Metabolomika antarktičkog lišaja Stereocaulon glabrum. ispitivanje metaboličkog odgovora na rehidrataciju i sunčevu svjetlost nakon 15 godina od prikupljanja

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UNIVERSITY OF SPLIT
FACULTY OF CHEMISTRY AND TECHNOLOGY

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Ivana Ripić, 142

Sažetak: Na Antarktici, gdje vladaju ekstremni uvjeti, mogu preživjeti samo organizmi razvijenih strategija preživljavanja. Lišajevi, kao simbiotski organizmi, razvili su izvanredne mehanizme prilagodbe kako bi preživjeli te teške uvjete. U ovom radu istražuje se metabolički odgovor antarktičkog lišaja *Stereocaulon glabrum* na rehidraciju i izloženost sunčevim zrakama nakon 15 godina od trenutka prikupljanja. Uzorci lišaja, prikupljeni s antarktičkog područja, rehidrirani su pod kontroliranim laboratorijskim uvjetima i izloženi sunčevoj svjetlosti tijekom 2 i 9 dana. Dodatno, provedena je analiza dehidriranog *S. glabrum* kako bi se dobio metabolički profil nakon 15 godina čuvanja u mraku i pri konstantnoj temperaturi. U ovom radu korišten je moćni tandem LC-MS/MS kao metoda za kvalitativnu i kvantitativnu analizu sekundarnih metabolita. Ukupno su identificirana 23 sekundarna metabolita, od kojih su glavni lobarinska kiselina, strepsilin, burgeanska kiselina i atranol.

Ključne riječi: lišajevi, *Stereocaulon glabrum*, sekundarni metaboliti, LC-MS/MS

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Keywords: lichens, *Stereocaulon glabrum,* secondary metabolites, LC-MS/MS

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OBJECTIVES

- Determination of metabolic profile of lichen *Stereocaulon glabrum* in a dormant stay
- Determination of the changes of the lichen *Stereocaulon glabrum* after re-wetting
- Optimization of extraction method for the lichen sample
- Extraction of lichen *Stereocaulon glabrum* using a Soxhlet apparatus with solvents of different polarities
- Qualitative and quantitative analysis of secondary metabolites using LC-MS/MS

SAŽETAK

Na Antarktici, gdje vladaju ekstremni uvjeti, mogu preživjeti samo organizmi razvijenih strategija preživljavanja. Lišajevi, kao simbiotski organizmi, razvili su izvanredne mehanizme prilagodbe kako bi preživjeli te teške uvjete. U ovom radu istražuje se metabolički odgovor antarktičkog lišaja *Stereocaulon glabrum* na rehidraciju i izloženost sunčevim zrakama nakon 15 godina od trenutka prikupljanja. Uzorci lišaja, prikupljeni s antarktičkog područja, rehidrirani su pod kontroliranim laboratorijskim uvjetima i izloženi sunčevoj svjetlosti tijekom 2 i 9 dana. Dodatno, provedena je analiza dehidriranog *S. glabrum* kako bi se dobio metabolički profil nakon 15 godina čuvanja u mraku i pri konstantnoj temperaturi. U ovom radu korišten je moćni tandem LC-MS/MS kao metoda za kvalitativnu i kvantitativnu analizu sekundarnih metabolita. Ukupno su identificirana 23 sekundarna metabolita, od kojih su glavni lobarinska kiselina, strepsilin, burgeanska kiselina i atranol.

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ABSTRACT

On Antarctica, where extreme conditions are present, only organisms that developed specific survival strategies can survive. Lichens, as symbiotic organisms, have developed remarkable adaptability strategies to withstand these harsh conditions. This study explores the metabolic response of the Antarctic lichen *Stereocaulon glabrum* to rehydration and sunlight exposure after being preserved for 15 years since its collection. The lichen samples, collected from the Antarctic region, were rehydrated under controlled laboratory conditions and subjected to sunlight exposure for 2 and 9 days, respectively. In addition to that, analysis of dehydrated *S. glabrum* was done to obtain the metabolic profile after 15 years of being kept in dark and at constant temperature. For this study, a powerful tandem LC-MS/MS was used for the qualitative, as well as the quantitative, analysis of the secondary metabolites. In total, 23 secondary metabolites were identifed of which the major are lobaric acid, strepsilin, bourgeanic acid and atranol.

Key words: lichens, *Stereocaulon glabrum*, secondary metabolites, LC-MS/MS

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1.Introduction

1.1. Lichens

Lichen is a symbiotic association of a mycobiont, which is often a fungi, and a photobiont. Green algae and cyanobacteria most commonly act as photobionts.[1] It is estimated that there are around 20,000 different lichen species. Mycobionts present in lichens mostly belong to Ascomycetes, around 98%. The remaining 2% are Deuteromycetes and Basidiomycetes.[2] On the other hand, only a few photobionts are involved in lichen formation. The majority of them belong to *Chlorococcales* (generas *Trebouxia, Chlorella, Pleurococus, Myrmecia, Coccomyxa*). Others are *Ulotrichales* and cyanobacteria (*Nostoc, Calothrix, Gloeocapsa, Scytonema*).[2] While fungi present in lichen cannot survive independently, some photobionts occur both in free-living states and lichenized. Green-algae *Trebouxia* and *Pseudotrebouxia* are the singular photobionts only found in lichenized form.[1,3]

Some authors may argue that lichens are not an example of a symbiosis as the mycobiont is a structurally dominant partner, but mostly they are considered to be a symbiotic association in which each partner has its own role. Mycobiont provides with water and mineral nutrient and has a protective role. On the other hand, photobiont provides with sugars or other carbohydrates through the process of photosynthesis. [4]

1.1.1 Lichen morphology

Lichens consist of a thallus which is a plant body that lacks leaves, stems and roots. Lichen's thallus is made of a layered structure where layers may vary (Figure 1). Lichens can have only two layers (algal and medulla), or they can also have a protective layer on the surface, called cortex. In addition to protection, the cortex also serves as a storage for pigments that give the colour to the lichens. The algal layer is a layer where fungi's hyphae are loosely interlaced with algae. Algal layer can be located near the surface, so that light can easily reach it, or in the centre of the thallus.[3,5] Below algal layer there is a wide medulla that acts as a water and carbohydrates storage. Some types of lichens under the medulla also have a lower cortex which is attached to the attachment organs or rhizinae.[3]

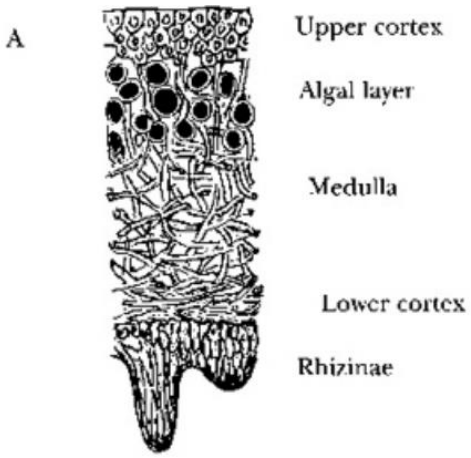


Figure 1. Layers of lichen's thallus: upper cortex, algal layer, medulla, lower cortex and rhizinae[3]

According to the shape and organization of the thallus, three types of thaulls growth can be distinguished: fruticose, foliose and crustose.

Fruticose lichens can have an upright shrubby, branchy or cup-like growth. Size of the fruticose lichens can range from several meters to a few millimetres.[1] Fruticose lichens are not necessarily attached to the substrate (e.g. *Cladonia*) but, if they are, they are either attached by the holdfast or they form cushion-like structures of separated lobes.[5] Examples of fruticose lichens are those of genera *Cladonia, Sphaerophorus* and *Stereocaulon.*

Foliose lichens are leaf-like and flat. Two types of foliose lichens can be differentiated. The ones that are only attached to the substrate with holdfast are called umbilicate foliose lichens (e.g. *Umbilicaria*). The other type, called laciniate foliose lichens, are attached to the substrate either with the lower surface or by the rhizines (e.g. *Lobaria, Parmelia*).[1,5]

Crustose is a lichen growth form that only forms upper cortex. They attach very strongly to the substrate with the hyphae of the medulla forming a crust. Examples of crustose lichens are *Lepraria, Vezdaea, Verrucaria* etc. [1,3] If crustose lichen forms elongated lobes on the edges of the thallus those lichens are then considered placodioid (e.g. *Lecanora*).[5]

Besides these three main growth types there are some intermediate growth forms. Squamulose lichens are considered the intermediate between crustose and foliose lichens. These lichens have squamules, scale-like plates that form the thallus. They can be attached to the substrate with their lower surface, like foliose lichens, or only on one edge. Like crustose lichens, they almost never form a lower cortex.

Some lichens can also have horizontal as well as vertical thallus i.e. thallus with twofold character. Horizontal thallus can only be present in the early stage of the growth and later disappear after which a lichen with a vertical thallus only remains. In the genus *Stereocaulon* a part of the horizontal thallus grows vertically which then forms into vertical thallus called pseudopodetium.

Two more subtypes of growth forms can be distinguished, hairlike lichens and gelatinous lichens. Hair-like lichens, as the names indicates, have the hair-like structure formed by thin layer of hyphae intertwined with algae (e.g. *Usnea longissima*). Gelatinous lichens have cyanobacterial photobiont which forms the thallus, hence the name (e.g. *Collema furfuraceum*).[5]

1.1.2. Lichen's resilience, survival mode/strategies

Lichens developed mechanisms to adapt to a wide range of stressors, both abiotic and biotic ones, which allows them to survive in extreme conditions, like those in the Artic, Antarctica or high altitude ecosystems. Extreme temperatures, drought, absence of light (different light intensity/exposure) or intense UV radiation are some abiotic factors that lichens are exposed to. Different level of sun exposure can result in less pigmented lichens or, in the case of foliose lichens, less branched thallus.[5,6] One of the most important characteristics of lichens is that they can live both in water rich and water poor areas. When the availability of the water is low, the poikilohydric nature of lichen is shown. Poikilohydry has been referred to as one of the two possible adaptions of plants, but also of briophytes and lichens, to drought conditions.[7] Essentially, poikilohydric organisms are Dessication-Tolerant (DT) and not drought-tolerant. This means that they have the ability to adapt their metabolism and internal structures to low-water availability situations, but also to resume their activity when water is again available. It is crucial for lichens and other poikylohydric organisms to preserve internal organ structures, specially cell integrity when water from cytoplasm is lost. And their ability to re-wet and resume metabolism it is of no less importance which can take from minutes to few hours.[8] Because of this lichens can survive long periods of drought by switching to anabiotic state. In other words, the cortex dries out and shrinks which prevents the cell damage. Polyols are thought to play a crucial role in stabilizing membranes through establishing hydrogen bonds with the hydrophilic heads of lipids outside of the membrane. This will prevent vitrification and loss of cell wall integrity. After the water conditions improve, lichens can activate their metabolism in a short period of time.[6] Lichens can also extract

moisture from rain, fog, vapor or dew and use it for the photosynthesis.[1,9] Besides poor water conditions or absence of light, they can survive in a wide range of temperatures, from tens of degrees below zero to temperatures up to 70 °C. Lichens can also serve as food to different animals (biotic stressors) which causes the change of phenotype in lichens which often leads to errors in taxonomic systematization.[5]

1.1.3. Lichen metabolites

Metabolites can be divided into primary and secondary. First ones (amino acids, carbohydrates, lipids, etc.) are a product of primary metabolism which lichen –and any other living being, needs in order to survive, for growth, reproduction and development. In addition to primary metabolites, lichens and the rest of living organisms produce a large amount of secondary metabolites. In the case of lichens, they are mostly produced by the mycobiont, while the primary metabolites are produced by mycobiont as well as the photobiont. The classical approach assumed that secondary metabolites are not critical for survival, but they represent and advantage. It was considered that many secondary metabolites are the result of ecological adaptations, helping them also with protection from insects and animals and with survival in extreme conditions. It is worth to note that the amount of secondary metabolites in lichens is highly dynamic and depends on factors such as light exposure, humidity, temperature and altitude. Generally, main secondary metabolite biosynthetic pathways are: shikimic acid, mevalonic acid and acetate-polymalonate pathways. Out of these three, most of the lichen compounds are synthesised in the acetate-polymalonate pathway. Those compounds are of aromatic nature: depsides, depsidones, monoaromatic phenols, dibenzofurans, diphenylethers, anthraquinones etc.[2,4] The general structures of these basic backbones are shown in the Figure 2.

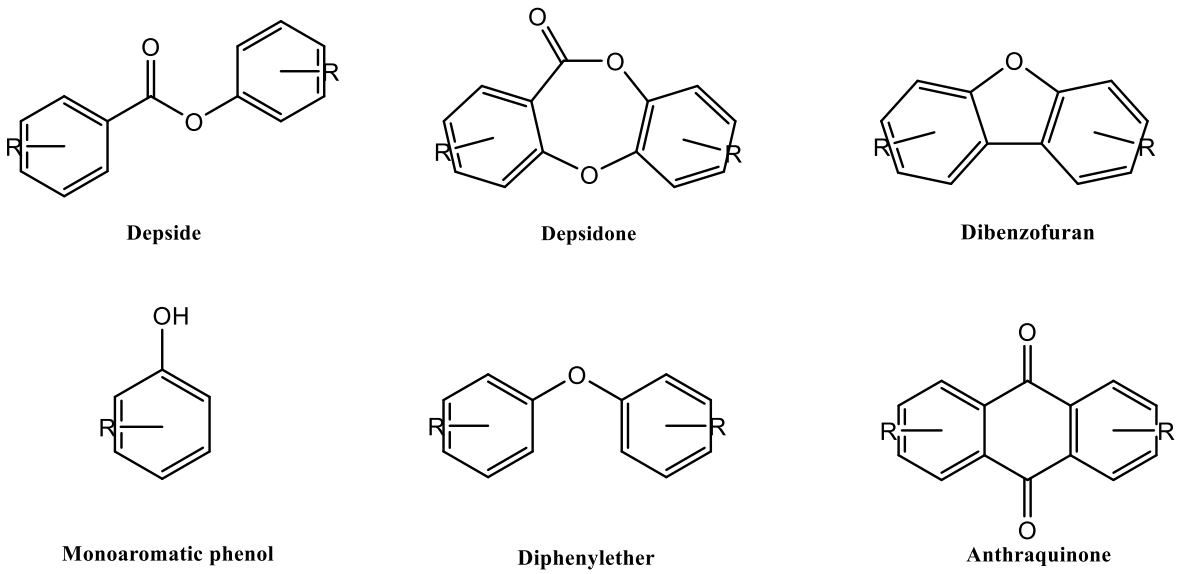


Figure 2. Structures of secondary metabolites synthesised in acetate-polymalonate pathway.

1.1.4. Antarctic lichens

The continent of Antarctica (Figure 3) is mostly covered in snow or ice, which makes it a hostile environment for any kind of life. Most of the vegetation is found coastwise or on the surrounding islands and archipelagos and consist of lichens, mosses and algae. Only two species of higher plants can be found in the temperate and snow-free zones: *Deschampsia antarctica* and *Colobanthus quitensis*.[10] Two climatic zones can be distinguished in Antarctica: the continental and the maritime zone. The continental Antarctica is a desert that includes all of the continent and the east coast of Antarctica. It has low precipitation and, as a consequence, the terrestrial life diversity is very limited, consisting mostly of mosses, algae and lichens which are the most abundant. Within continental Antarctica some regions have more diverse biota (e.g. Thurston Island, Harrow Peaks, Northern Victoria Land, etc.) and within those areas the lichen genera *Candelaria, Physcia* and *Xanthoria* are the most commonly found.[11]

In the maritime Antarctica the climate is milder, with temperatures that can exceed 0ºC during the summer, and rainfall is more common. Moreover, the climatic change has caused that episodes of temperatures between 0ºC and 5 ºC are becoming more common every year. Those conditions allow richer and more diverse biota of which lichens are the most abundant ones. Geographically, maritime Antarctica includes west coast which also include the Antarctic Peninsula, South Shetland, South Orkney and South Sandwich

Islands. Fruticose and foliose lichens are predominantly present. Some of the most common genera found in this region are *Usnea*, *Haematomma* and *Umbilicaria.*[10,11]

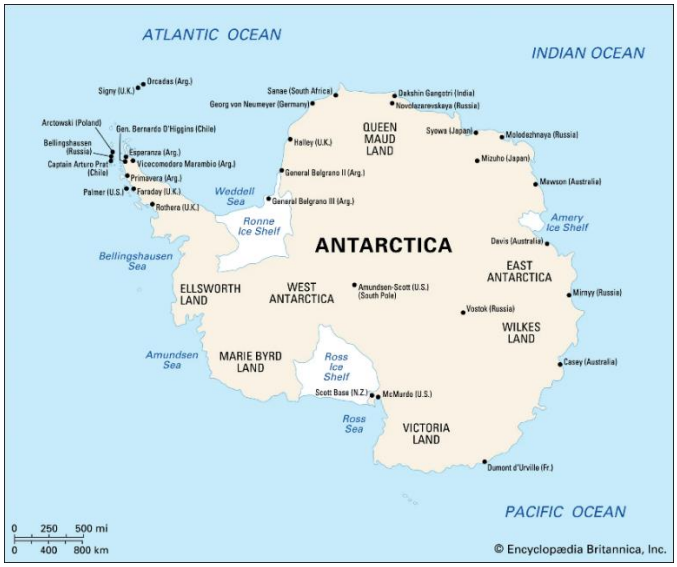


Figure 3. Map of Antarctica[12]

Some of the lichens that are found on Antarctica along with their locality (Antarctic/subantarctic region) and if they're endemic species are shown in Table 1.

