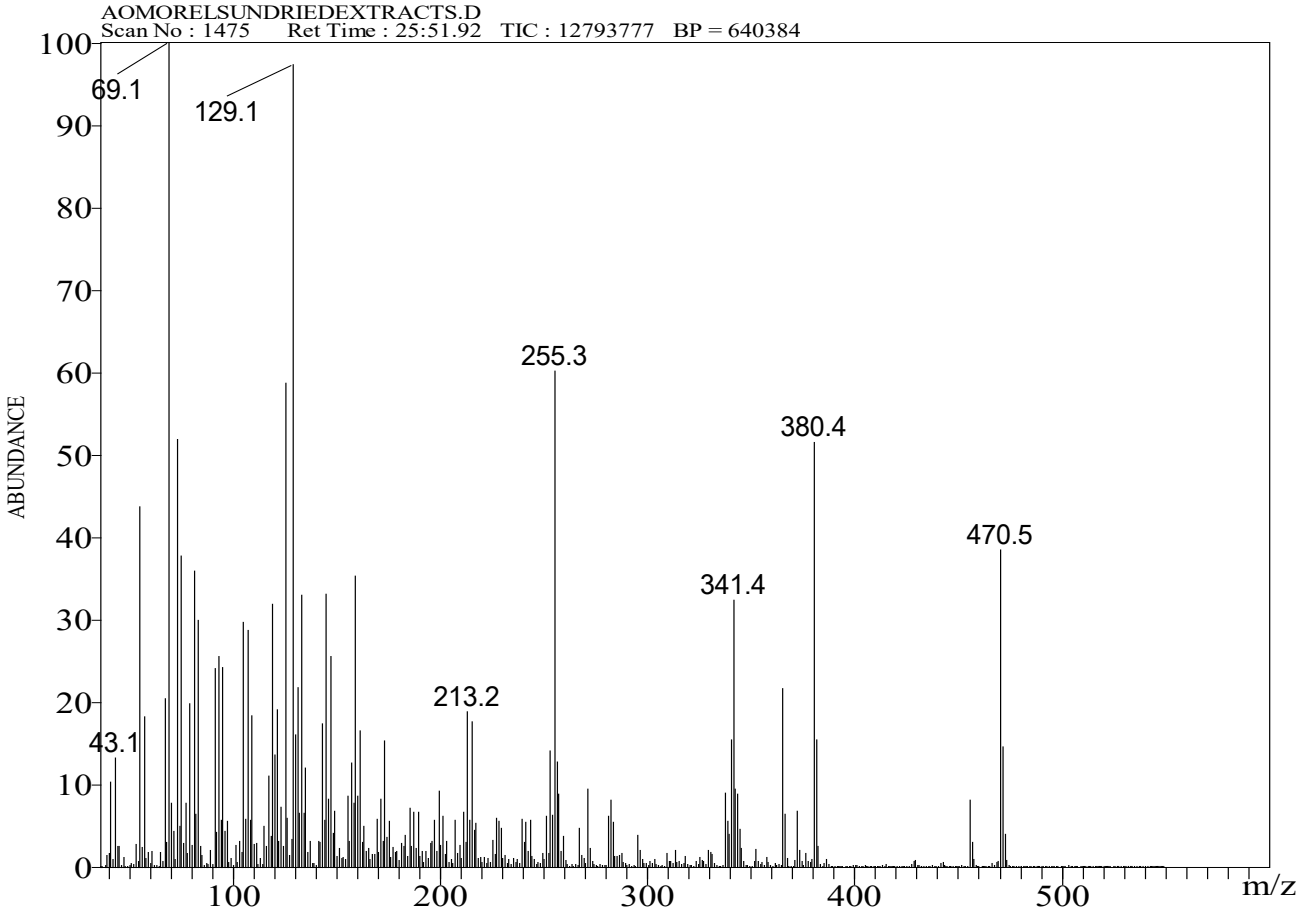


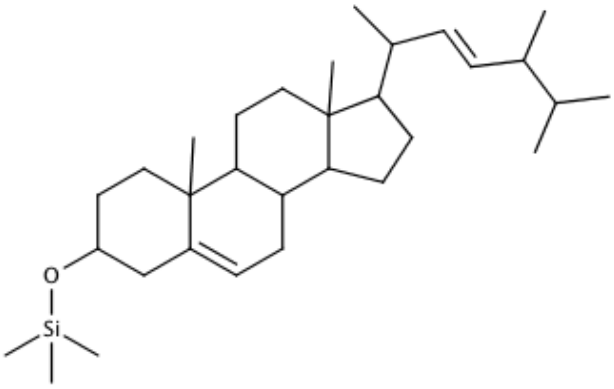
Mass Spectrum for Dried Oyster Mushrooms (**4**).



