

## Primary study of sterols composition of *Rhodiola sachalinensis* by using GC/MS

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## GC/MS를 이용한 고산 홍경천의 스테롤 구성에 대한 초기연구

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**요약:** 고산 홍경천에 포함된 스테로이드 화합물의 구성을 흡착 컬럼 크로마토그래피 정제와 GC/MS를 이용하여 측정하였다. 스테롤은 에탄올과 디클로로메탄을 각각 초음파와 Soxhlet의 용매로 사용하여 추출하였다. 추출물은 클로로포름과 물로 액-액 추출을 수행하여 분배하였으며, 실리카 컬럼으로 정제하였으며, BSTFA를 사용하여 silyl유도 반응을 수행하였다. GC/MS를 이용하여 고산 홍경천에서  $\beta$ -sitosterol, stigmasterol과 cycloartenol을 포함한 18가지 자유 스테롤과 9가지 포함체 스테롤을 검출할 수 있었다. 그 중에서 cholest-5-ene-3-ol, cholesterol, stigmasterol,  $\beta$ -sitosterol은 스테롤 표준품으로 확인하고 정량분석을 수행하였다. 대부분의 스테롤은 클로로포름 분액에서 검출되었고, C<sub>29</sub>는 여러 그룹 중에서 가장 많은 그룹이었다.  $\beta$ -sitosterol은 가장 많이 함유된 성분이며 상대함량은 45.94%이고, 차례로 ergost-7-ene-3-ol (11.33%), 4,14-dimethyl-ergosta-8,24(28)-diene-3-ol (7.07%), stigmasterol (6.09%), cycloartenol (5.43%)과 4-methyl-cholest-5-ene-3-ol (5.39%)이었다.

**Abstract:** The steroid compounds in *Rhodiola sachalinensis* were determined with adsorption column chromatographic purification and GC/MS. Sterols were extracted by sonication and Soxhlet with ethanol and dichloromethane, respectively. The extract was partitioned with chloroform and water using liquid-liquid extraction, and purified with a silica column after the sterols had been converted to the corresponding silyl derivatives with BSTFA. Eighteen free sterols, including  $\beta$ -sitosterol, stigmasterol and cycloartenol, and nine sterol conjugates were found from *Rhodiola sachalinensis* by GC/MS. Among them, cholest-5-ene-3-ol, cholesterol, stigmasterol,  $\beta$ -sitosterol were confirmed and quantified with sterol standards. Most sterols were presented in the chloroform part, with C<sub>29</sub> being the most abundant group in this sterol group.  $\beta$ -sitosterol was the most abundant compound with a relative content of 45.94% followed by ergost-7-ene-3-ol (11.33%), 4,14-dimethyl-ergosta-8,24(28)-diene-3-ol (7.07%), stigmasterol (6.09%), cycloartenol (5.43%) and 4-methyl-cholest-5-ene-3-ol (5.39%).

**Key words :** *Rhodiola sachalinensis*, Sterols, Derivatives, Sonication, Soxhlet, GC/MS

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## 1. Introduction

*Rhodiola*, one of the most popular traditional Chinese medicines, mainly distributes in the northern hemisphere with about 90 species recorded worldwide, of which more than 70 species are found in China. Because of its potent biological activity, *Rhodiola* has been widely used for a long time in China, Serbia, Mongolia, and Russia to increase physical endurance, work productivity, and longevity and to treat fatigue, asthma, haemorrhage, anaemia, impotence, and nervous system disorders.<sup>1-2</sup> This root has been used to treat anti-hypoxia, anti-microwave radiation, anti-oxidant, anti-cancer, enhancement of learning and memory, and to extend human life.<sup>3-5</sup> The major bioactive components in *Rhodiola* species are phenylethanol derivatives (rhodioloside or salidroside, tyrosol), flavones (quercetin, hyperoside, kaempferol, rhodiolin, rhodionin, triclin, acetylrodalgin, catechins and proanthocyanins), phenylpropanoids (rosavin, rosin and rosarin) and others including phenolic acids, ethyl gallate, ethereal oil, organic acids and lipids, etc.<sup>6</sup>

The plant sterols that naturally occur in traditional Chinese medicines commonly contain one or more double bonds in the ring structure and various hydrocarbon groups attached to the rings. Sterols and their fatty-acid esters are essentially water insoluble. The hydroxyl group is usually attached to carbon-3; it is often esterified with a fatty acid in plants. The hydrocarbon chain of the fatty-acid substituent varies in length, usually from 16 to 20 carbon atoms, and can be saturated or unsaturated. Some sterols such as sitosterol and stigmasterol in plants are degraded to alcohols by yeast, and then taken up by various organisms.