Table 1. Representation of the lichens found on the Antarctica and their distribution across the world. [13]

Taxon	Antarctica/ Subantarctica	World	Endemic to Antarctica	Taxon	Antarctica/ Subantarctica	World	Endemic to Antarctica
Acarospora				***Buellia***			
A. convoluta	+		+	*B. anisomera*	+		+
A. badiofusca	+		+	*B. cladocarpiza*	+		+
A. gwynnei	+		+	*B. darbishirei*	+		+
A. macrocyclos	+		+	*B. falklandica*	+		+
Amandinea				*B. granulosa*	+		+
A. coniops	+	+		*B. grimmiae*	+		+
A. petermannii	+		+	*B. illaetabilis*	+		+
A. augusta	+		+	*B. perlata*	+		+
A. babingtonii	+		+	*B. russa*	+		+
A. isabellina	+		+	*B. subpedicellata*	+		+
A. latermarginata	+		+	***Caloplaca***			
Arthonia				*C. buelliae*	+		+
A. subantarctica	+			*C. johnstonii*	+		+
Arthopyrenia praetermissa	+		+	*C. athallina*	+		+
Austrolecia antarctica	+		+	*C. cirrochrooides*	+		+
Bacidia				*C. millegrana*	+		+
B. rhodochroa	+		+	*C. psoromatis*	+		+
B. stipata	+		+	*C. regalis*	+		+
B. tuberculata	+		+	*C. schofieldii*	+		+
Bellenerea pullata	+		+	*C. siphonospora*	+		+
Bouvetiella pallida	+		+	***Candelariella***			

Taxon	Antarctica/ Subantarctica	World	Endemic to Antarctica	Taxon	Antarctica/ Subantarctica	World	Endemic to Antarctica
Bryonora peltata	+		+	*C. flava*	+		+
				C. murrayi	+		+
Carbonea assentiens	+		+	*Lepraria straminea*	+		+
Catillaria corymbosa	+		+	*Leptogium puberulum*	+		+
Cladonia				*Massalongia intricata*	+		+
C. borealis	+	+	+	*Notolecidea ochyrae*	+		+
C. galindezii	+		+	***Pertusaria***			
C. pleurota	+	+	+	*P. corallophora*	+		+
Haematomma erythromma	+		+	*P. excludens*	+	+	
Himantormia lugubris	+		+	*P. signyae*	+		+
Huea				*Poeltidea perusta*	+		+
H. cerussata	+		+	*Porpidia skottsbergiana*	+		+
H. coralligera	+		+	*Protopannaria austro-orcadensis*	+		+
Hymenelia glacialis	+		+	***Psoroma***			
Lecania				*P. hypnorum*	+	+	
L. brialmontii	+		+	*P. saccharatum*	+	+	
L. gerlachei	+		+	*Ramalina terebrata*	+		+
L. racovitzae	+		+	***Rhizocarpon***			
Lecanora				*R. grande*	+	+	
L. dancoensis	+		+	*R. geographicum*	+	+	

Taxon	Antarctica/ Subantarctica	World	Endemic to Antarctica	Taxon	Antarctica/ Subantarctica	World	Endemic to Antarctica
L. expectans	+		+	*Rhizoplaca aspidophora*	+		+
L. griseosorediata	+		+	***Stereocaulon***			
L. physciella	+		+	*S. alpinum*	+	+	
Lecidea andersonii	+		+	*S. glabrum*	+	+	
Lecidella siplei	+		+	*S. vesuvianum*	+	+	
Thelenella antarctica	+		+	***Verrucaria***			
Trimmatothelopsis antarctica	+		+	*V. elaeoplaca*	+		+
Umbilicaria				*V. tessellatula*	+		+
U. antarctica	+		+	***Xanthoria***			
U. kappeni	+		+	*X. candelaria*	+	+	
U. decussata	+	+		*X. elegans*	+	+	
Usnea				*Zahlbrucknerella marionensis*	+		+
U. aurantiaco atra	+	+					
U. antarctica	+	+					

1.1.5. *Stereocaulon* genus

Stereocaulon genus (Figure 4) is a fruticose-type lichens distributed worldwide from tropical to both polar regions. (Antarctica and Arctic).[14] In the Antarctic region it is abundant in Antarctic Peninsula and sub-Antarctic islands, while it's poorly distributed on the continental Antarctica.[15] Lichens of this genus can grow on metal-rich soils, rocks or surrounded by moss. The genus includes around 130 species and all of them have primary crustose thallus which disappears at an early stage and secondary fruticose thallus.[16]

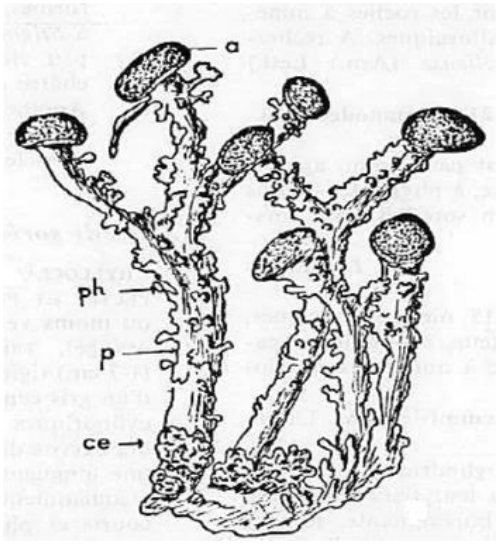


Figure 4. Morphologic characteristics of *Stereocaulon* genus. a-apothecia; ph-phyllocadia; p-pseudopodetium; ce-cephalodia. [16]

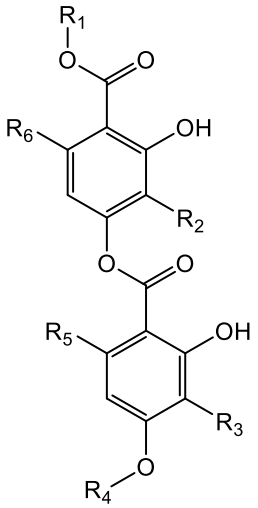
1.1.5.1. Chemical study techniques

Although it has cosmopolitan distribution and it produces a lot of secondary metabolites, genus *Stereocaulon* has not been thoroughly investigated. The chemical constituents were mostly investigated for chemotaxonomic purposes using thin layer chromatography (TLC) to identify the major compounds. This type of identification should be considered as tentative, as in some cases compounds of similar polarity cannot be distinguished, even with the use of different reagents and solvent systems. With the development of the new methods, mostly chromatographic techniques, secondary metabolites and their properties are now studied in more depth. To isolate the secondary metabolites from *Stereocaulon* species, dried samples of *Stereocaulon* lichens are usually extracted or macerated with solvents such as methanol,[17] hexane, acetone or ethyl acetate.[18] With the development of other techniques, such as HPLC, 13C and 1H NMR and MS, more and more compounds have been isolated and characterized.[19,20] More recently the tandem use of high

performance liquid chromatography (HPLC) and mass spectrometry (MS) has led to a more global approach named "metabolomics" in which the secondary metabolite's composition of the organism is studied as a whole. This approach has been successfully used in the study of proteins ("proteomics") since there are strong MS databases of proteins. However, the development of metabolomics is still in its first steps due to the lack of potent MS databases and the fact that secondary metabolites are much more smaller molecules than proteins and thus, the coincidence of a given molecular mass for more than one compound is more likely to occur. NIST library of volatile compounds is an example of a strong database suitable for use in this type of studies. However, for lichens there is no a strong library of MS data which can support the metabolomic approach, even though some advances based on Artificial Inteligence are being currently developed for lichens.[21]

1.1.5.2. Chemical composition of Stereocaulon species

Major secondary metabolites present in the genus *Stereocaulon* are depsides (Figure 5), depsidones (Figure 6), dibenzofurans (Figure 7), diphenylethers (Figure 8), monoaromatic phenols (Figure 9), steroids and terpenoids (Figure 10), plus some miscellaneous compounds (Figure 11). These compounds are known to have antimitotic [22], antibacterial [23], anti-inflammatory [24] and antioxidant [25] properties among others.



Atranorin: R_1=CH_3, R_2=CH_3, R_3=CHO, R_4=H, R_5=CH_3, R_6=CH_3

Cladoniodesin: R_1=CH_3, R_2=CH_3, R_3=COOH, R_4=H, R_5=CH_3, R_6=CH_3

Lecanoric acid: R_1=H, R_2=H, R_3=H, R_4=H, R_5=CH_3, R_6=CH_3

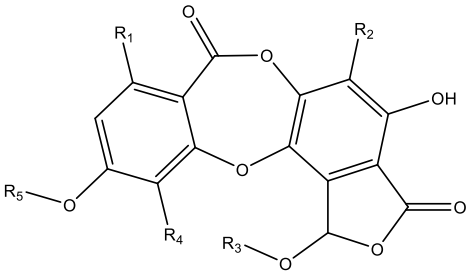
Anziaic acid: R_1=H, R_2=H, R_3=H, R_4=H, R_5=C_5H_{11}, R_6=C_5H_{11}

Perlatoic acid: R_1=H, R_2=H, R_3=H, R_4=CH_3, R_5=C_5H_{11}, R_6=C_5H_{11}

Miriquidic acid: R_1=H, R_2=H, R_3=H, R_4=CH_3, R_5=$C_2H_4COC_2H_5$, R_6=C_5H_{11}

Methyl-3-methyllecanorate: R_1=H, R_2=CH_3, R_3=H, R_4=H, R_5=CH_3, R_6=CH_3

Figure 5. Depsides found in *Stereocaulon*



Norstictic acid: R_1=CH_3, R_2=CH_3, R_3=H, R_4=CHO, R_5=H

Stictic acid: R_1=CH_3, R_2=CH_3, R3=H, R_4=CHO, R_5=CH_3

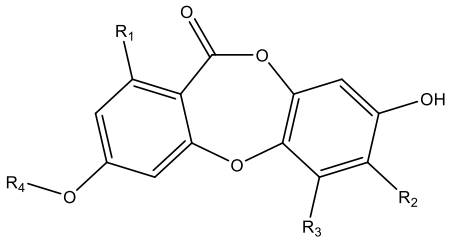
Cryptostictic acid: R_1=CH_3, R_2=CH_3, R_3=H, R_4=CH_2OH, R_5=CH_3

Menegazziaic acid: R_1=CH_3, R_2=CH_3, R_3=H, R_4=OH, R_5=CH_3

Peristictic acid: R_1=CH_3, R_2=CH_3, R_3=H, R_4=COOH, R_5=CH_3

Constictic acid: R_1=CH_3, R_2=CH_2OH, R_3=H, R_4=CHO, R_5=CH_3

Vesuvianic acid: R_1=CH_3, R_2=CH_3, R_3=C_2H_5, R_4=CHO, R_5=CH_3

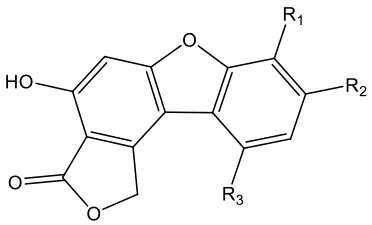


Lobaric acid: R_1=COC_4H_9, R_2=COOH, R_3=C_5H_{11}, R_4=CH_3

Norlobaric acid: R_1=COC_4H_9, R_2=H, R_3=C_5H_{11}, R_4=H

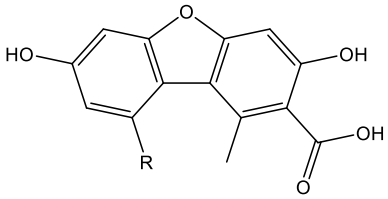
Colensoic acid: R_1=C_5H_{11}, R_2=COOH, R_3=C_5H_{11}, R_4=CH_3

Figure 6. Depsidones found in *Stereocaulon*



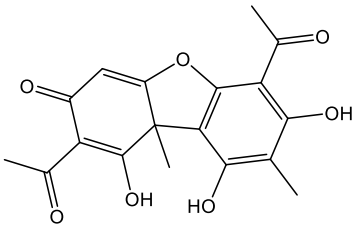
Strepsilin: R_1=H, R_2=OH, R_3=CH_3

Porphyrilic acid: R_1=COOH, R_2=CH_3, R_3=OH



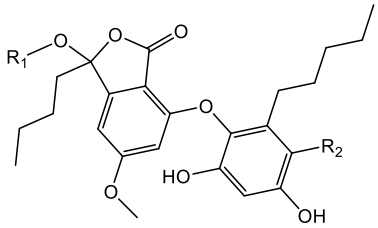
Isostrepsilic acid: R=CH_2OH

Hypostrepsilalic acid: R=CHO



Usnic acid

Figure 7. Dibenzofurans found in *Stereocaulon*

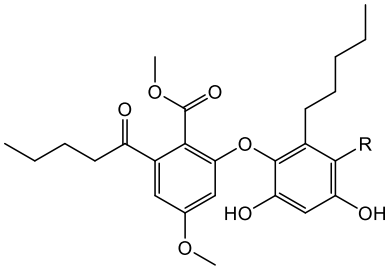


Lobarin: R_1=H, R_2=COOH

Methyl lobarin: R_1=CH_3, R_2=COOH

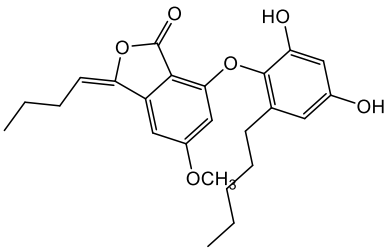
Sakisacaulon A: R_1=H, R_2=H

Methyl sakisacaulon: R_1=CH_3, R_2=H



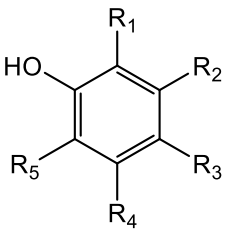
Esterified lobarin: R=COOH

Esterified sakisacaulon: R=H



Anhydrosakisacaulon

Figure 8. Diphenylethers found in *Stereocaulon*



Atranol: R_1=CHO, R_2=OH, R_3=H, R_4=CH_3, R_5=H

Methyl orsellinate: R_1=$COOCH_3$, R_2=CH_3, R_3=H, R_4=OH, R_5=H

Methyl haematommate: R_1=$COOCH_3$, R_2=CH_3, R_3=H, R_4=OH, R_5=CHO

Ethyl haematommate: R_1=$COOC_2H_5$, R_2=CH_3, R_3=H, R_4=OH, R_5=CHO

Methyl orcinol carboxylate: R_1=$COOCH_3$, R_2=CH_3, R_3=H, R_4=OH, R_5=CH_3

4,6-dihydroxy-2-methoxy-3-methylacetophenone: R_1=$COCH_3$, R_2=OCH_3, R_3=CH_3, R_4=OH, R_5=H