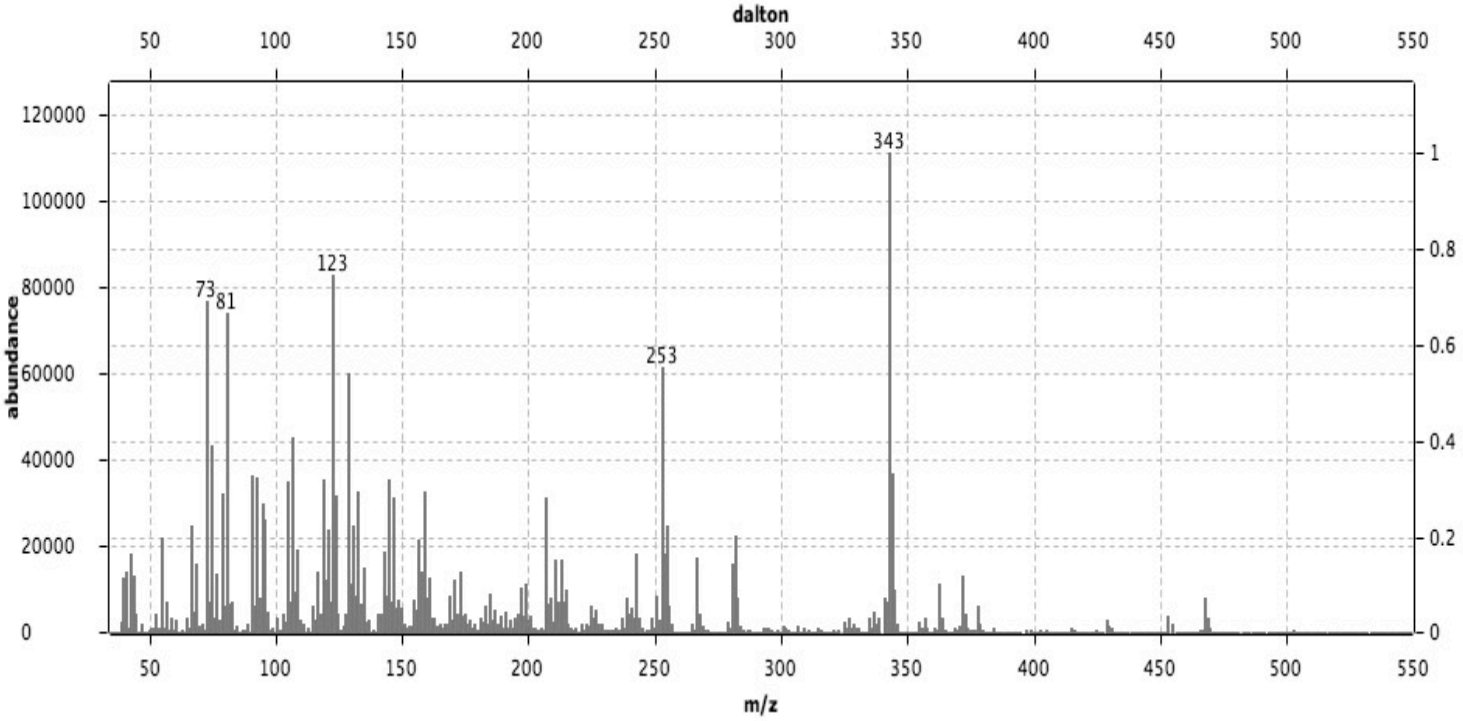


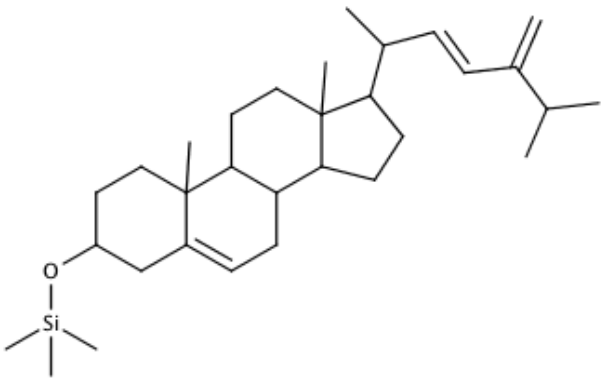
Mass Spectrum for Sundried Morel Mushrooms (**5**).