A universal role of sterols is to functions as part of membrane structures and lipids. In the metabolic pathway, sterol intermediates are precursors of other important biological compounds such as chlorophyll in plants, vitamins A, D, E, and K. Sterols are important regulators of membrane functions and metabolic pathways. Sterols are also an important part of the unsaponifiable matter of fats and oils in

the human diet.<sup>7-10</sup>

The many effective components in *Rhodiola sachalinensis* have been extensively studied in the last decades.<sup>2,11</sup> However, few studies have attempted to determine the sterol composition from *Rhodiola sachalinensis*. In this study, sterols in *Rhodiola sachalinensis* were extracted by sonication and Soxhlet, isolated by column chromatography, and then identified by GC/MS after they were converted into corresponding silyl derivatives.

## 2. Experimental

### 2.1. Chemicals and Reagents

The *Rhodiola sachalinensis* samples used in this study were collected from the Yanji Food and Drug Administration, Jilin province, China in July of 2007. *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA: 99% in 1% TMCS) was purchased from Chem Service (USA). Cholest-5-ene-3-ol, cholesterol, stigmasterol, and  $\beta$ -sitosterol standards were obtained from Steroids (USA). Ethanol, chloroform, ethyl acetate, acetone, dichloromethane, hexane, methanol, and petroleum ether were all of HPLC grade and purchased from Aldrich (St. Louis MO, USA). Anhydrous sodium sulfate and all glassware were burned at 400 °C for 12 hr and washed with solvent before use. Silica gel (75-150  $\mu$ m) was purchased from Qingdao Hailong dried silica-gel factory (Shandong, China).

### 2.2. Instrumental Analysis

The sterols were separated using a Shimadzu GC2010 (Tokyo, Japan) with DB-5MS capillary column (30 m $\times$ 0.32 mm i.d.; coated film thickness: 25  $\mu$ m). One microliter of sample was introduced in splitless mode using an autoinjector AOC-5000 from Shimadzu. The temperature of the injection port was 290 °C. The oven temperature was held at 80 °C for 1 min, then elevated to 100 °C at 20°C/min, from 100 to 200 °C at 10 °C/min, and from 200 to 300 °C at 20 °C/min, and finally held at 300 °C for 20 min. A Shimadzu QPMS 2010 mass spectrometer was interfaced with the chromatographic system at an

interface temperature of 290 °C. Helium was used as the carrier gas at a flow rate of 2.30 mL/min. Electron ionization mode was applied to the mass spectrometry analysis. Ion source temperature was 200 °C. The electron multiplier voltage was 1250 kV and the energy of ionizing electron was 70eV. Scan mode was used with sampling rate of 0.2 s.

### 2.3. Extraction

Ten grams of dry *Rhodiola sachalinensis* immersed in 200 mL of dichloromethane was extracted for 12 hr with Soxhlet equipment by six cycles per hour. Ten grams of dry *Rhodiola sachalinensis* immersed in 100 mL of 95% ethanol was extracted by

sonication at room temperature for 30 min.

### 2.4. Purification

The Soxhlet extract was concentrated to dryness by rotary evaporator. It was dissolved in 1 mL of chloroform was performed column chromatography. The collected fraction was concentrate to dryness by rotary evaporator. It was dissolved in acetone and was subjected to GC/MS for identification. To achieve the best analysis results, the four replicate analysis were performed.

After the sonication extract was filtered, 50 mL of water was added to the solution, and the solution was then concentrated to 50 mL by using a rotary evaporator.