Figure 9. Monoaromatic phenols found in *Stereocaulon.*

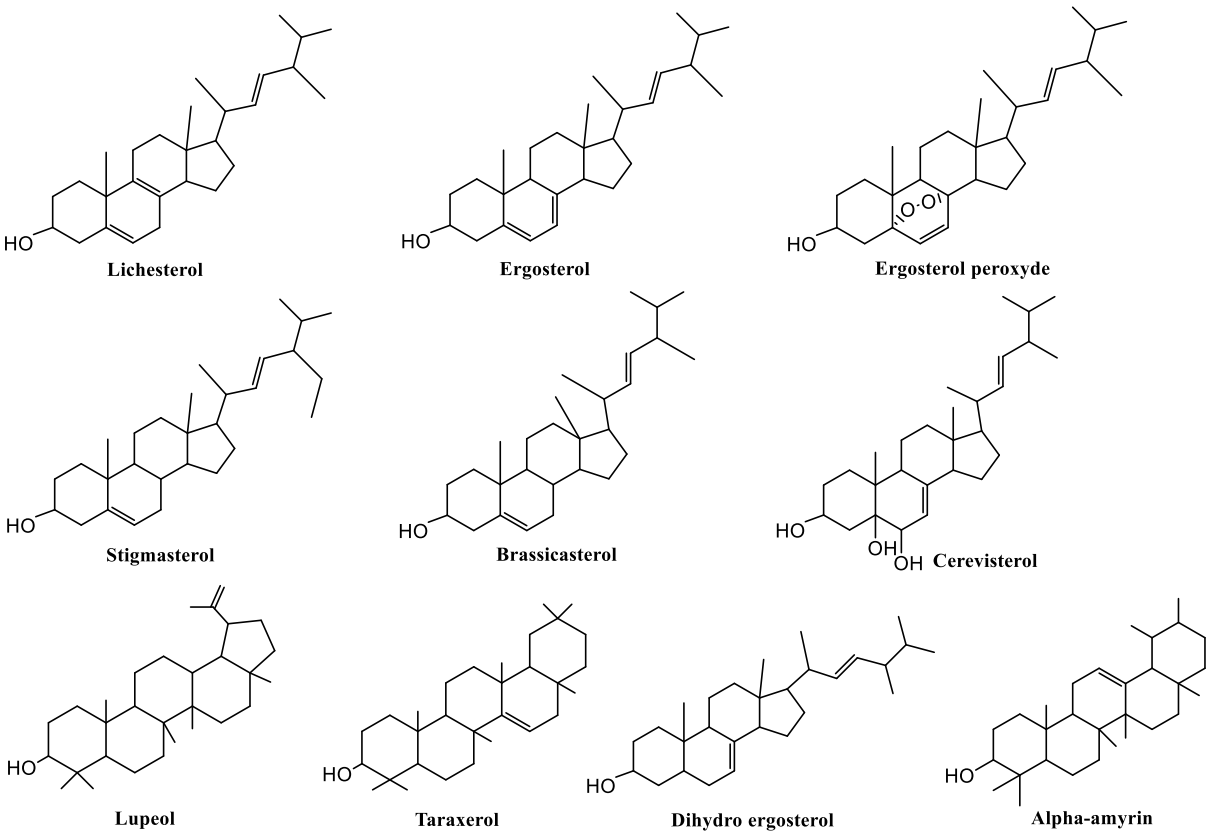


Figure 10. Steroids and terpenoids found in *Stereocaulon.*

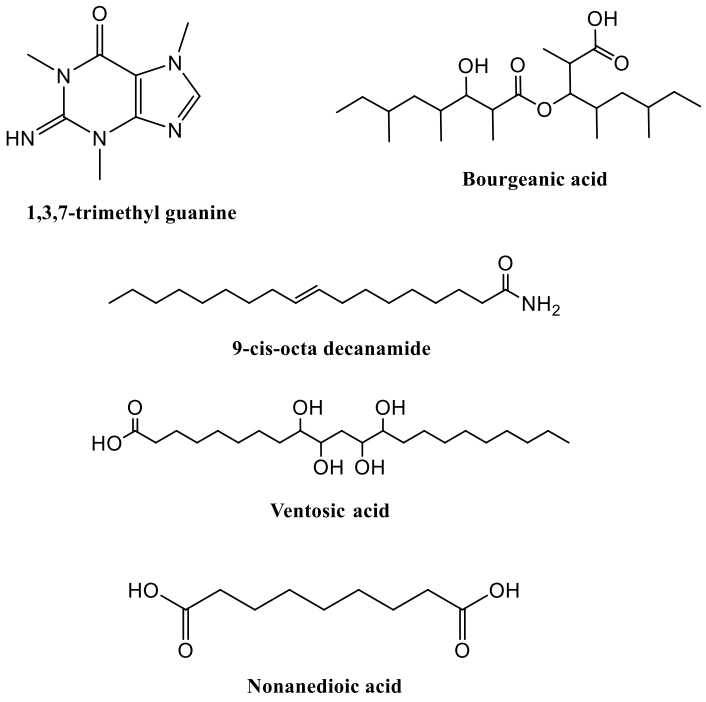


Figure 11. Miscellaneous compounds found in *Stereocaulon.*

1.1.5.3. *Stereocaulon glabrum*

Stereocaulon glabrum (Figure 12) has thallus from 5 to 7 cm high. Pseudopodetia or secondary thallus can be variable, from being little divided to being strongly divided. Stem is smooth and can be grey white to orange brown. Cephalodia contains cyanobacteria *Nostoc*. It is considered to be a homogeneous species. It grows along Southernmost South America, Tristan da Cunha Island (South Central Atlantic Ocean) and Antarctica.[11] On Antarctica it is mostly distributed on South Georgia, South Orkney Islands, South Shetland Islands and Antarctic Peninsula. It can be found in a variety of habitats: soil, moss-covered ground, rocks etc. and at different altitudes from sea level to 1200 m.[15]

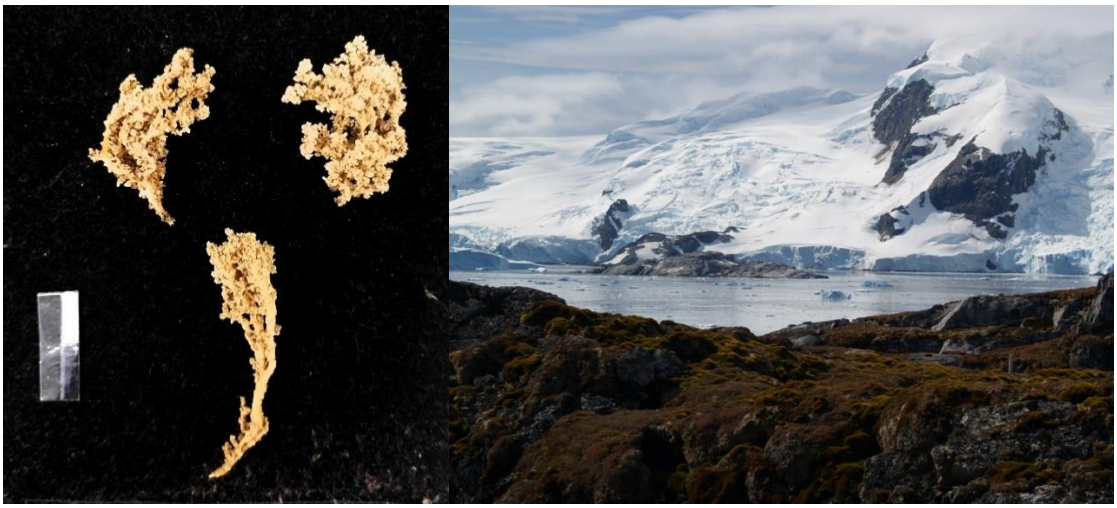


Figure 12. *Stereocaulon glabrum.* Left: Voucher specimen kept at the Dept. of Organic Chemistry, Faculty of Science, University of Cadiz; right: habitat of *S. glabrum*: Caleta Cierva (Paradise Bay), Antarctic Peninsula (Photo: © Juan Carlos García Galindo).

1.2. Metabolomics

Metabolomics is relatively young field of "omics" which focuses on the analysis of the compounds produced by the living cells or metabolome. The metabolome is defined as the complete set of metabolites produced by the cell in relation to its metabolic processes. For a compound to be considered a metabolite it needs to meet certain criteria. It needs to be synthesised by the cell and have a purposeful function in an organism, e.g. in defence. It also interacts with enzymes and through the chemical reaction alters its properties. Genomics and proteomics enhance our comprehension of the genome of all living organisms, whereas metabolomics provides a comprehensive view of the processes occurring in a living organism at a given moment.

The biggest obstacle in metabolomics is that up-to-date there is no single method that can be used for the metabolome analysis. Rather, a combination of different methods, mainly including tandem chromatographic techniques coupled with other identification techniques, such as mass spectrometry or NMR spectroscopy are commonly used. The nonexistence of a single method for the analysis makes it more difficult to compare the results between different laboratories and create a standardized procedure for this type of analysis. Also, one of the problems in analysis is that the metabolome consists of hundreds if not thousands, chemically different compounds. From high polar compounds to low polar compounds, this mixture of such different compounds makes it harder to extract and isolate them all and basically determine the complete metabolome. Within

metabolome analysis, metabolome profiling and metabolome fingerprinting can be distinguished. Metabolome profiling implies the analysis of a specific group of metabolites e.g., amino acids. On the other hand, metabolome fingerprinting implies analysis of NMR or MS spectra of all metabolites synthesised by the cell. [26]

As it is already mentioned, the diversity of metabolites poses a challenge in the analysis. The methods that are mostly used are chromatography and mass spectrometry, often together as GC-MS or LC-MS and LC-MS/MS systems. When choosing between available methods it is important to decide what kind of information is intended to be obtained with the analysis.

For the identification of secondary metabolites methods that are mostly used are chromatography (Thin-layer Chromatography, High-Performance Liquid Chromatography), mass spectrometry (MS) and nuclear magnetic resonance (NMR).

1.2.1. Chromatography

Chromatography is an analytical separation method of a mixture based on the distribution of the components of the mixture between a mobile and stationary phase. The technique allows the separation of the compounds in the mixture based on the rates at which they are carried through the stationary phase. The rates for each compound depend upon the differential affinity of each compound between the mobile and the stationary phase. The separated compounds can then be further analysed.[27]

While there are quite a few chromatography methods developed, gas chromatography (GC) and liquid chromatography (LC) are the most widely used in metabolome analysis. [26]

1.2.2. Mass spectrometry (MS)

Mass spectrometry is a broadly used technique in chemical analysis. It can be used on its own but also in tandem with some other techniques such as chromatography. Molecules are firstly ionized and then they are separated based on their mass to charge ratio (m/z) and afterwards detected. [26]

1.2.3. Liquid chromatography-mass spectrometry (LC-MS)

The LC is often coupled with MS to get the qualitative as well as the quantitative analysis while using a small amount of a sample. [28] MS is used because it acts as a selective

detector for the liquid chromatography. Combining these two methods allows the analysis of samples with a greater amount of compounds in a short period of time.

1.2.4. Nuclear magnetic resonance (NMR)

NMR spectroscopy is a powerful method that gives the information about how many distinct atoms are in a studied molecule. The criteria for an atom to be "seen" by the NMR is that its nucleus has to have a magnetic moment. Many of the nuclei can be studied with the NMR but ^{1}H and ^{13}C are the most used ones and the most interesting for the organic chemist. From the information gained by NMR, a structure of the molecule of the interest can be determinated.[29]

2. EXPERIMENTAL PART

2.1. Lichen samples

Samples of *Stereocaulon glabrum* were collected in Paradise Bay on Antarctic Peninsula. The collecting point was Highlander Point (64º 09' 00" S, 60o 53' 00" W) on 25th of January 2007. by Dr. sc. Juan Carlos García Galindo. The lichen can be found in patches in the snow-free cliffs facing the bay, and far from the intertidal zones. *S. glabrum* grows mixed with other lichen species and mosses (Figure 13). The lichen was stored in paper bags and kept in dark, at constant temperature of 15ºC and dried for 15 years.

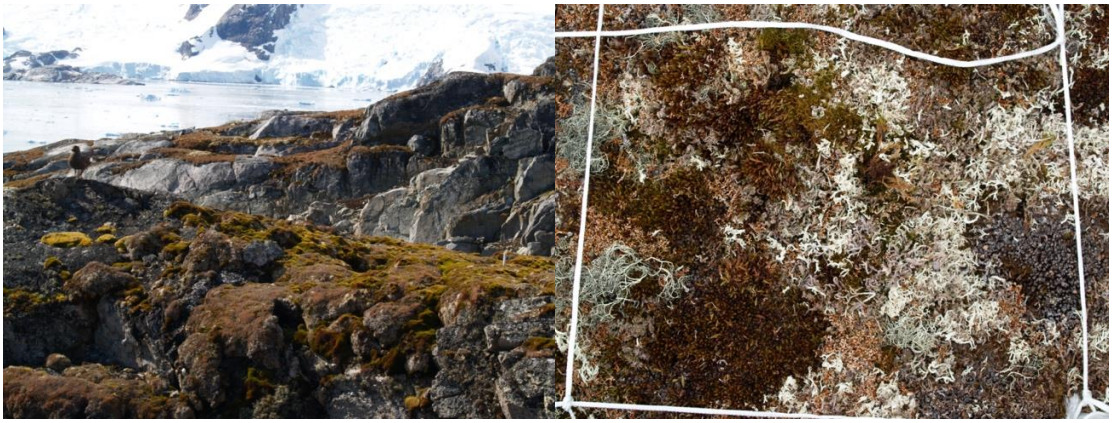


Figure 13. Left: sampling location at Caleta Cierva (Paradise Bay); right: patch of *S. glabrum* growing along with other *Stereocaulon*, lichen species and mosses. (Photo: © Juan Carlos García Galindo).

2.2. Optimization of the extraction methodology

To determine the right extraction method a preliminary study was carried out. Since the amount of the lichen is limited, there were two goals of the preliminary study:

- Selection of the best extraction methodology to obtain the better rate of secondary metabolites extraction
- Determine minimum amount of sample that will be used without compromising future studies on this species

The procedure used is as follows: 2 samples of 1g each were taken. The lichen was cut into smaller pieces and put into packets made of filter paper. For the extraction, we selected two classical methodologies that usually give good yields with non-volatile

metabolites: ultrasound assisted extraction and Soxhlet extraction. In order to obtain the whole profile of secondary metabolites, a set of solvents of increasing polarities were used, from the least polar hexane to more polar acetone and in the end isopropanol with the highest polarity out of the three.

In the available literature authors mostly used methanol for the extraction.[30] In this experiment, methanol was not used to prevent the methylation of the compounds. Since we want to know the exact structures of the present compounds, isopropanol was chosen as the solvent for the preliminary study due to its high boiling point and polarity, but also because in case of etherification or esterification, the adducts would be easily detected.

2.3. Rehydration experiments

Three samples of 1 g each of the dry lichen *S. glabrum,* were placed in glass beakers. Samples two and three were sprayed with deionized water and placed in the sun for 2 days and 9 days, respectively (Figure 14). Sample No. 1 was set as time 0 to record the secondary metabolites' lichen profile in the dehydrated stated after 15 years. When the time of each experiment was finished (0 h, 48 h and 9 days), each sample was cut into smaller pieces with the scissors and placed in a packet made of filter paper for extraction. Experiments were run in triplicate.

Figure 14. Lichens were sprayed with deionized water and placed in the sun for 2 days or 9 days.

2.4. Extraction

The ultrasound extraction of the lichen samples was performed on VWR Ultrasonic Cleaner for total of 30 minutes (Figure 15). It was done in intervals of 5 min to maintain

the optimal conditions of temperature. The samples were extracted sequentially with 45 mL of the following solvents: hexane, acetone and ethanol.

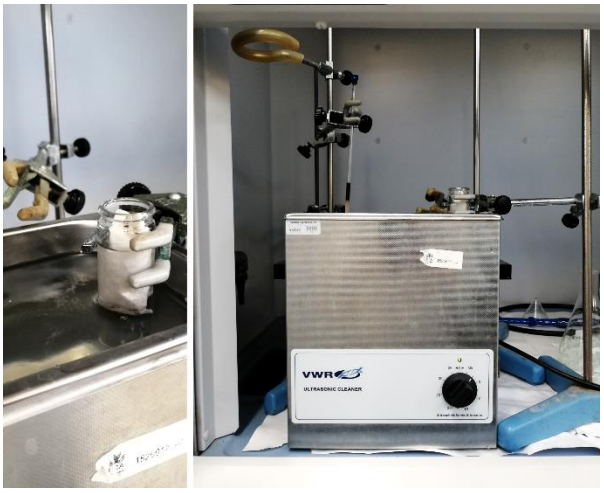


Figure 15. Ultrasound assisted extraction of the lichen.