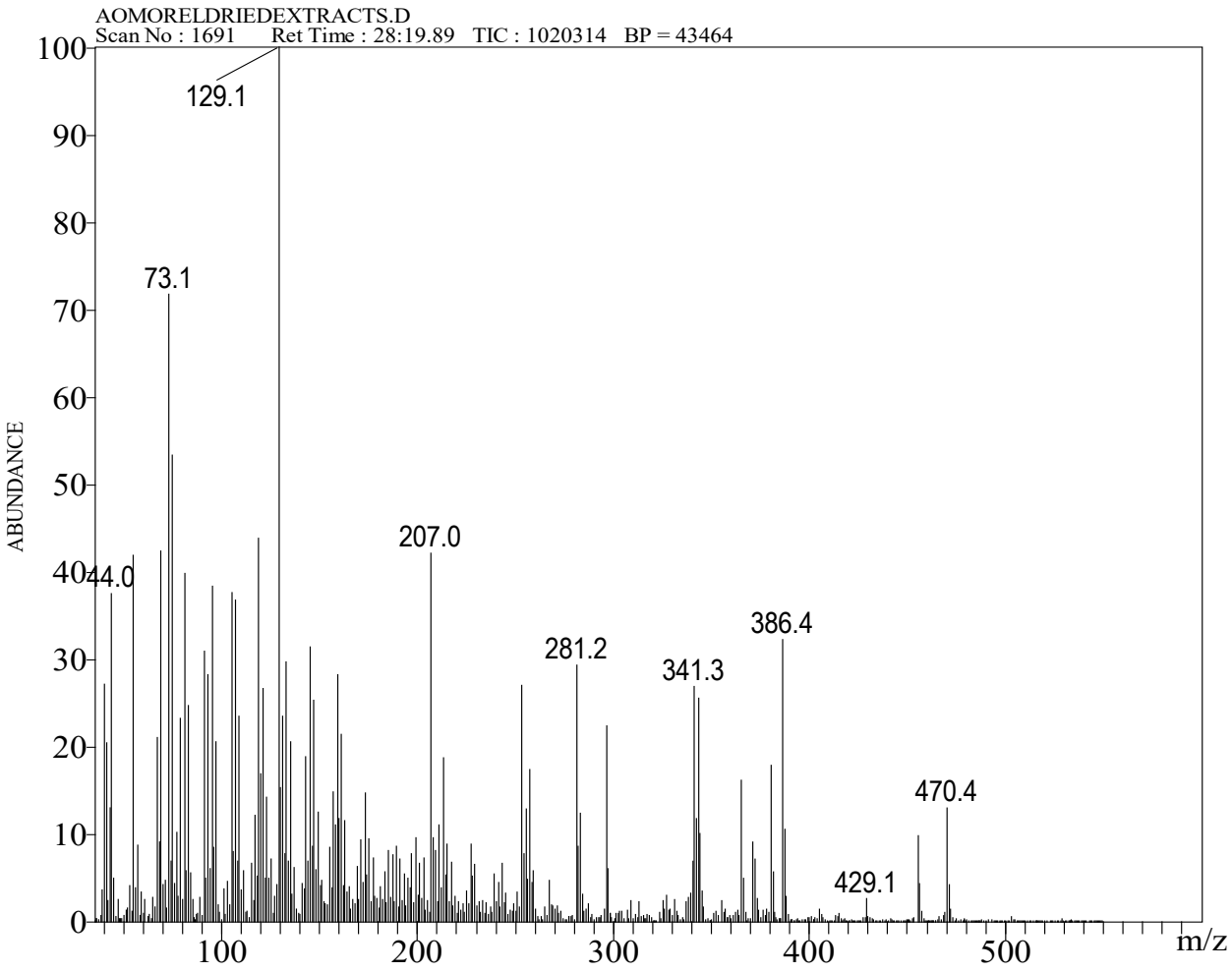


Mass spectrum for Sundried Morel Mushrooms (**13**).





Mass spectrum for Dried[a] Morel Mushrooms (**10**).