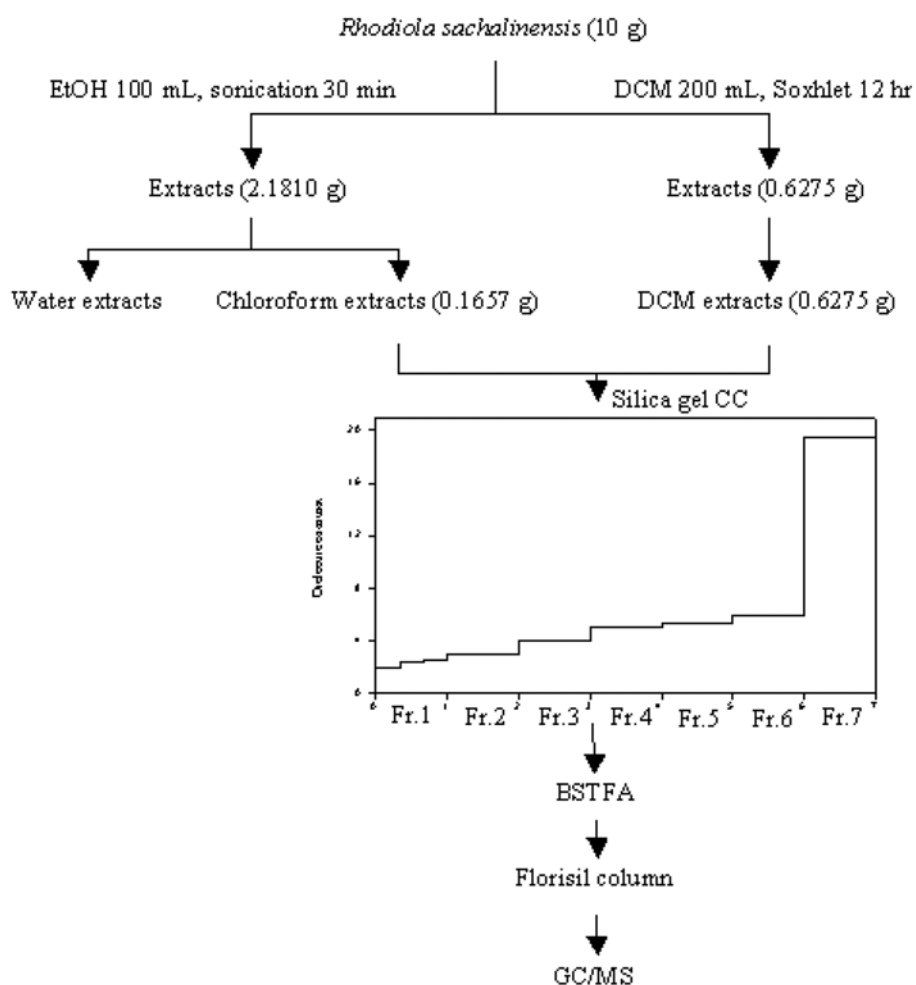


Fig. 1. Purification procedure of the sterol extract from *Rhodiola sachalinensis*.

The resulting solution was further partitioned with 50 mL of chloroform. The solution was separated into a chloroform and a water layer. The organic solutions collected were concentrated to 1 mL, for column chromatography, it was dried with anhydrous sodium sulfate.

The chloroform extracts and Soxhlet extracts were subjected into silica gel column (50 g), respectively. Solvent gradient elution was performed with petroleum ether, ethyl acetate, and methanol (stepwise, 0%-100%-0% for ethyl acetate). The solvent gradient elution program was following: petroleum ether, petroleum ether:ethyl acetate=10:1, 5:1, 3:1, 1:1, 1:3, 1:5, 0:10, and ethyl acetate:methanol=10:1, 5:1, 3:1, 1:1. And then, elution volume was fixed 50 mL. Seven fractions were obtained from chloroform and Soxhlet extracts, respectively (*Fig. 1*). These were concentrated to 1 mL with rotary vacuum evaporator. These extracts were dissolved in acetone and were injected to GC/MS after silyl derivatization by rotary vapor evaporator.

### 2.5. Derivatization

The 100  $\mu$ L of BSTFA was added to the extract for silyl derivatives. The derivatization reaction was completed after more than 30 min.<sup>12-13</sup> According to article related with clean up of sterols,<sup>13</sup> silyl derivative of extract samples were purified with florasil column kit, in which hexane was used as elution solvent, and the eluent was directly injected into the GC-MS for determination of sterols after it was concentrated to 1 mL.

## 3. Results and Discussion

### 3.1. Purification

Organic extracts from crude plants were containing the various chloroplasts, dietary fiber, protein, mineral, various vitamins, and lipids *etc.* From the dielectric constants of the solvents selected and sterols, it is easily found that the polarity of the sterols were similar with this of chloroform solvent. Base on principle of "like dissolve like", the ethanol extraction of *Rhodiola sachalinensis* was partitioned with

chloroform. And then, the water part was stored for further study. In order to obtain more pure sterol fraction, the absorption column chromatographic separation was conducted on the chloroform part.