The Soxhlet extraction of the lichen samples was carried on for 15 cycles (Figure 16). The samples were sequentially extracted with 100 mL of the following solvents: hexane, acetone and isopropanol. Isopropanol was used instead of ethanol (used before for ultrasound assisted extraction) because the solvent was not HPLC-grade and contained an important amount of additives.

Figure 16. Soxhlet extraction of the samples.

After extraction, each extract was concentrated using rotary evaporator and then transferred into vials. The hexane extracts were transferred using chloroform, acetone extracts using a mixture of chloroform and methanol. Ethanol extracts were dissolved with methanol, while isopropanol extracts were dissolved using a mixture of methanol and chloroform.

2.5. LC-MS/MS

2.5.1 LC-MS/MS sample preparation

Usnic acid (UA) was used as internal standard and a calibration curve with usnic acid was performed. The concentrations made were 0.025, 0.1, 0.25, 1, 2.5, 10 and 25 ppm. A stock solution of 25 ppm of UA was prepared by dissolving 2.5 mg of UA in 100 mL of acetonitrile. The remaining concentrations were made by successive dilutions with acetonitrile.

Sample's final concentration was set to 10 ppm, which according to previous experience of the research group (unpublished data) will give the best results in the analysis without saturation of the signal. Process was as follows:

- In a first step, samples were dissolved with different amounts of acetonitrile (ACN) to obtain a stock solution with a concentration of 25,000 ppm in water. Solution was accomplished with the aid of a water bath and an ultrasound bath whenever necessary. In the case of hexane extracts, an initial amount of chloroform was added to pre-dissolve the sample, while in the case of acetone and isopropanol extracts, they were pre-dissolved in methanol. Also, it isworth mentioning that the acetone and the isopropanol extracts didn't dissolve completely, blurriness was still present even after using water bath and the ultrasound.
- Stock solutions (25 mg/mL) were then diluted with ACN to get an initial concentration of 1000 ppm. The second dilution was made from the first one by diluting it 10 times with ACN to obtain a 100 ppm concentration.
- The final concentration, of 10 ppm was obtained by dilution with H_2O (0,1% formic acid). In parallel, a solution of UA in ACN (25 ppm) was prepared and a volume of this solution was added to obtain 2 mL of a final aqueous: ACN solution 70:30 of 10 ppm of the extract and 1 ppm of UA.

Table 2. Sample preparation procedure.

Initial extract	Stock solution (25 mg/mL) (SS)	First dilution (1000 ppm) (FD)	Second dilution (100 ppm) (SD)	Third dilution (10 ppm) (TD)
Mass=X	V(ACN)=X/25	40 µL (SS) 960 µL (ACN)	100 µL (FD) 900 µL (ACN)	200 µL (SD) 1,720 µL (H_2O, 0,1% formic acid) 80 µL (UA solution)

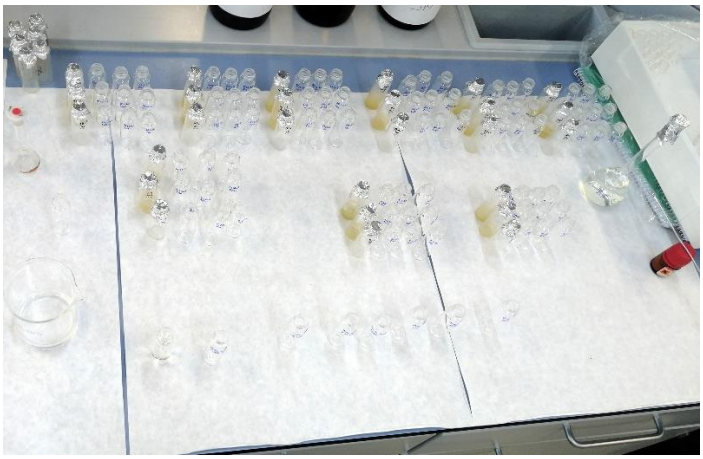


Figure 17. Preparation of the samples for the LC-MS/MS analysis.

2.5.2. LC-MS/MS analysis

LC-MS/MS was performed on QTOF XEVO-G2S WATERS instrument. Column used for liquid chromatography was BEH C18 1,7μm Acquity UPLC Waters. Injected volume was 5 μL of 10 ppm samples. Solvents used were water (0.1% formic acid) and ACN (0.1% formic acid).

Conditions of Mass spectrometer:

- Cone voltage: 25V
- Mass interval: 100-1200 m/z

3. Results and discussion

3.1. Preliminary study

After the preliminary study, the results of two different methods were compared (shown in Table 3). As it's seen in the Table 3, the larger amount of extracts was obtained using Soxhlet apparatus in the case of all three of the solvents. The limited amount of sample available prevented to run an optimization of ultrasound assisted extraction such as temperature, cycles, potency, etc. Because of that, for the future experiments that method was chosen. Also, the obtained amount of extracts was enough for the future LC-MS/MS analysis so it was concluded that 1g of sample was enough.

Table 3. Ratio of obtained extract and the sample

	Ultrasound assisted extraction	Soxhlet extraction
Mass sample	m_{s1}=1.0038 g	m_{s2}=1.0103 g
Mass of hexane extract	m_{Hx1}= 0.0119 g	m_{Hx2}= 0.0377 g
Ratio	0.0119	0.0313
Mass of acetone extract	m_{Ac1}=0.0304 g	m_{Ac2}=0.0313 g
Ratio	0.0303	0.0310
Mass of ethanol extract	m_{EtOH1}=0.1766 g	m_{EtOH2}=0.4744 g
Ratio	0.1759	0.4696

3.2. Soxhlet extraction

Results of Soxhlet extraction of *S. glabrum* are summarized in Table 4.

Table 4. Results of Soxhlet extraction.

	Hexane extract (mg)	Acetone extract (mg)	Isopropanol extract (mg)	Total (mg)
0 h –Replicate No. 1	20.9	20.3	38.2	79.4
0 h –Replicate No. 2	15.4	23.1	37.6	76.1
0 h –Replicate No. 3	19.0	21.2	33.2	73.4
Total	55.3	64.6	109.0	**228.9**
48 h –Replicate No. 1	17.7	27.1	56.9	101.7
48 h –Replicate No. 2	16.4	17.4	63.3	97.1
48 h –Replicate No. 3	15.4	26.9	61.2	103.5
Total	49.5	71.4	181.4	**302.3**

	Hexane extract (mg)	Acetone extract (mg)	Isopropanol extract (mg)	Total (mg)
9 d –Replicate No. 1	19.2	22.7	57.3	99.2
9 d –Replicate No. 2	22.0	27.1	53.2	102.3
9 d –Replicate No. 3	20.8	29.9	51.3	102.0
Total	62	79.7	161.8	**303.5**

Accordingly, the average mass obtained for each extract and condition is summarized in Table 5 and figure 18.

Table 5. The average mass obtained for each extract

	Hexane extract (mg)	Acetone extract (mg)	*Iso*propanol extract (mg)	Total (mg)
0 h	18.4 ± 2.8	21.5 ± 1.4	36.3 ± 2.7	**76.3**
48 h	16.5 ± 1.1	23.8 ± 5.6	60.5 ± 3.3	**100.8**
9 d	20.7 ± 1.4	26.6 ± 3.6	53.9 ± 3.1	**101.2**
Total	55.6 ± 5.3	71.9 ± 10.6	150.7 ± 9.1	

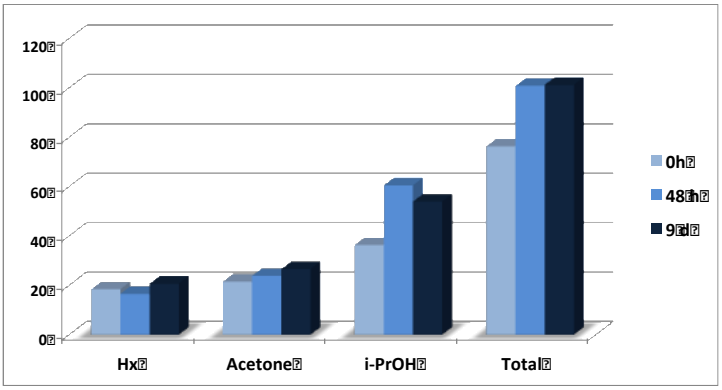


Figure 18. Average masses of secondary metabolites for each extract and total mass obtained under the three conditions tested: 0h, 48 h and 9 days.

The data clearly shows the increase of the secondary metabolites content after 48 h, which is especially clear for the most polar components (*iso*propanol extract) without a noticeable difference between the 48 h and 9 days. It is worth to note that this increment it is not observed in the hexane extract, which contains the lower polarity compounds. In the hexane extract the standard deviation does not allow to differentiate the three weights obtained. In any case, these first results are in good accordance with the literature, which shows that the period of time the lichen needs to resume their activity after re-wetting can

be a matter of few hours to 24-48 h [6] and support the hypothesis that after 15 years of being stored in dark and dry conditions the lichen is still viable and can resume its metabolic activity.

3.3. LC-MS/MS analysis

A total number of 23 secondary metabolites of *Stereocaulon glabrum* were detected using LC-MS/MS by any of the two modes used (positive or negative). Some of them were detected in the positive mode as Na-adducts. The global results are shown in the table 6 below.

Table 6. List of identified compounds detected in *S. glabrum*.

Compound/ time of re-hydration	Hexane			Acetone			Isopropanol		
	0h	48 h	9 d	0h	48 h	9 d	0h	48 h	9 d
strepsilin	+	+	+	+	+	+	+	+	+
9-cis-octadecanamide	+	+	+	+	+	+	+	+	+
bourgeanic acid	+	+	+	+	+	+		+	
lichesterol/ergosterol	+	+	+	+			+	+	
anhydrosakisacaulon	+	+	+	+	+	+	+	+	+
norlobaric acid	+		+						
methyl orcinol carboxylate/ 4,6-dihydroxy-2-methoxy-3-methylacetophenone	+	+			+	+			
stictic acid	+								
cerevisterol	+								
ethyl-haematommate		+							
colensoic acid		+	+						
lobaric acid	+		+	+	+	+	+		+
lobarin				+	+	+			
isostrepsilic acid			+	+	+	+			
miriquidic acid				+		+			
methyl lobarin/ esterified lobarin				+		+			
cryptostictic acid	+	+	+	+	+	+	+	+	+
methyl-orsellinate			+	+	+	+	+	+	+
anthraquinone	+	+			+	+		+	+

Compound/ time of re-hydration	Hexane			Acetone			Isopropanol		
	0h	48 h	9 d	0h	48 h	9 d	0h	48 h	9 d
atranol	+			+	+	+	+	+	+
nonanedioic acid	+	+	+	+	+	+	+	+	+
cladoniodesin	+	+	+	+	+	+	+	+	+
anziaic acid or sakisacaulon				+		+			

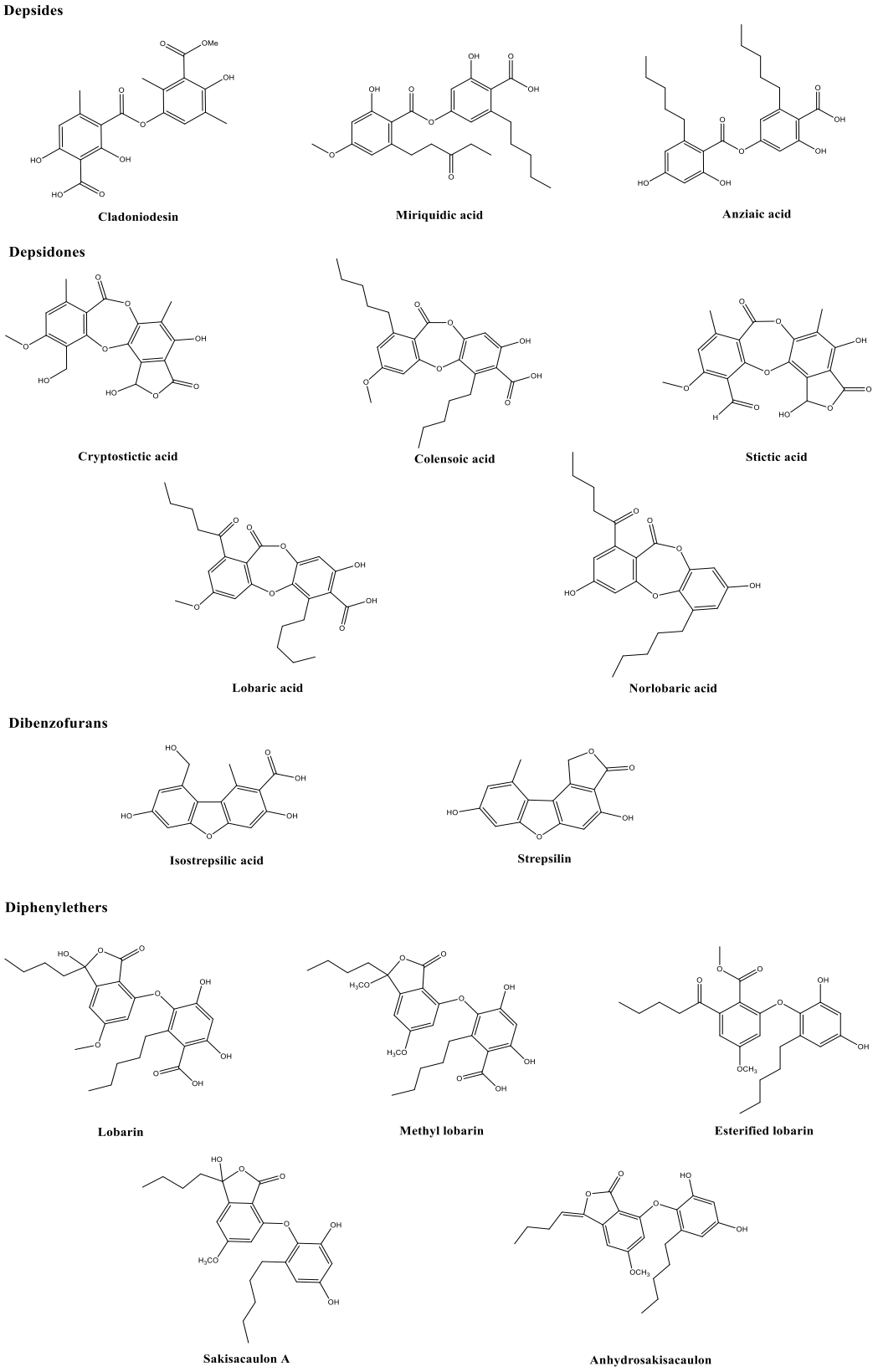


Figure 18. Depsides, depsidones, dibenzofurans and diphenylethers identified in *S.glabrum*

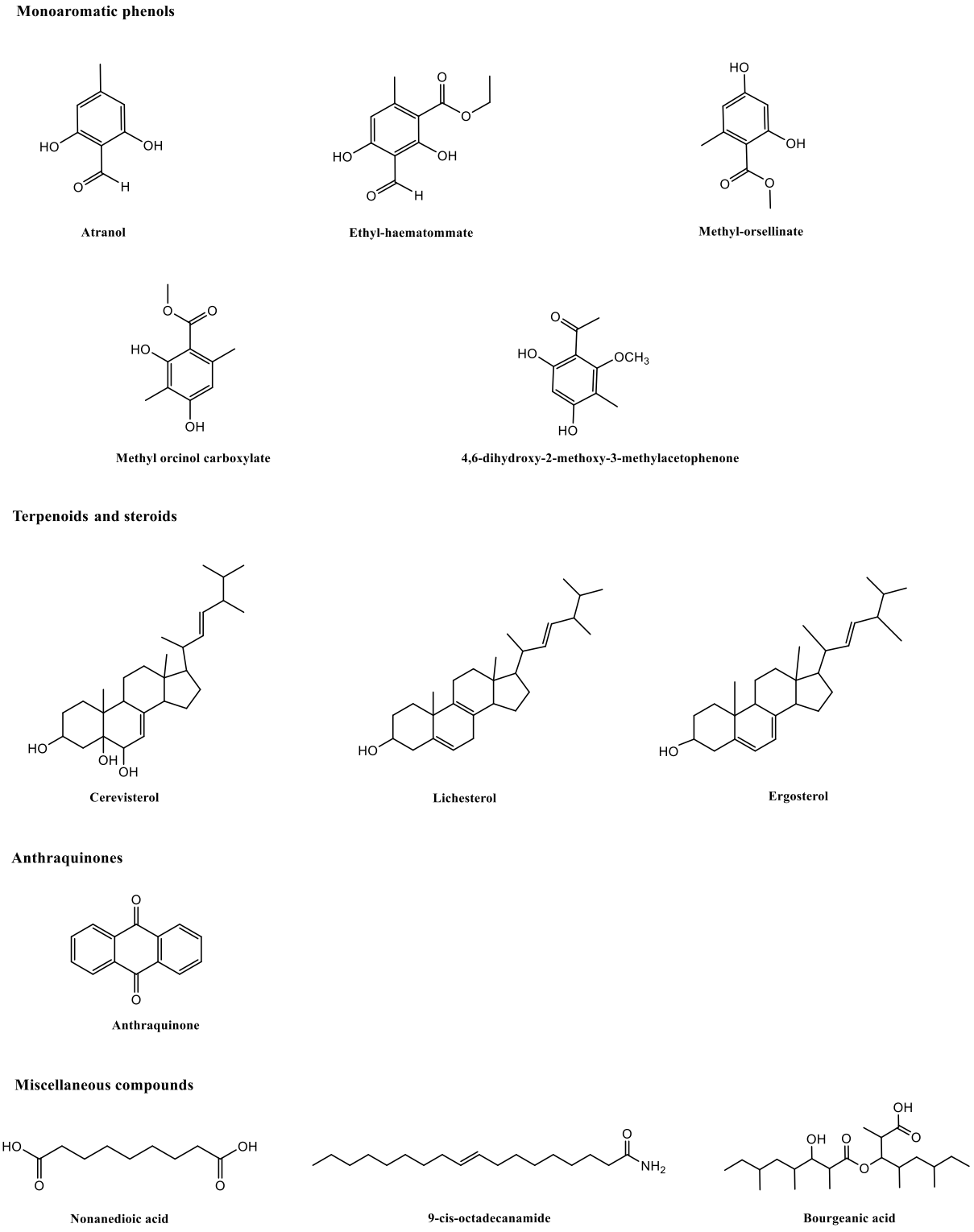


Figure 19. Monoaromatic phenols, terpenoids/steroids, anthraquinones and miscellaneous compounds identified in *S.glabrum*.