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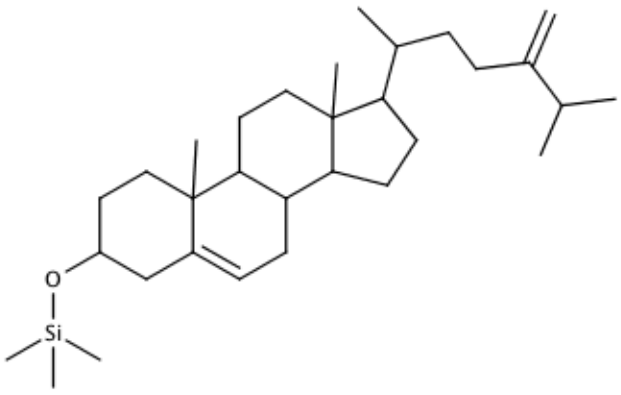
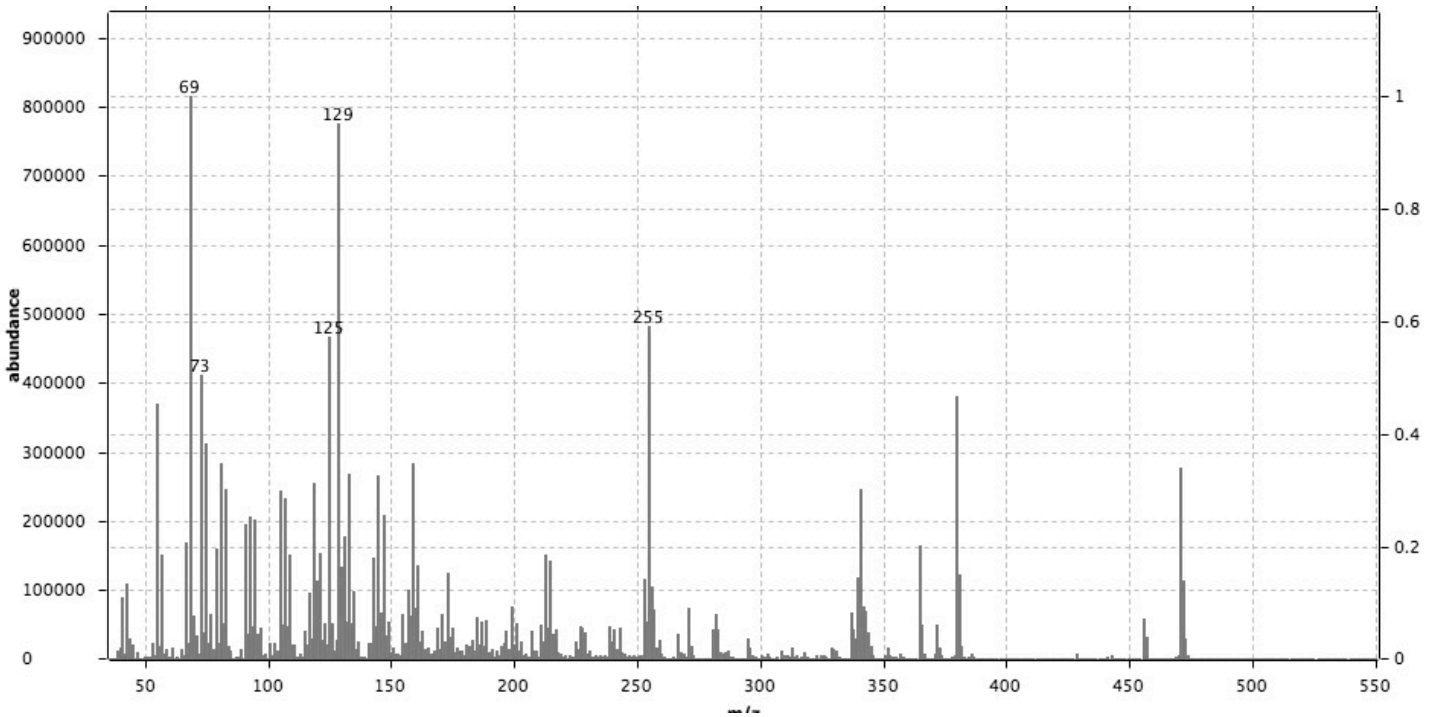
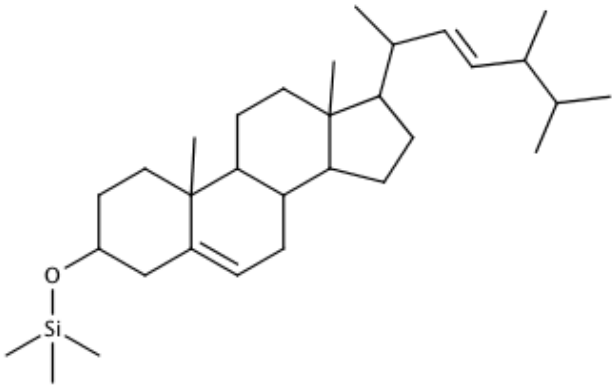
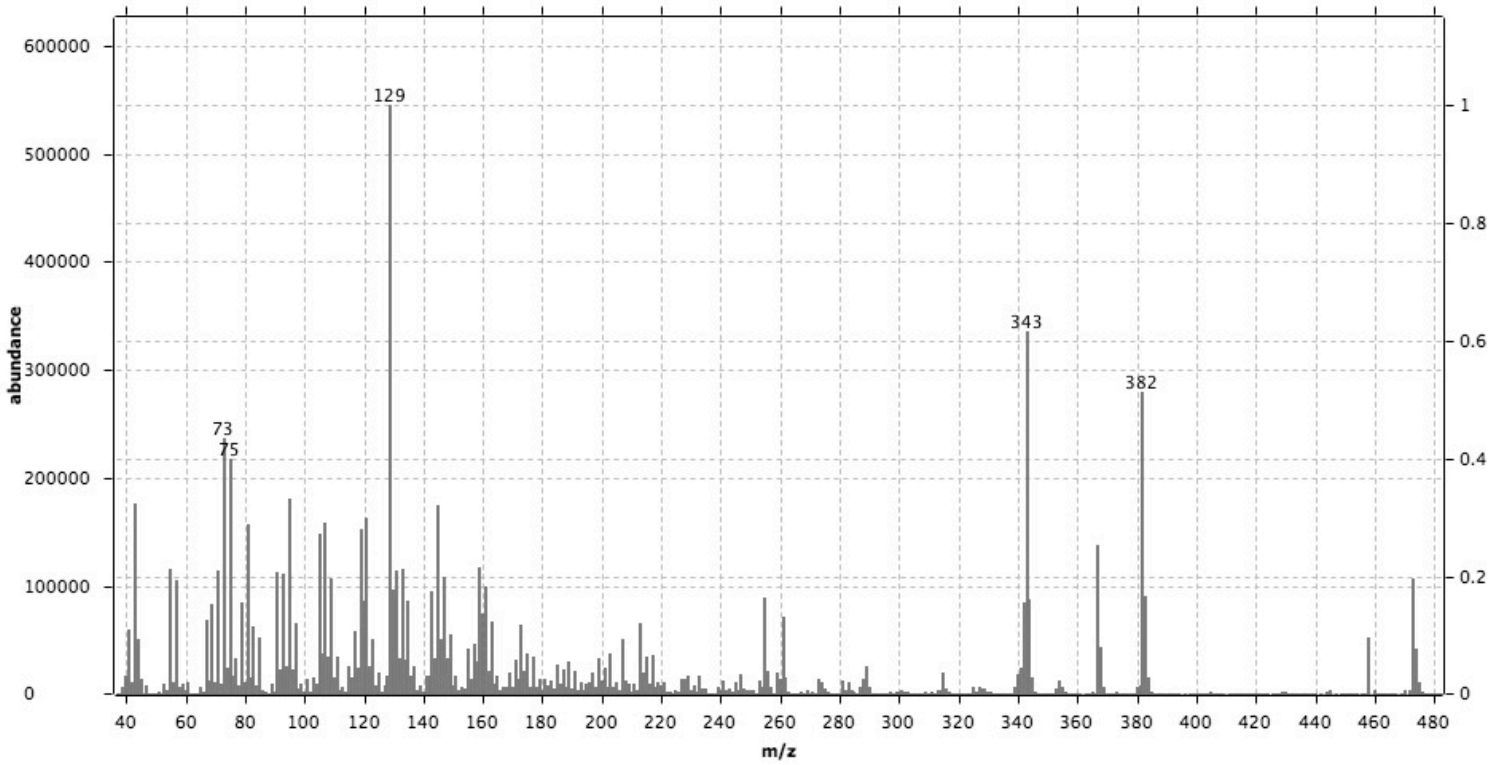