In the adsorption chromatography, the absorbent and elution solvent were most important factors affecting on separation of target compounds from extract mixture. In the line of references,<sup>14-16</sup> silica gel was used for the separation of the organic extracts and then mixture of petroleum ether, ethyl acetate, and methanol were used as solvent gradient elution. Total seven elution fractions were obtained. Among them, most of sterols were eluted in the fourth fraction ranging from 50 to 100 mL with gradient from 50 to 75%. Total nine sterols were identified in the fraction. Those are  $\beta$ -sitosterol, ergost-7-ene-3-ol, 4-methyl-ergosta-7,24(28)-diene-3-ol, cholesterol, ergosta-5,7,22-triene-3-ol, ergosta-7,22-diene-3-ol, gorgost-5-ene-3-ol, lanost-7,9(11),24-triene-3-ol, and stigmaterol. Only two sterols such as lanosta-8,24-diene-3-ol, lanosta-9(11),24-diene-3-ol, were determined in the six fraction of chloroform extracts. Other seven sterols such as 4,14-dimethyl-ergosta-8,24(28)-diene-3-ol, cycloartenol, 4,4-dimethyl-cholesta-8,24-diene-3-ol, 4,23,24-trimethyl-ergost-22-ene-3-ol, cholest-5-ene-3-ol, ergost-8(14)-ene-3-ol, and 4-methyl-cholest-5-ene-3-ol were determined in the other fraction consisting with petroleum ether and ethyl acetate solvent gradient elution.

In summary, the silica gel column chromatography was used successfully for separation and purification of the sterols from the chloroform extract of *Rhodiola sachalinensis*.

### 3.2. Sterol chemicals and composition profiles

Eighteen free sterols (*Table 1*) were identified by GC/MS after the purification procedure. The total ion currents of these sterols are shown in *Fig. 2*. The most abundant compound was  $\beta$ -sitosterol (45.94%), which also was found as major sterol in other plant such as *Kalanchoe marmorata*, *Kalanchoe petitiiana*, *Kalanchoe daigremontiana*, and *Kalanchoe spathulata etc.*<sup>17-19</sup> Other major constituents were ergost-7-ene-3-ol (11.33%), stigmaterol (6.09%), cycloartenol

Table 1. Chemical constituents of the free sterols from *Rhodiola sachalinensis*

No.	Compound	Retention Time (min)	Relative area by sonication extraction (%)	Similarity (%)	Relative area by Soxhlet extraction (%)	Similarity (%)
1	Cholest-5-ene-3-ol	21.919	0.75	81	0.97	82
2	Cholesterol	23.875	0.91	80	1.64	79
3	Ergosta-5,7,22-triene-3-ol	24.379	0.62	66	t	-
4	Ergost-7-ene-3-ol	24.977	11.33	77	11.63	77
5	4-methyl-cholest-5-ene-3-ol	25.383	5.39	77	3.18	84
6	Ergost-8(14)-ene-3-ol	25.724	1.04	79	t	-
7	Stigmasterol	25.791	6.09	82	7.92	80
8	4,14-dimethyl-ergosta-8,24(28)-diene-3-ol	26.003	7.07	69	t	-
9	-sitosterol	26.058	45.94	80	60.30	80
10	Lanosta-8,24-diene-3-ol	26.472	3.37	80	t	-
11	Ergost-7,22-dien-3-ol	26.626	1.64	73	1.21	73
12	4-methyl-ergosta-7,24(28)-diene-3-ol	26.736	3.69	86	5.47	84
13	Cycloartenol	26.959	5.43	82	2.02	82
14	Lanosta-9(11),24-diene-3-ol	27.591	1.39	70	t	-
15	Gorgost-5-ene-3-ol	27.891	1.78	74	1.24	75
16	4,4-dimethyl-cholesta-8,24-diene-3-ol	29.276	1.12	65	0.91	62
17	Lanost-7,9(11),24-triene-3-ol	29.302	1.22	62	1.13	64
18	4,23,24-trimethyl-cholest-22-ene-3-ol	31.599	1.26	69	1.37	62

t: trace compound

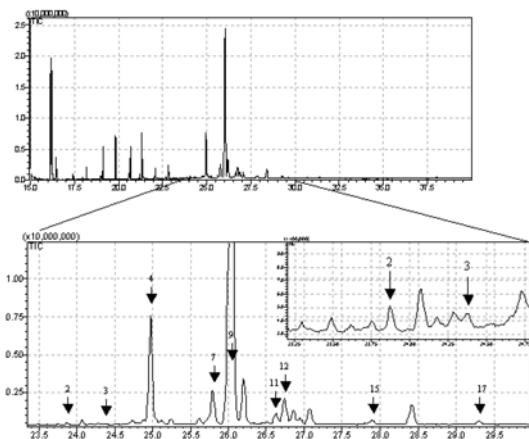


Fig. 2. Total ion currents of the several derived sterols from *Rhodiola sachalinensis*.