For the quantitative analysis, usnic acid was used as a standard. Calibration curve (Figure 20) was used with the range of concentrations from 0.1 ppm to 10 ppm.

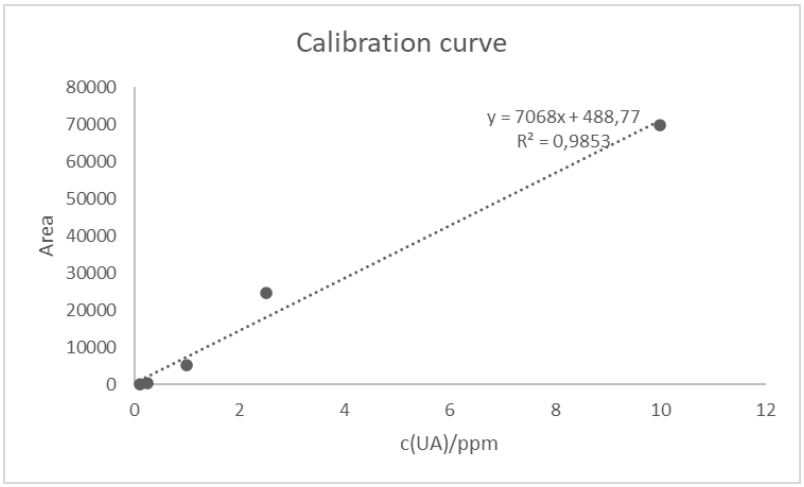


Figure 20. Calibration curve of usnic acid.

Table 7. Quantitative analysis of compounds identified in *S. glabrum*

0h sample							
Compound	Retention time (min)	Amount (mg)	% of compound in sample	Compound	Retention time (min	Amount (mg)	% of compound in sample
Strepsilin	6.94	1.24E-01 ± 0.02	0,.01%	Lobaric acid	9.87	2.09E+00 ± 0.65	0.21%
9-*cis*-octadecanamide	11.51	3.81E-02 ±0.01	<0.01%	Lobarin	9.87	9.44E-03	<0.01%
Bourgeanic acid	8.64	2.77E+00 ± 1.20	0.28%	Isostrepsilic acid	9.87	2.32E-01 ± 0.08	0.02%
Lichesterol/Ergosterol	13.00	1.07E-02	<0.01%	Miriquidic acid	9.87	1.70E-02	<0.01%
Anhydrosakisacaulon	6.93	1.47E-02	<0.01%	Methyl lobarin/Esterified lobarin	9.87	4.49E-03	<0.01%
Norlobaric acid	9.54	3.92E-03	<0.01%	Cryptostictic acid	0.23	2.43E-02 ± 0.01	<0.01%
Methyl orcinol carboxylate/4,6-dihydroxy-2-	9.97	5.26E-03	<0.01%	Methyl-orsellinate	16.37	8.17E-03	<0.01%

methoxy-3-methylacetophenone							
Stictic acid	9.97	3.68E-03	<0.01%	Anthraquinone	9.75	7.05E-03	<0.01%
Cerevisterol	10.43	4.54E-03	<0.01%	Atranol	0.22	2.1E+00 ± 0.32	0.21%
48 h sample							
Compound	Retention time (min)	Amount (mg)	% of compound in sample	Compound	Retention time (min)	Amount (mg)	% of compound in sample
Strepsilin	6.94	1.97E-01 ± 0.02	0.02%	Colensoic acid	9.77	4.59E-03	<0.01%
9-*cis*-octadecanamide	11.51	5.89E-02 ± 0.01	0.01%	Lobaric acid	9.88	1.82E-01 ± 0.01	0.02%
Bourgeanic acid	8.59	2.80E+00 ± 0.75	0.28%	Isostrepsilic acid	9.88	2.34E-02	<0.01%
Lichesterol/erogsterol	13.02	1.48E-02 ± 0.01	<0.01%	Cryptostictic acid	0.24	6.35E-02 ± 0.03	0.01%
Anhydrosakisacaulon	6.93	2.72E-02 ± 0.01	<0.01%	Anthraquinone	9.72	9.36E-03 ± 0.01	<0.01%
Methyl orcinol carboxylate/4,6-	9.94	1.06E-02	<0.01%	Atranol	0.23	3.49E+00 ± 0.42	0.35%

dihydroxy-2-methoxy-3-methylacetophenone							
Ethyl-haematommate	9.74	4.3E-03	<0.01%				
9 days sample							
Compound	Retention time (min)	Amount (mg)	% of compound in sample	Compound	Retention time (min)	Amount (mg)	% of compound in sample
Strepsilin	6.92	1.72E-01 ± 0.01	0.02%	Lobarin	9.86	1.08E-02	<0.01%
9-*cis*-octadecanamide	11.49	5.91E-02	0.01%	Isostrepsilic acid	9.86	2.50E-01 ± 0.11	0.02%
Bourgeanic acid	11.07	1.87E+00 ± 0.37	0.19%	Miriquidic acid	9.86	1.14E-02	<0.01%
Lichesterol/Ergosterol	13.02	5.53E-03	<0.01%	Methyl lobarin/ Esterified lobarin	9.86	6.86E-03	<0.01%
Anhydrosakisacaulon	6.93	2.62E-02	<0.01%	Cryptostictic acid	0.24	7.01E-03 ± 0.04	<0.01%
Norlobaric acid	9.51	3.24E-03	<0.01%	Methyl-orsellinate	16.37	6.15E-03	<0.01%
Methyl orcinol carboxylate/4,6-	9.93	6.36E-03	<0.01%	Anthraquinone	9.71	2.18E-02	<0.01%

dihydroxy-2-methoxy-3-methylacetophenone							
Colensoic acid	9.72	2.97E-03	<0.01%	Atranol	0.23	3.02E+00 ± 1.10	0.30%
Lobaric acid	9.87	1.54E+00 ± 1.54	0.15%				

The major compounds in all of the samples were bourgeanic acid, atranol and lobaric acid.

At time = 0 h major compound is bourgeanic acid, comprising 0.28% of the sample, followed by lobaric acid (0.21%) and atranol (0.21%). Chromatogram and mass spectrum are shown in Figures 21 and 22. Besides the above-mentioned compounds, strepsilin (0.01%) and isostrepsilic acid (0.02%) were also quantified and present in significant amounts, while the rest of compounds shown in Table 7 were present only in traces.

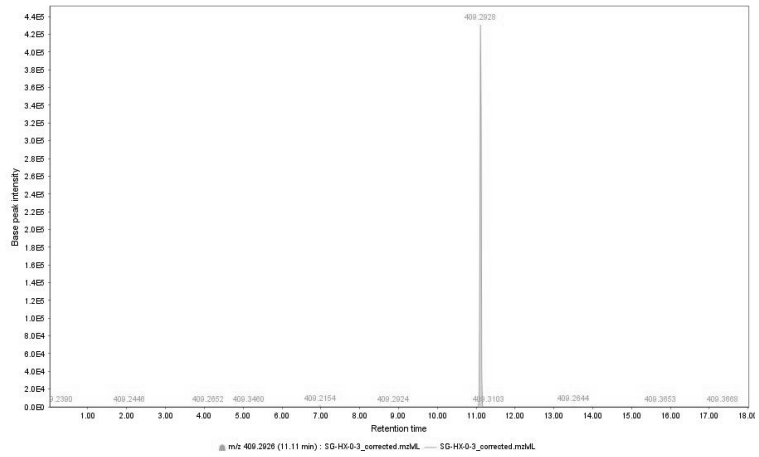


Figure 21. Chromatogram of a bourgeanic acid detected in positive mode as [M+Na].

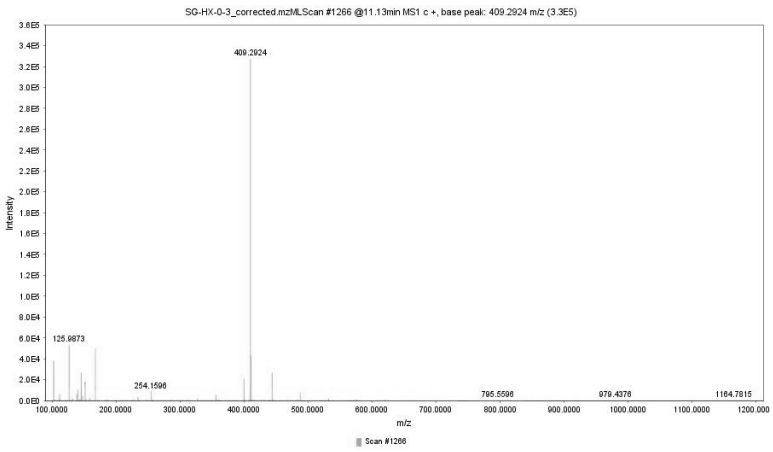


Figure 22. Mass spectrum of bourgeanic acid detected at m/z 409.2924.

At t = 48 h, atranol was the major compound comprising 0.35% of the sample. It was detected in a positive mode as a Na-adduct (Figure 23) at m/z 175.0598 (retention time = 0.23 min). Bourgeanic acid (0.28%) was the second major compound. Lobaric acid (0.02%), strepsilin (0.02%), 9-*cis*-octadecanamide (0.01%) and lobaric acid (0.01%) were also detected in amounts that were quantified. The rest of compounds were detected as traces.

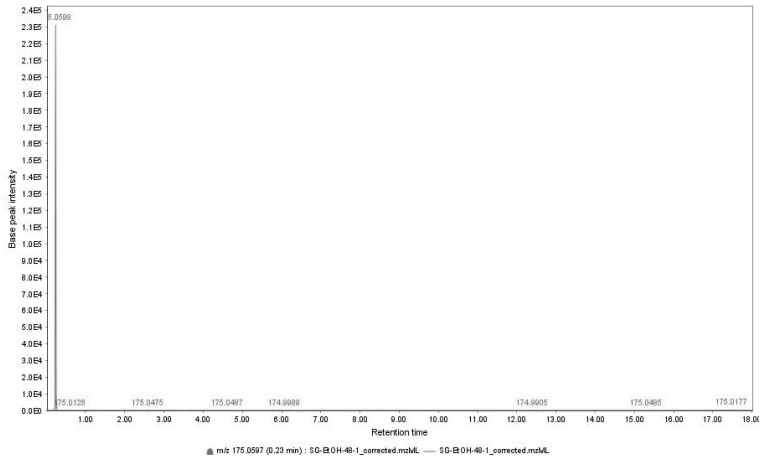


Figure 23. Chromatogram of atranol detected as [M+Na].

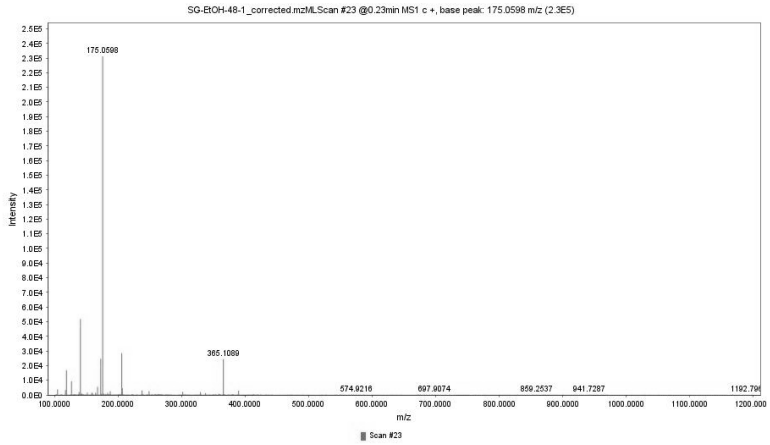


Figure 24. Mass spectrum of atranol with m/z 175.0598

Finally, at t = 9 days, the major compound was again atranol (0.30 %), with bourgeanic acid being the second major compound (0.19%). Lobaric acid was detected as the third major compound as [M+H], but also as Na-adduct, at retention time of 9.87 min, comprising 0.15% of the sample. The chromatogram and mass spectrum are shown in Figures 25 and 26. Other significant compounds detected are isostrepsilic acid (0.2%), strepsilin (0.02%) and 9-*cis*-octadecanamide (0.01%).

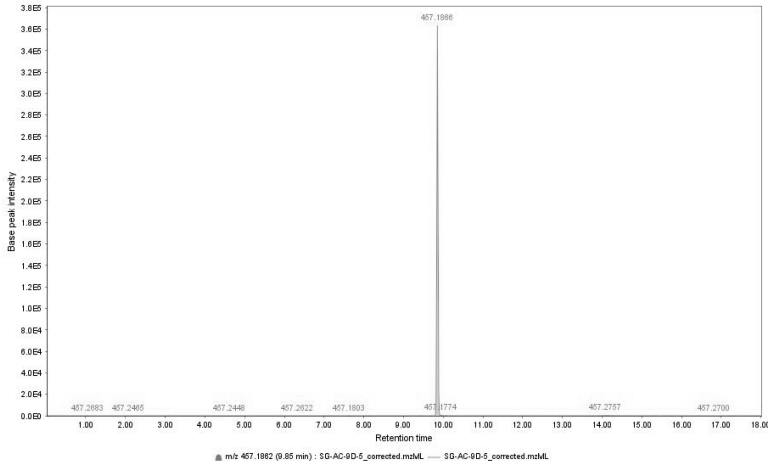


Figure 25. Chromatogram of lobaric acid detected in positive mode as [M+H].

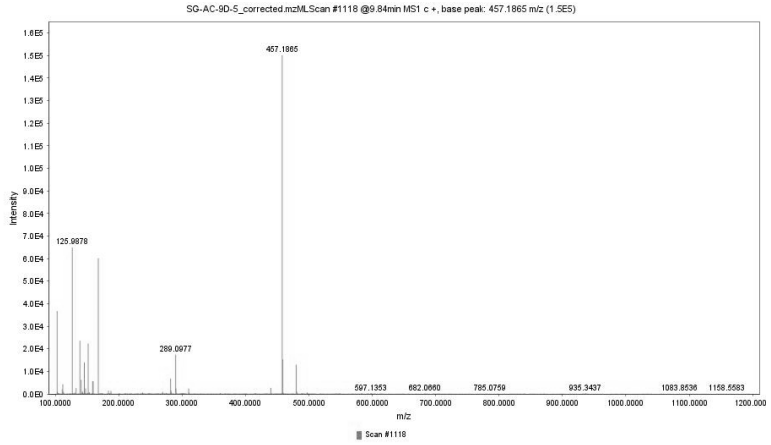


Figure 26. Chromatogram of lobaric acid with m/z 457.1865.