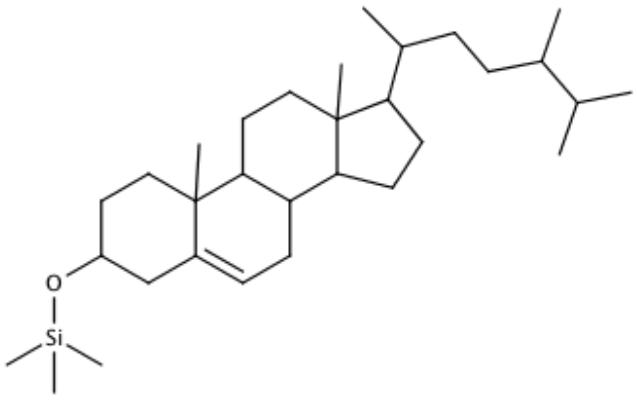
Mass spectrum for Standards (**5**).



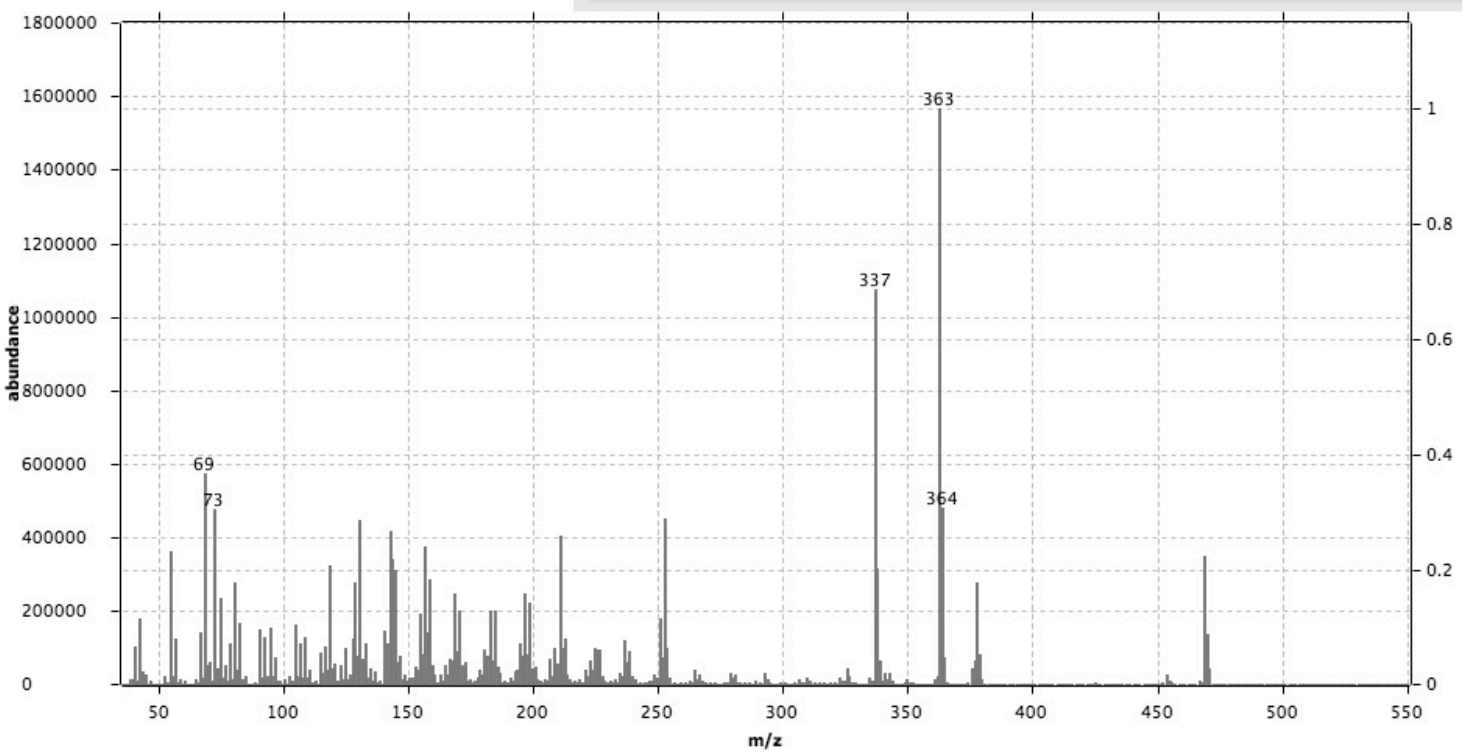


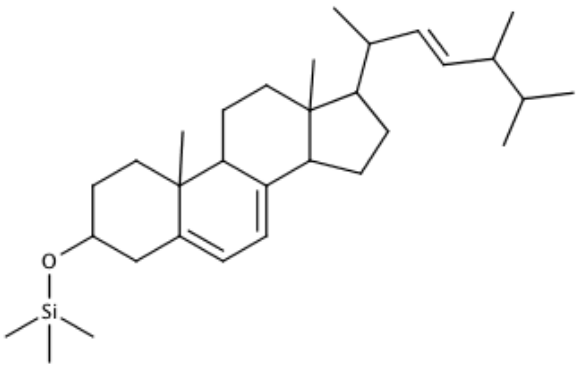