(2) Cholesterol trimethylsilyl ether; (3) (22E)-3-[(trimethylsilyl)oxy] ergosta-5,7,22-triene; (4) 3-[(trimethylsilyl)oxy] ergost-7-ene; (7) Stigmasterol trimethylsilyl ether; (9)  $\beta$ -sitosterol trimethylsilyl ether; (11) (22E)-3-[(trimethylsilyl)oxy] ergosta-7,22-diene; (12) 4-methyl-3-[(trimethylsilyl)oxy] ergosta-7,24(28)-diene; (15) 3-[(trimethylsilyl)oxy] gorgost-5-ene; (17) 3-[(trimethylsilyl)oxy] lanost-7,9(11),24-triene

(5.43%), and cholesterol (0.91%). These components were identified by mass spectrums. Compare the

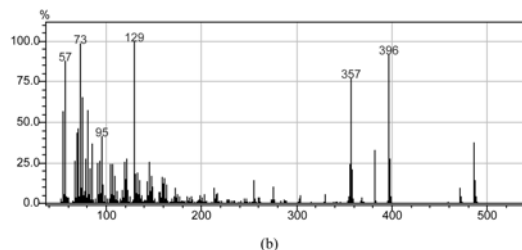
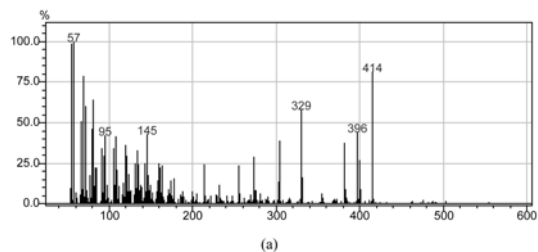


Fig. 3. Mass spectrums of  $\beta$ -sitosterol (a) and -sitosterol trimethylsilyl ether (b).

spectrums of  $\beta$ -sitosterol and  $\beta$ -sitosterol trimethylsilyl ether (Fig. 3), the ion fragment and ion intensities were different at  $m/z$  129, 414, 486, etc. Fig. 4 shows the chromatogram and mass spectrum of 4-methyl-cholest-5-ene-3-[(trimethylsilyl)oxy] in derived

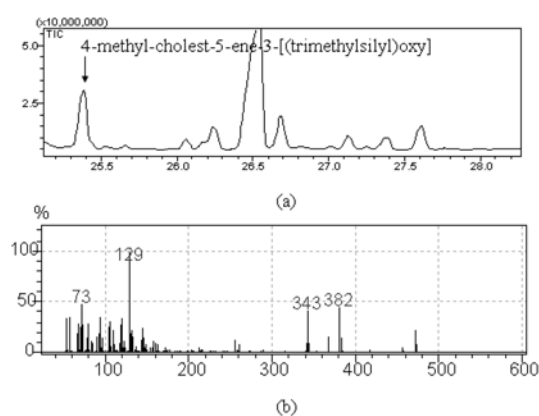


Fig. 4. Chromatogram (a) and mass spectrum (b) of 4-methyl-cholest-5-ene-3-[(trimethylsilyl)oxy].

Fr. 3 of chloroform extracts. The retention time of 4-methyl-cholest-5-ene-3-[(trimethylsilyl)oxy] was 25.383 min. The major ion fragments of 4-methyl-cholest-5-ene-3-[(trimethylsilyl)oxy] were at  $m/z$  129, 343, 382, etc. Other free sterols and conjugated sterols were identified in different fraction of chloroform extracts by mass spectrum. For example,  $\beta$ -sitosterol acetate was identified in several fractions contain the Soxhlet extracts. The retention time of  $\beta$ -sitosterol acetate was 23.758 min. The major ion fragments of  $\beta$ -sitosterol acetate were at  $m/z$  105, 131, 255, 396, etc. Most free sterols were conjugated with other components at position of 3. From these major ion peaks and ion intensities were identified 18 free