The spectra of other detected compounds can be found in Supplementary.

4. Conclusions

In this study, three sets of experiments were performed in order to determine how long does it take for a lichen to resume the biosynthesis of secondary metabolites after being kept in dark and at constant temperature for 15 years. Each sample was extracted with solvents of different polarities to obtain the complete metabolic profile. For qualitative and quantitative analysis, LC-MS/MS was used. In total, 23 secondary metabolites were identified.

- At the initial time (t = 0 h), 18 compounds were identified, which can be set as the original state of the *S. glabrum*. Bourgeanic acid is the major compound, followed atranol and lobaric acid. Strepsilin and isostrepsilic acid were also present in significant amounts.
- In the first treatment (t = 48 h) after exposure to sunlight and moisturing for 48 hours, 13 compounds were identified and the total amount of major compounds mostly remained the same as in the first case. However, the profile changed, being now atranol the major compound. Cryptostictic acid and 9-*cis*-octadecanamide were now detected in amounts that could be quantified. Strepsilin remained at the same level, but isostrepsilic acid was now detected is traces.
- In a lichen sample exposed to sunlight for 9 days, 17 compounds were identified with slightly increased amounts of some major compounds (atranol, bourgeanic acid, lobaric acid) which is in good accordance with the hypothesis that the lichen is resuming is metabolic activity.

To summarize, *S. glabrum* can resume its metabolic activity after the re-wetting and exposure to sunlight. The amount of secondary metabolites increases after re-hydration and exposure to sunlight and the metabolomic study shows that the profile of secondary metabolites detected changes as the lichen resumes its activity.

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6. Supplementary

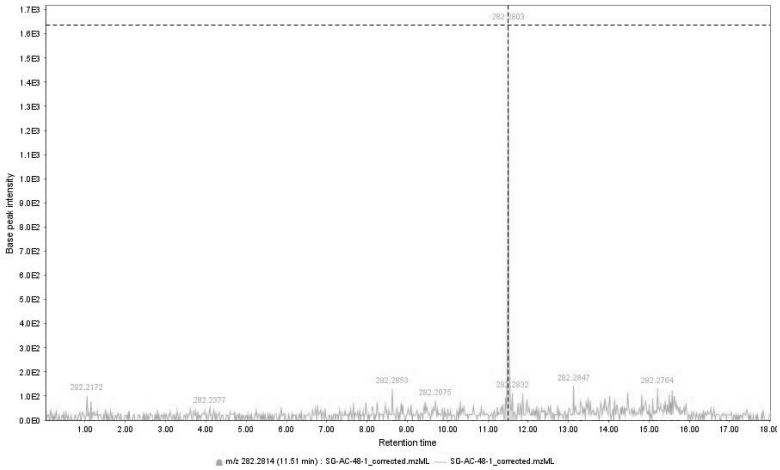

Figure S1. Chromatogram of 9-*cis*-octadecanamide.

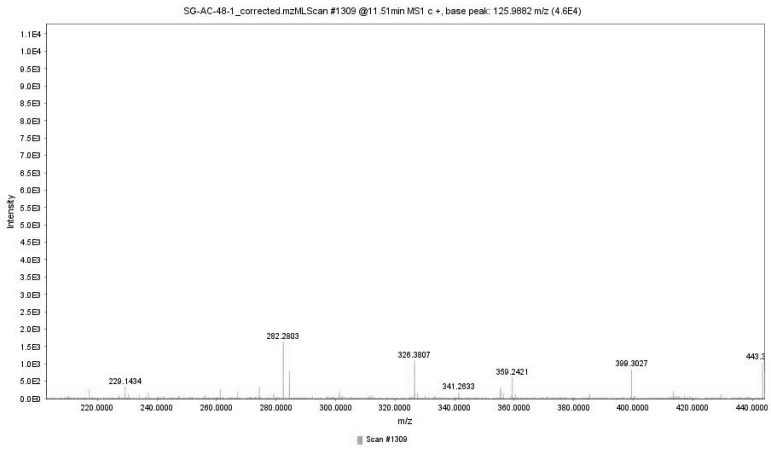

Figure S2. Mass spectrum of 9-*cis*-octadecanamide.

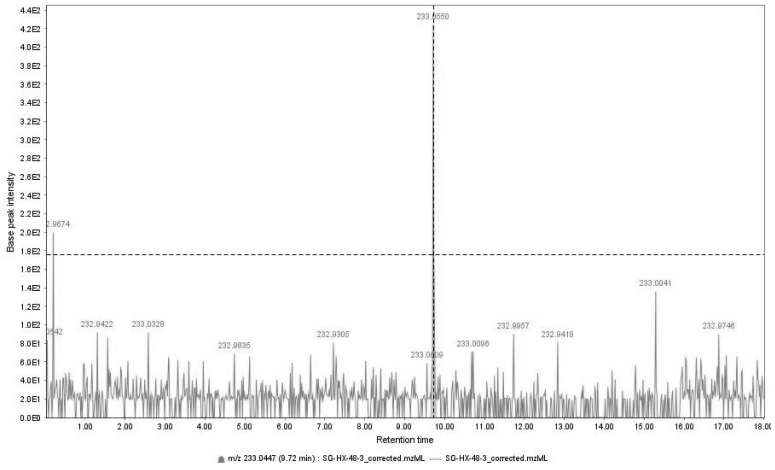

Figure S3. Chromatogram of anthraquinone detected as [M+Na].

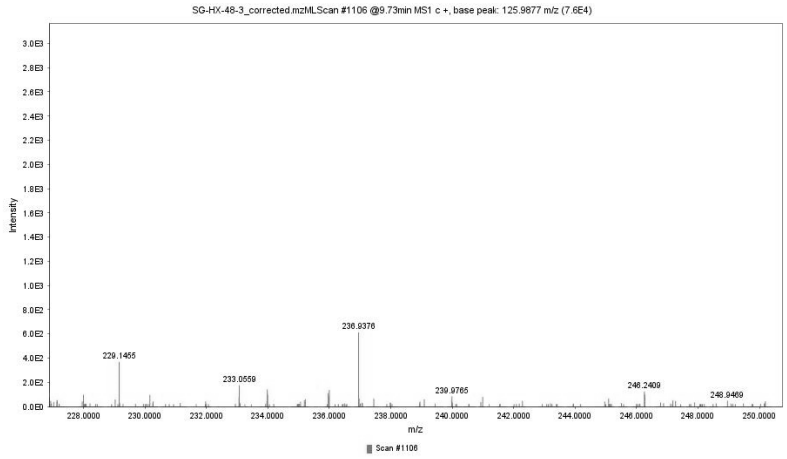

Figure S4. Mass spectrum of anthraquinone with m/z 233.0559.

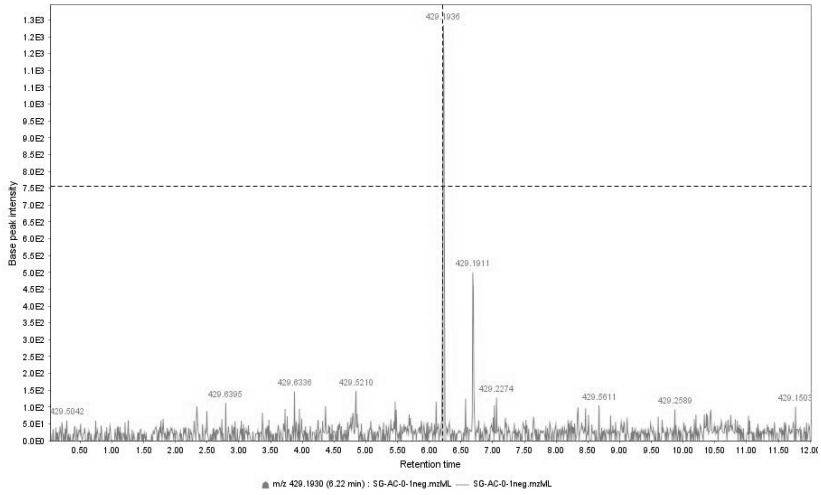

Figure S5. Chromatogram of anziaic acid or sakisacaulon A detected as [M-H]

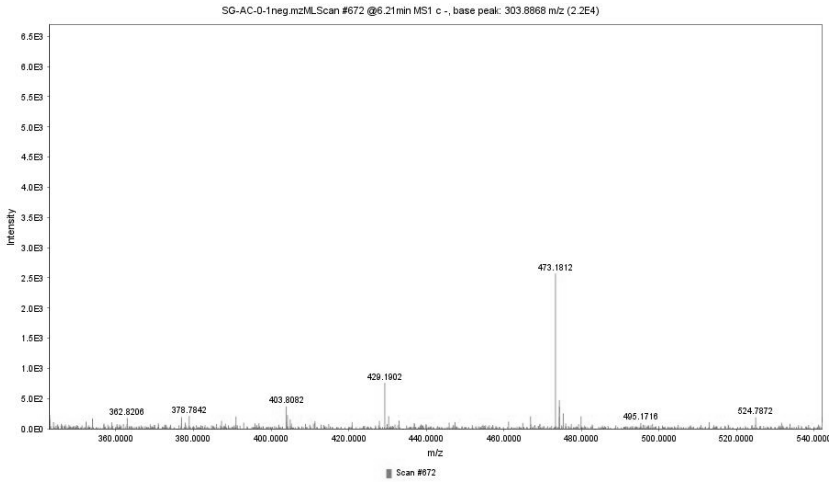

Figure S6. Mass spectrum of anziaic acid or sakisacaulon A with m/z 429.1902.

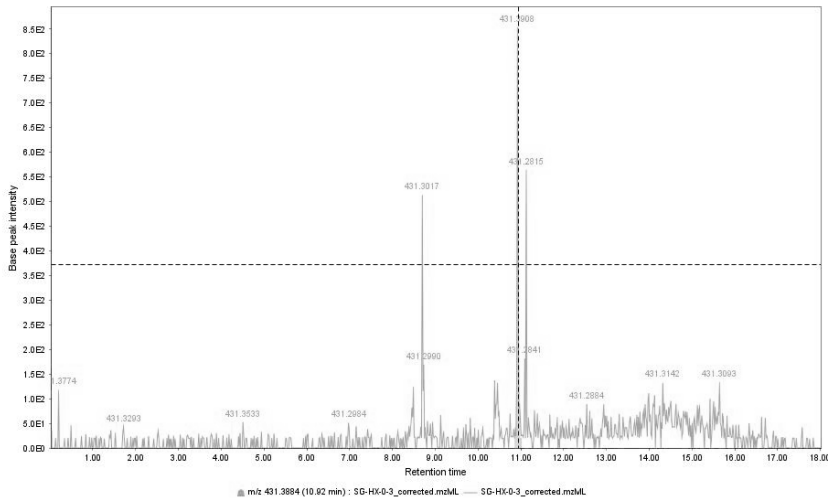

Figure S7. Chromatogram of cerevisterol detected as [M+H].

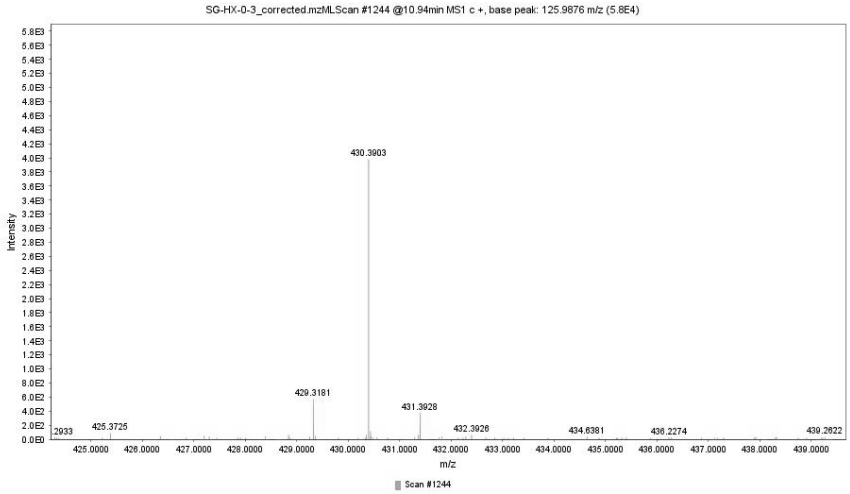

Figure S8. Mass spectrum of cerevisterol with m/z 431.3928.

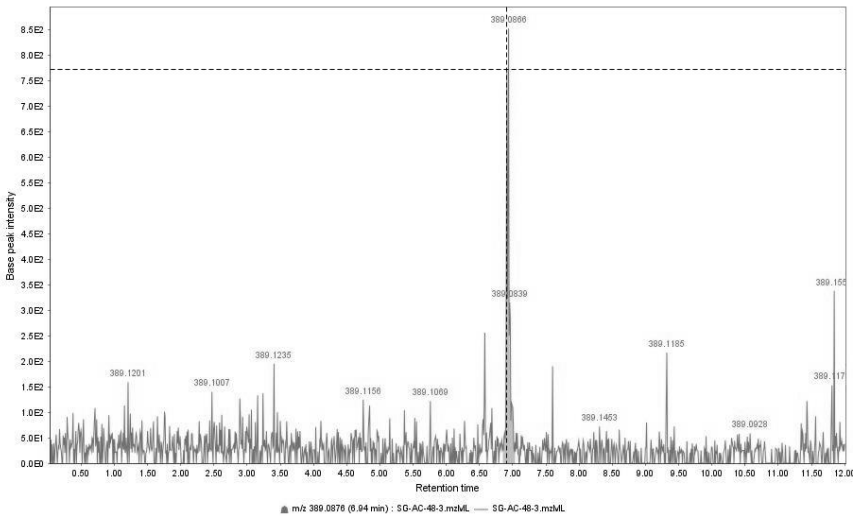

Figure S9. Chromatogram of cladoniodesin detected as [M-H].

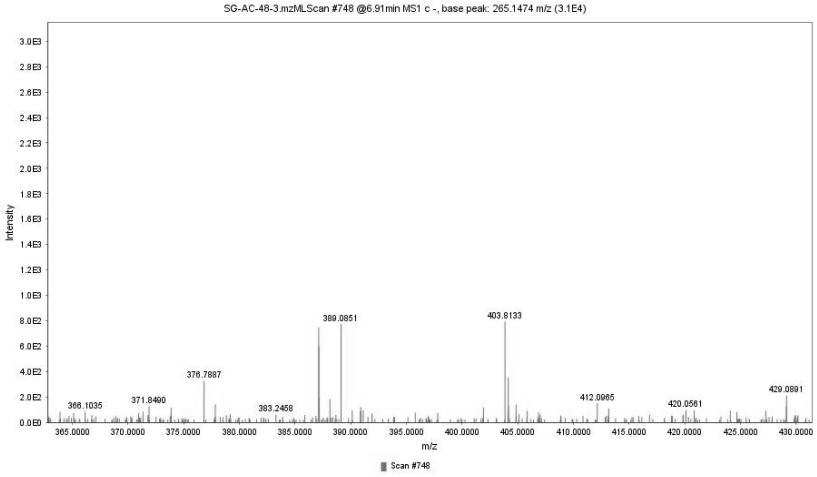

Figure S10. Mass spectrum of cladoniodesin with m/z 389.0851.

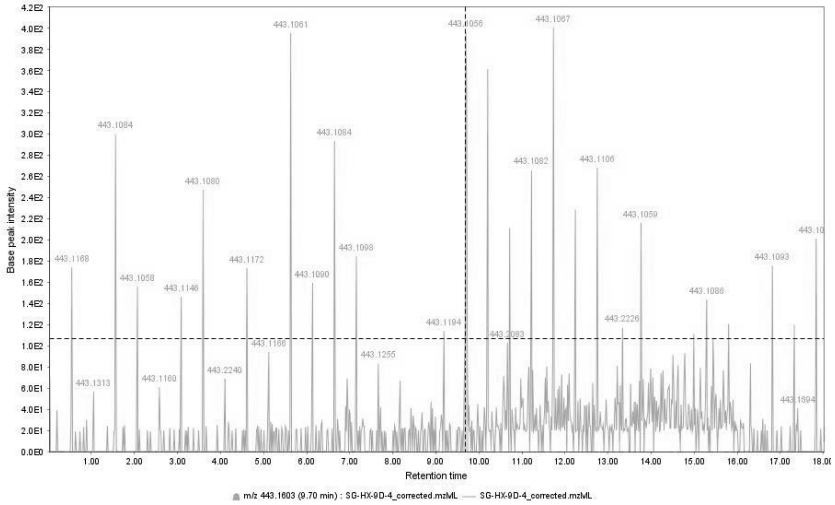

Figure S11. Chromatogram of colensoic acid detected as [M+H].