Mass spectrum for Standards (**6**).



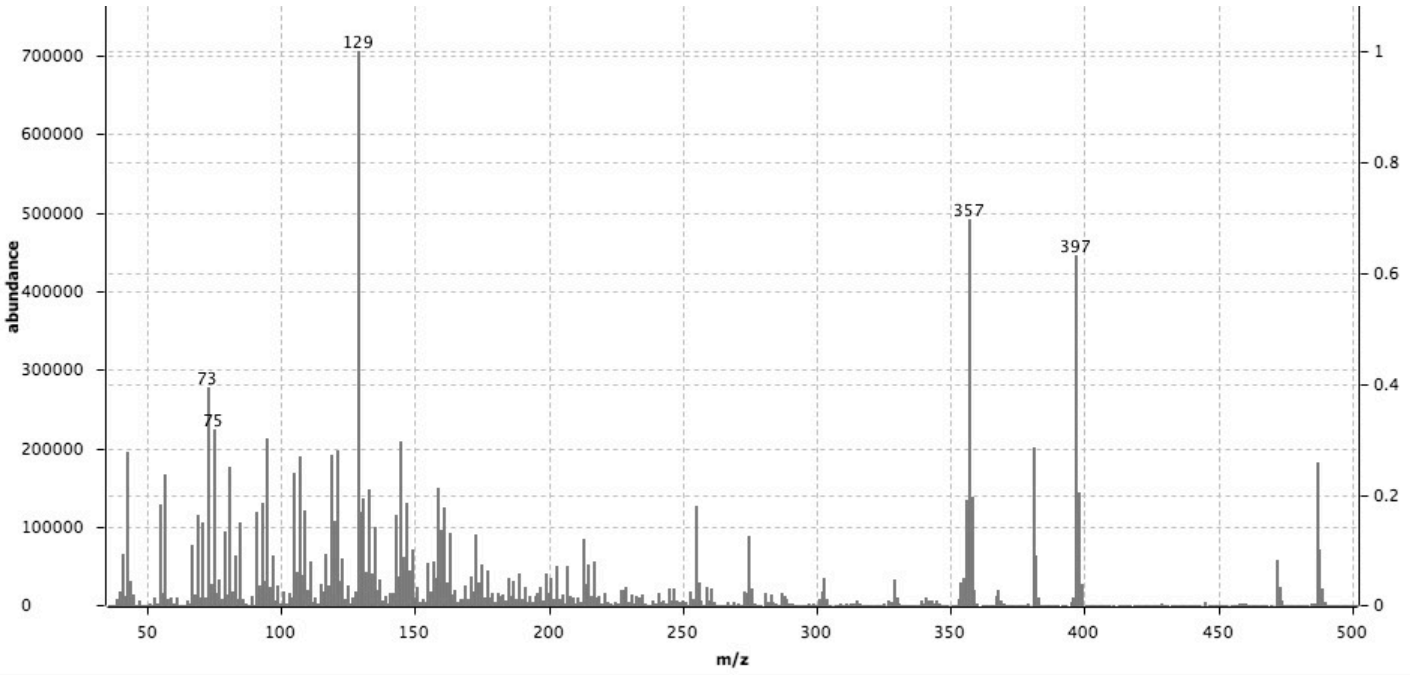


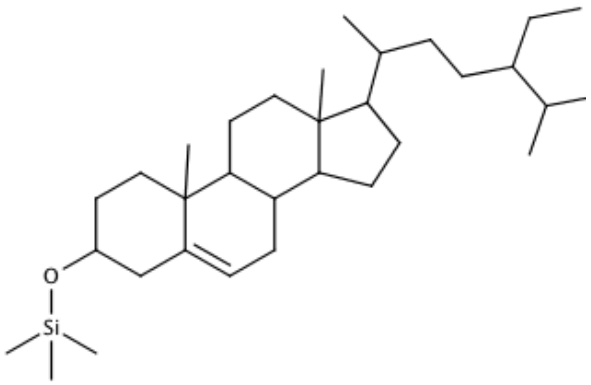
Mass spectrum for Standards (**1**).