Table 2. Free sterol contents from *Rhodiola sachalinensis* ordered according to the total number of carbons (summarized peak area in percentages)

Carbon numbers	Relative peak area by sonication extraction (%) <sup>*</sup>	Relative peak area by Soxhlet extraction (%) <sup>*</sup>
C <sub>27</sub>	1.63	1.85
C <sub>28</sub>	11.69	7.84
C <sub>29</sub>	68.72	82.63
C <sub>30</sub>	17.96	7.68
Total	100	100

<sup>\*</sup>Relative contents

sterols and 9 conjugated sterols.

Table 2 lists the free sterol contents from *Rhodiola sachalinensis* ordered according to the total number of carbons. These sterol contents were relative contents. The most abundant compound was C<sub>29</sub> free sterol, which include the  $\beta$ -sitosterol. The sterols were consisted with C<sub>27</sub>, C<sub>28</sub>, C<sub>29</sub> and C<sub>30</sub>, and their composition profile is 1.63, 11.69, 68.72 and 17.96%, respectively. To relative C<sub>29</sub>, the ratios of C<sub>27</sub>, C<sub>28</sub> and C<sub>30</sub> are 42.16, 5.88 and 3.83, respectively. The ratio of C<sub>29</sub> and C<sub>27</sub> was large about forty times than this of C<sub>30</sub> and C<sub>28</sub>. The similar results were reported in seeds of the six *Adansonia* species.<sup>20</sup>

The modification of free sterols into steryl esters with fatty acids is the most abundant conjugation form in the plants, and it is of biological importance since it is related with storage of sterols.<sup>21</sup> For

Table 3. Chemical constituents of the conjugated sterols from *Rhodiola sachalinensis*

No.	Compound	Retention Time (min)	Relative area by sonication extraction (%) <sup>*</sup>	Similarity (%)	Relative area by Soxhlet extraction (%) <sup>*</sup>	Similarity (%)
1	8,9-epoxy-3-(phenylmethoxy)-cholestan-7-ol	17.647	0.47	76	0.57	78
2	Ergosta-7,22-dien-3-ol acetate	22.153	t	t	0.43	76
3	Sitgmasta-5,22-dien-3-ol acetate	23.635	0.59	78	0.53	75
4	-sitosterol acetate	23.758	2.91	86	1.94	82
5	Stigmast-5-en-3-ol olete	23.837	2.52	78	0.44	79
6	7-oxocholesteryl isocaproate	27.001	0.37	60	t	t
7	11, 18-epoxylanostan-3-ol acetate	27.384	0.14	70	0.70	68
8	Ergosterol acetate	28.155	0.16	72	t	t
9	7-dehydrocholesteryl isocaproate	29.414	0.58	75	t	t

t: trace compound

<sup>\*</sup>Relative contents

conjugated sterols in the *Rhodiola sachalinensis*, the most abundant compound was  $\beta$ -sitosterol acetate (2.91%). Next abundant compounds were stigmast-5-en-3-ol acetate (2.52%), stigmasta-5,22-dien-3-ol acetate (0.59%), and 7-dehydroxycholesteryl isocaproate (0.58%) (Table 3). As reported by Benveniste,<sup>22</sup> a large number of phytosterol and phytostanol fatty acid esters have serum cholesterol-lowering properties and are inhibitors of cholesterol absorption in human small intestine. In the some manner, the conjugated sterol with free sterol from *Rhodiola sachalinensis* will be applied to the pharmaceuticals, nutrition, cosmetic industry.

In summary, C<sub>29</sub> free sterol was most abundant compounds and high levels of nine conjugated sterol were determined from *Rhodiola sachalinensis*.

### 3.3. Confirmation

In order to confirm the sterols determined, sterol standard mixture sample GC-MS analysis and Soxhlet extraction were carried out. And the results were compared with those obtained from above experiments. The compared parameters were retention time, fragment profile, numbers and the composition profile.