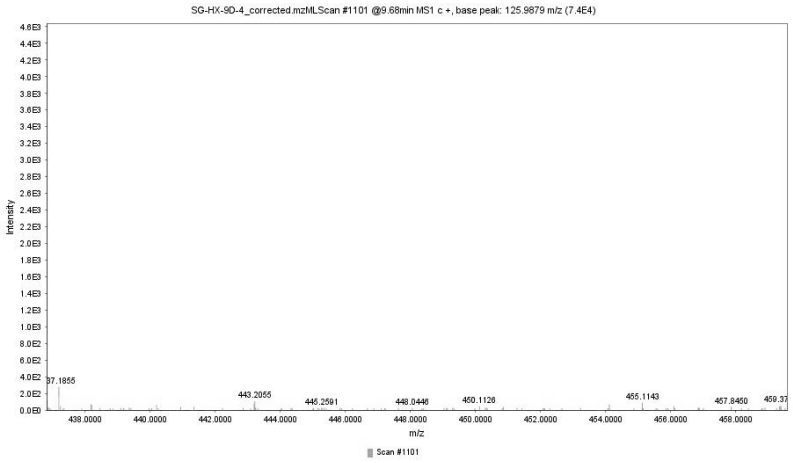

Figure S12. Mass spectrum of colensoic acid found in traces at m/z 443.2065.

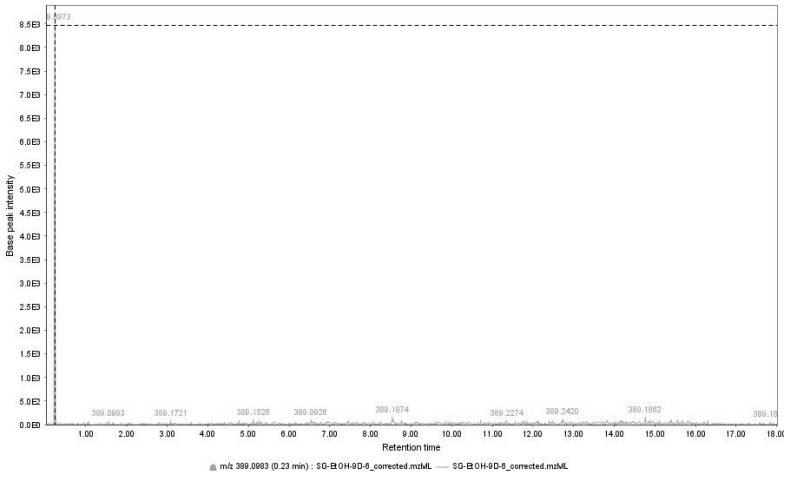

Figure S13. Chromatogram of cryptostictic acid detected as [M+].

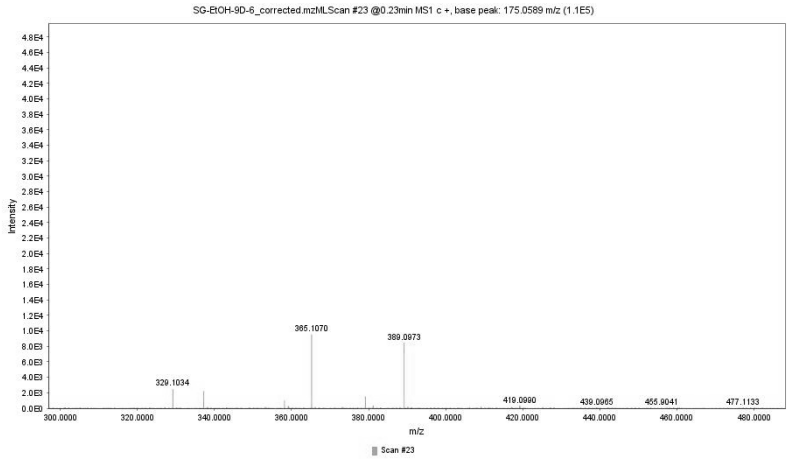

Figure S14. Mass spectrum of cryptostictic acid with m/z 389.0973.

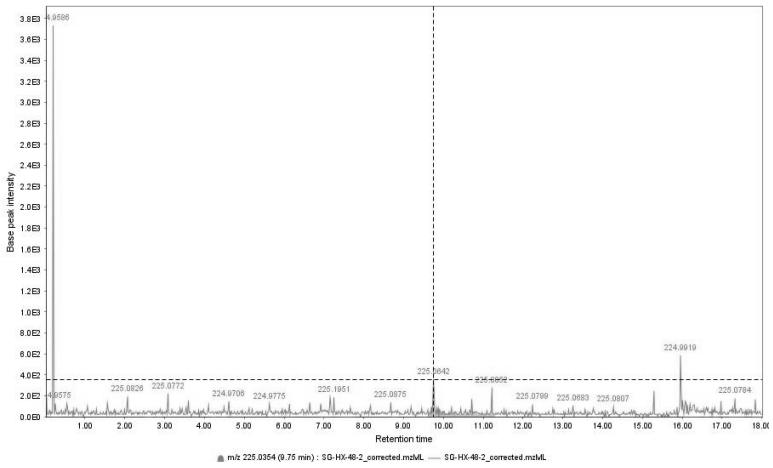

Figure S15. Chromatogram of ethyl-haematommate detected as [M+H].

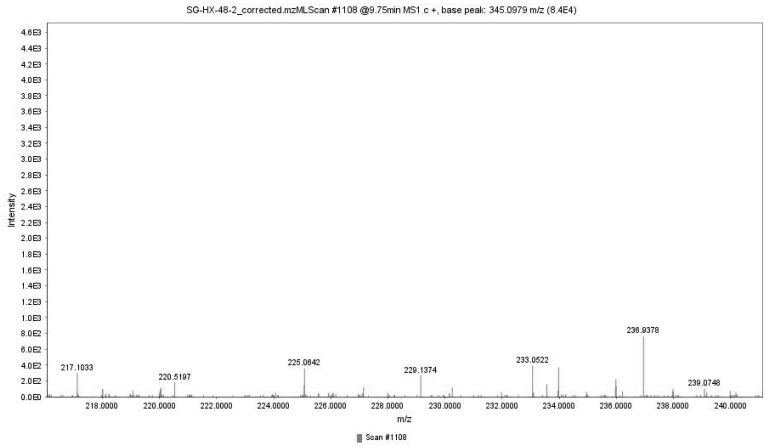

Figure S16. Mass spectrum of ethyl-haematommate with m/z 225.0642.

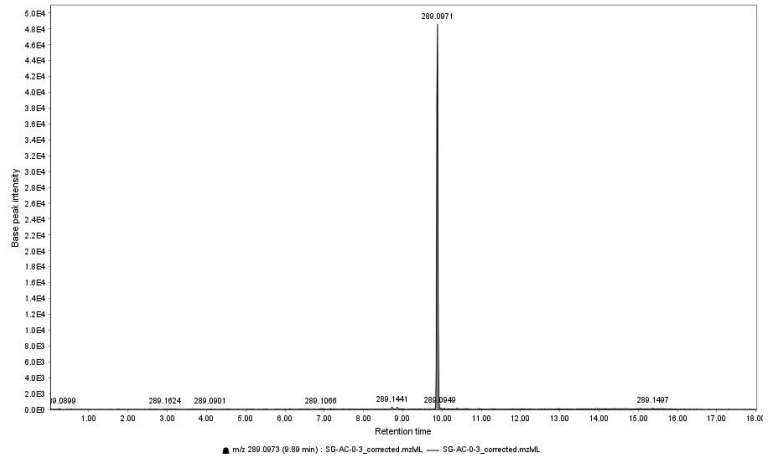

Figure S17. Chromatogram of isostrepsilic acid detected as [M+H].

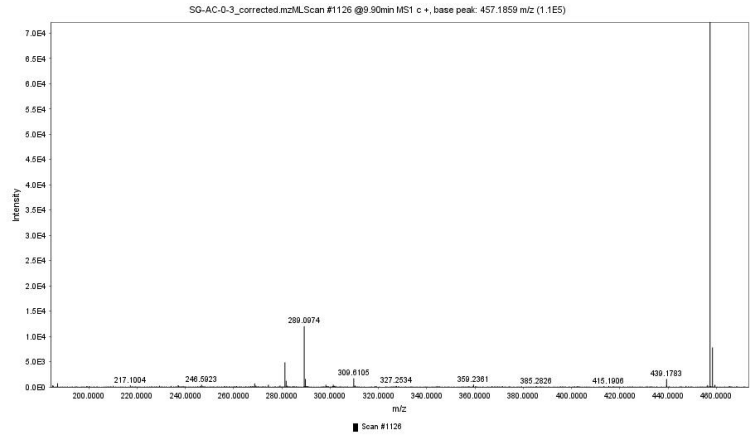

Figure S18. Mass spectrum of isostrepsilic acid at m/z 289.0974.

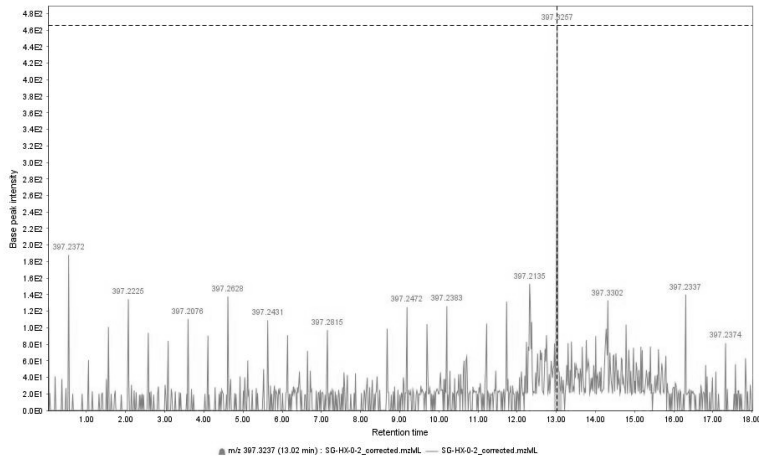

Figure S19. Chromatogram of lichesterol or ergosterol detected as [M+H].

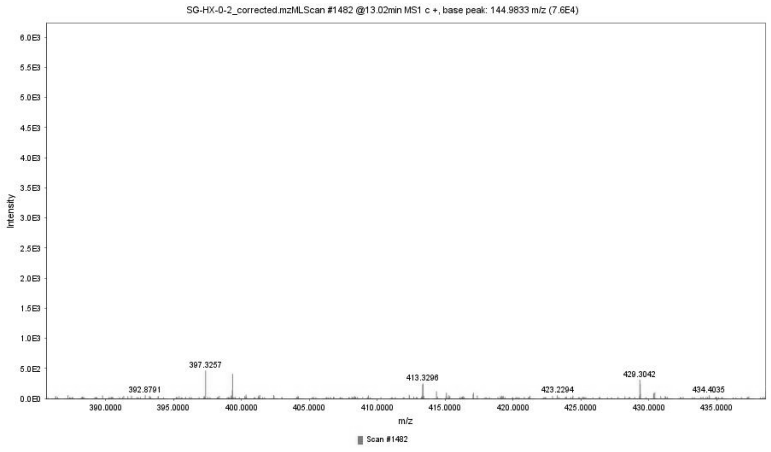

Figure S20. Mass spectrum of lichesterol or ergosterol with m/z 413.3296.

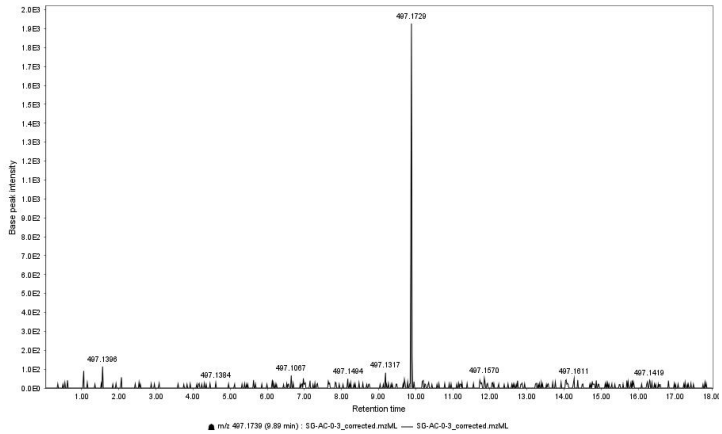

Figure S21. Chromatogram of lobarin detected as [M+Na].

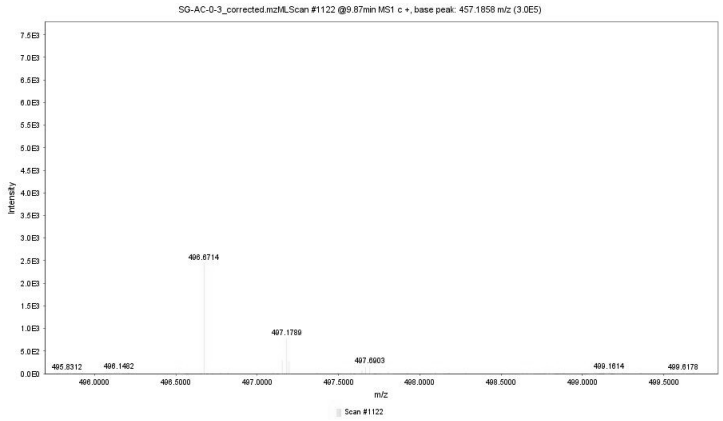

Figure S22. Mass spectrum of lobarin with m/z 497.1729.

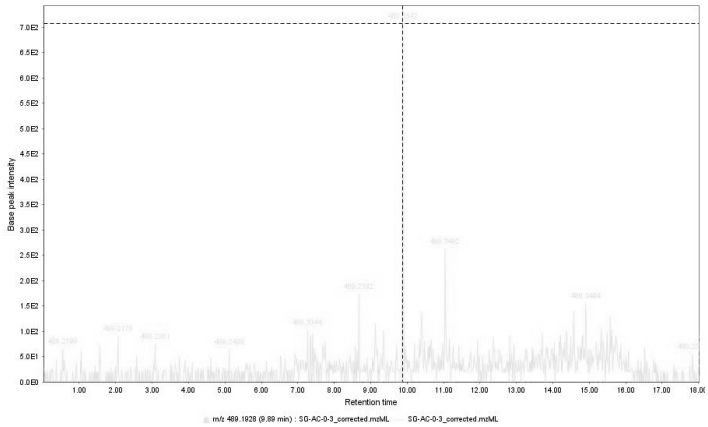

Figure S23. Chromatogram of methyl lobarin or esterified lobarin detected as [M+H].

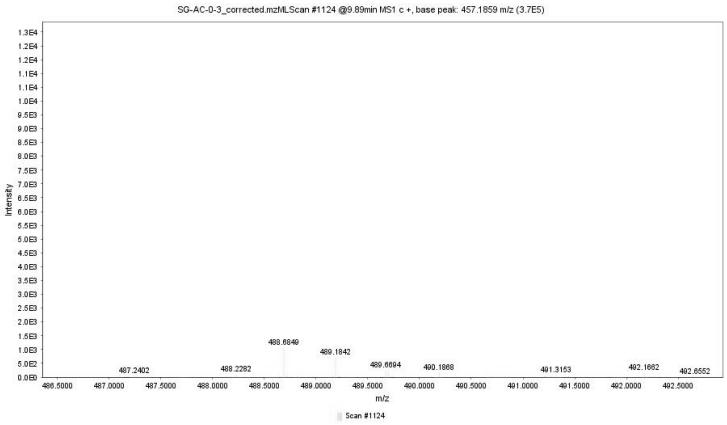

Figure S24. Mass spectrum of methyl lobarin or esterified lobarin with m/z 489.1842.