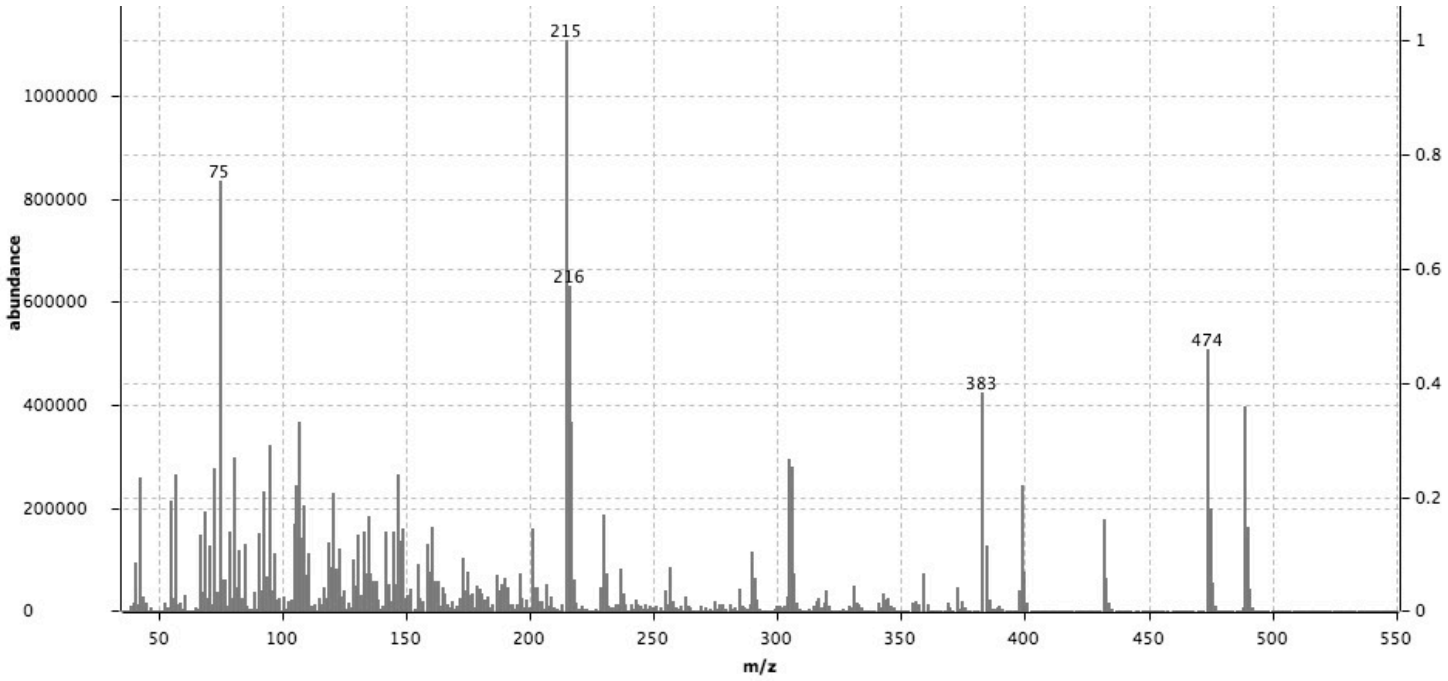


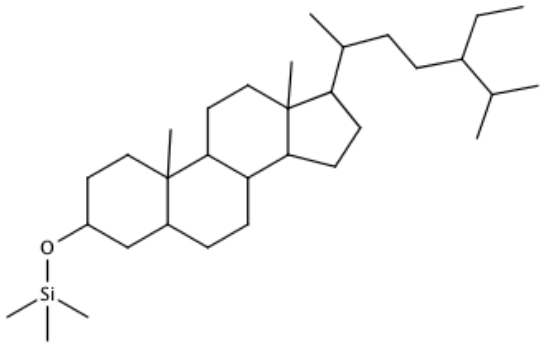
Mass spectrum for Standards (**8**).





Mass spectrum for Standards (**9**).





Mass spectrum for Standards (**7**).