Four sterol standards such as cholest-5-ene-3-ol, cholesterol, stigmasterol,  $\beta$ -sitosterol were used for confirmation of sterols identified from the *Rhodiola sachalinensis* extract. The GC-MS conditions were same with those in analysis of extract samples. Under six replicate experiments including four standards and three extract samples, the relative standard deviations (RSD, %) of retention times were 2.3, 4.5, 2.5 and 2.2% for cholest-5-ene-3-ol, cholesterol, stigmasterol,  $\beta$ -sitosterol, respectively. And the same

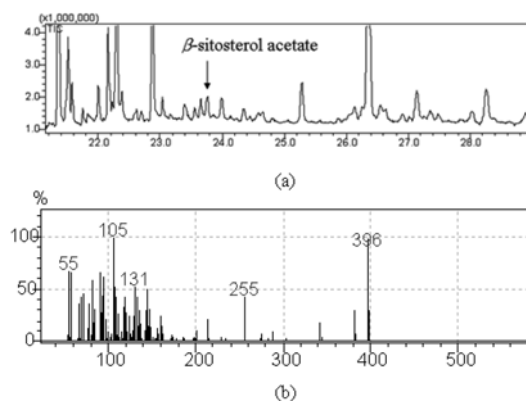


Fig. 5. Chromatogram (a) and mass spectrum (b) of  $\beta$ -sitosterol acetate.

mass spectra profiles of sterol silyl derivatives were obtained for each corresponding standards.

In order to confirm sterol numbers and composition profile, the chloroform extracts were obtained by the Soxhlet extraction. The whole experiment procedures for Soxhlet extract were same with those in the sonication experiment except extraction method. As shown in Table 1 and 3, mainly 13 and 6 kinds of free and conjugated sterols were determined from the Soxhlet extract, and the composition profile was very similar with this found in sonication extracts, respectively. This result is strongly supported by the similarity (average: 75) between sonication and Soxhlet method. In Soxhlet extract, the most and lowest abundant of compounds were also C<sub>29</sub> (82.63%) and C<sub>27</sub> (1.85%) free sterols (Table 2), respectively.

### 3.4. Comparison

*Rhodiola* plant belongs to Crassulaceae family.

Table 4. Chemical constituents of the sterols from Crassulaceae family

No.	Plants	Sterol profiles	Major compounds
1	<i>Rhodiola sachalinensis</i>	18	Sitosterol (45.94%); Ergost-7-ene-3-ol (11.33%)
2	<i>Kalanchoe pinnata</i> <sup>23</sup>	18	Stigmasta-5,24-dienol (30.20%); Sitosterol (27.40%); Stigmasterol (1.80%)
3	<i>Kalanchoe marmorata</i> <sup>17</sup>	11	24-ethylcholesterol (sitosterol, 73.50%)
4	<i>Kalanchoe petitiiana</i> <sup>17</sup>	20	24-ethylcholesterol (sitosterol, 52.00%); Cholesterol (2.20%);
5	<i>Kalanchoe daigremontiana</i> <sup>18</sup>	17	24-ethylcholesterol (sitosterol, 38.00%); Cholesterol (1.00%)
6	<i>Kalanchoe spathulata</i> <sup>19</sup>	3	Sitosterol (71.00%); Stigmasterol (23.00%)

The sterols composition of Crassulaceae family reported in literatures was hardly found. To the best of the authors knowledge, only four paper was reported that several sterols were determined from *Kalanchoe pinnata*, *Kalanchoe marmorata*, *Kalanchoe petitiiana*, *Kalanchoe daigremontiana*, and *Kalanchoe spathulata*.<sup>23,17-19</sup> Comparison and description on kinds, sterol profiles and major compounds were given Table 4. The sterol profiles of *Rhodiola sachalinensis* were similar to those of other plants belonging to the Crassulaceae family. Sitosterol was the most abundant constituent in all the plants, except for *Kalanchoe pinnata*,

#### 4. Conclusions

In this work, eighteen free sterol and nine conjugated sterols were determined for the first time from *Rhodiola sachalinensis* by sonication and Soxhlet extraction. The most abundant compound was  $\beta$ -sitosterol (45.94% in sonication; 60.30% in Soxhlet), which was also the main peak detected in fraction 4 of the chloroform extracts. The most abundant compound was C<sub>29</sub> free sterol in sonication samples (68.72%), which included  $\beta$ -sitosterol. The *Rhodiola sachalinensis* extracts had sterol profiles similar to those of other members of the Crassulaceae family; in addition, the most abundant constituent in *Rhodiola sachalinensis* was sitosterol, similar to the plants in the Crassulaceae family. These results will form a database for investigating the constituents of nature products and resources of pharmaceuticals, nutrition, and cosmetic products.

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