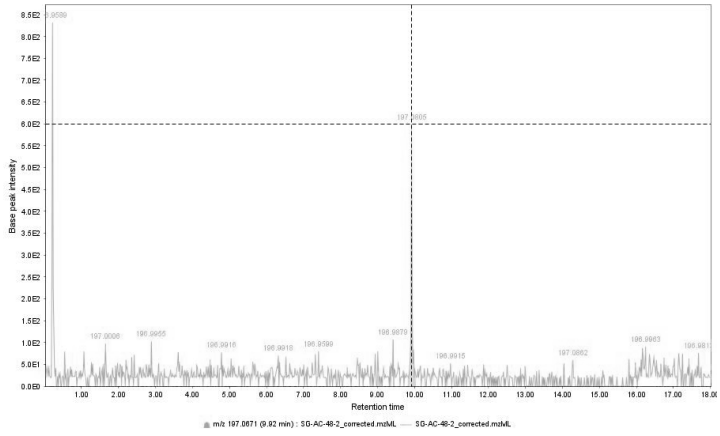

Figure S25. Chromatogram of methyl orcinol carboxylate or 4,6-dihydroxy-2-methoxy-3-methylacetophenone detected as [M+H].

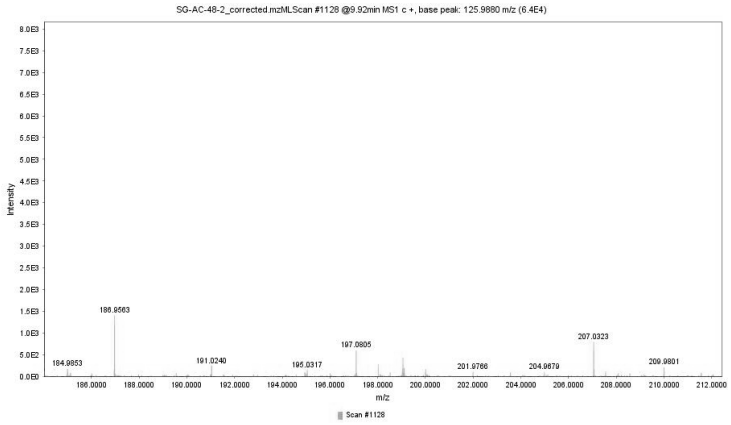

Figure S26. Mass spectrum of methyl orcinol carboxylate or 4,6-dihydroxy-2-methoxy-3-methylacetophenone with m/z 197.0805.

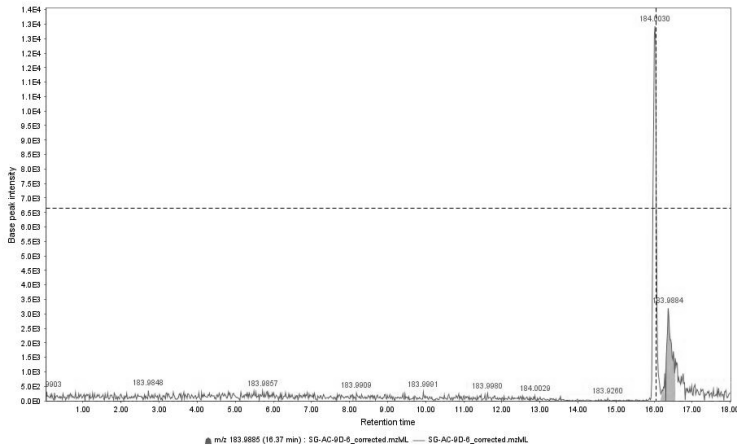

Figure S27. Chromatogram of methyl-orsellinate detected as [M+H].

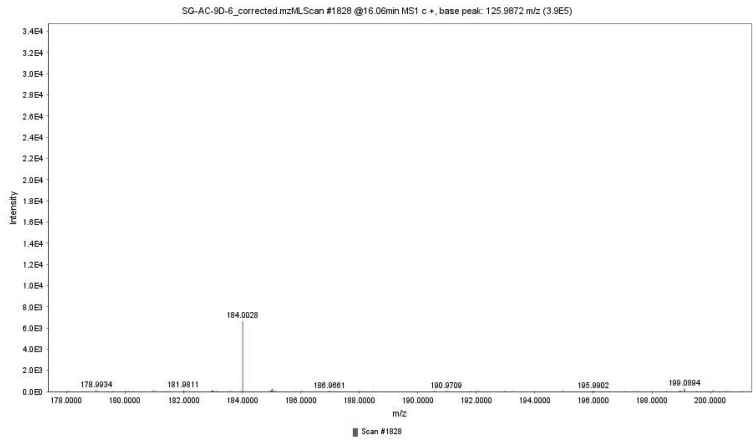

Figure S28. Mass spectrum of methyl-orsellinate with m/z 184.0028.

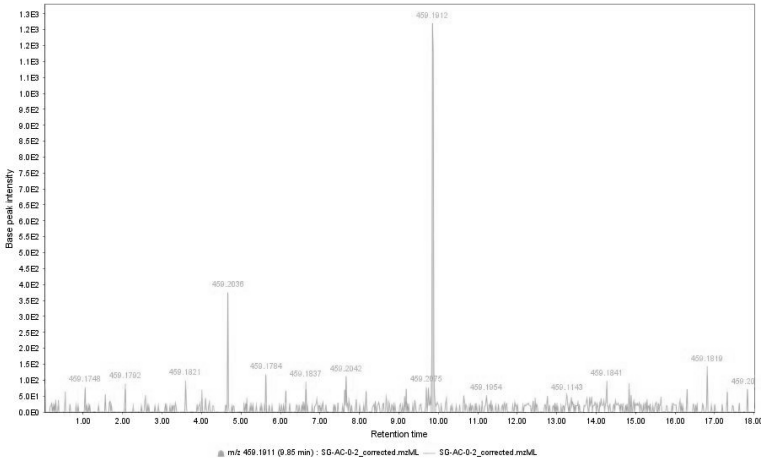

Figure S29. Chromatogram of miriquidic acid detected as [M+H].

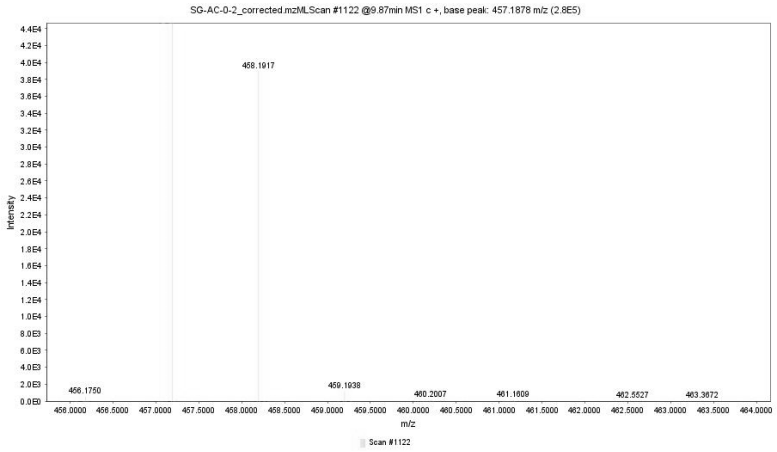

Figure S30. Mass spectrum of miriquidic acid with m/z 459.1938.

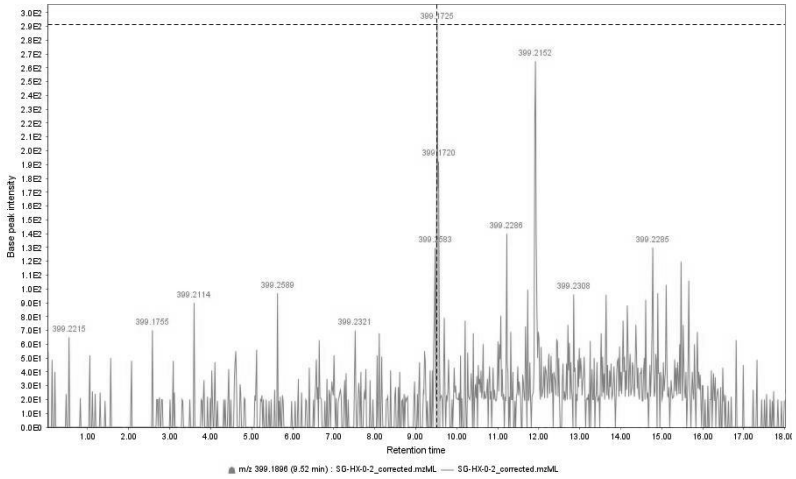

Figure S31. Chromatogram of norlobaric acid detected as [M+H].

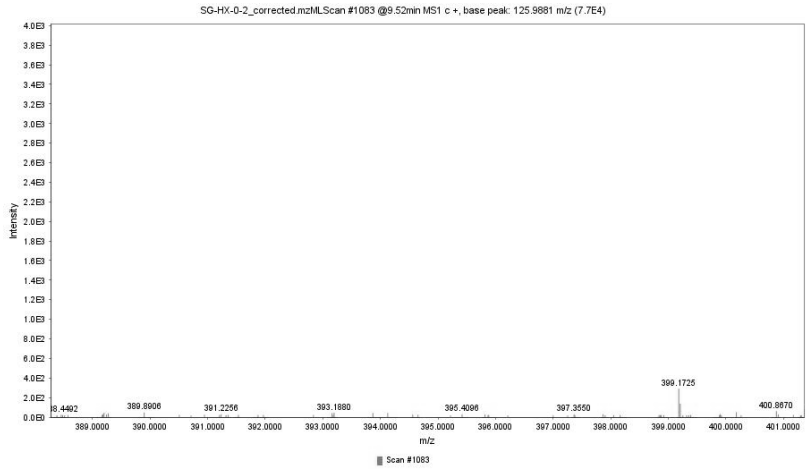

Figure S32. Mass spectrum of norlobaric acid with m/z 399.1725.

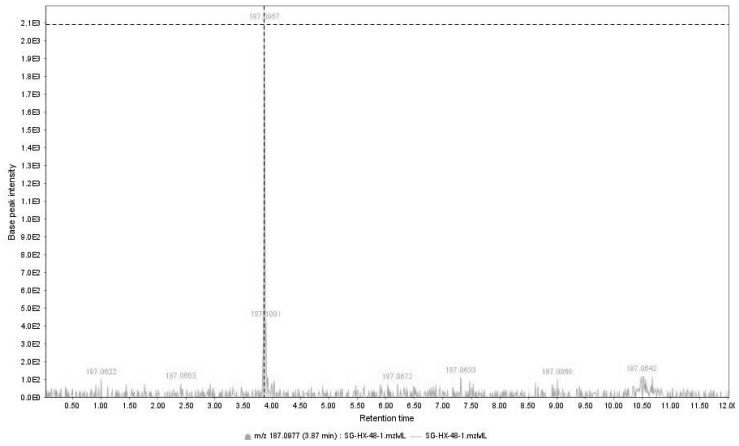

Figure S33. Chromatogram of nonanedioic acid detected as [M-H].

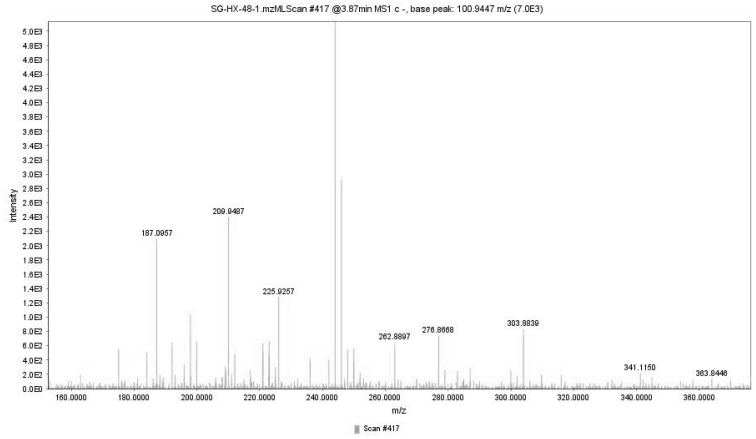

Figure S34. Mass spectrum of nonanedioic acid with m/z 187.0957.

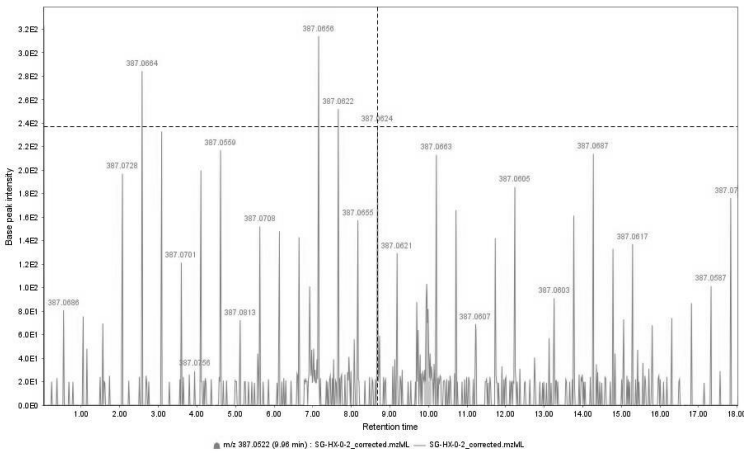

Figure S35. Chromatogram of stictic acid detected as [M+H].

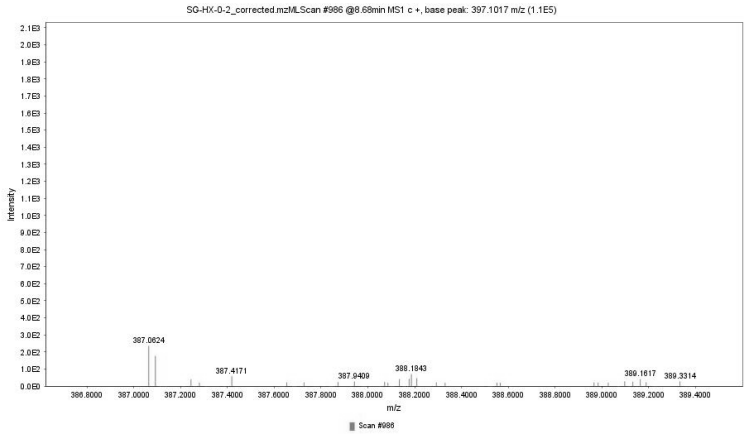

Figure S36. Mass spectrum of stictic acid with m/z 387.0624.

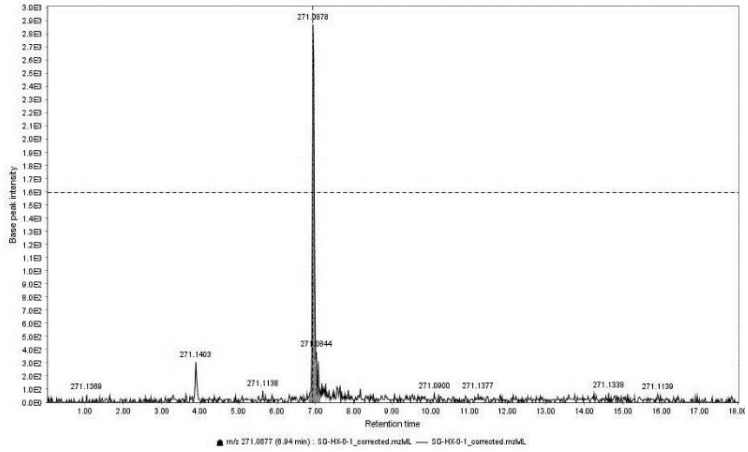

Figure S37. Chromatogram of strepsilin detected as [M+H].

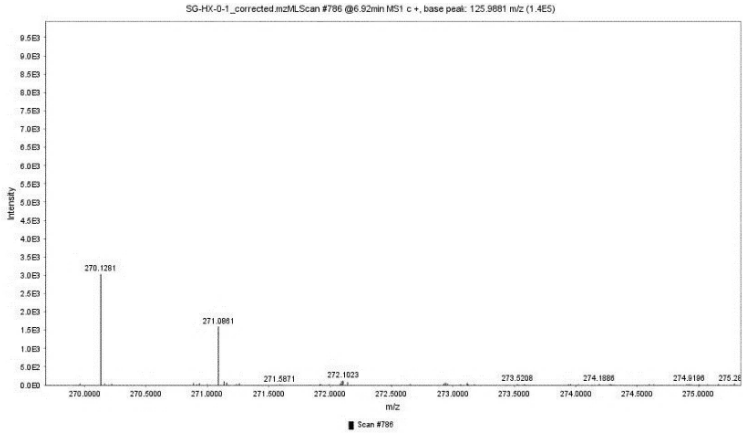

Figure S38. Mass spectrum of strepsilin with m/z 271.0861.