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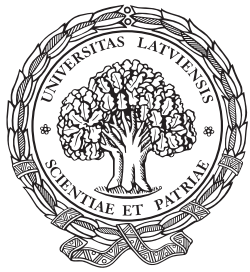

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COMPOSITION OF MOSSES, THEIR METABOLITES AND ENVIRONMENTAL STRESS IMPACTS

DOCTORAL THESIS

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CONTENTS

ABBREVATIONS

CP MAS ^{13}C NMR – cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance spectroscopy
DMSO – dimethylsulphoxide
DPPH – 2-diphenyl-1-picrylhydrazyl
BSTFA – N,O-Bis(trimethylsilyl)trifluoroacetamide
ETAAS – electro-thermal atomic absorption spectrometry
GC/MS – gas chromatography – mass spectrometry
HCA – hierarchical cluster analysis
FAAS – flame atomic absorption spectrometry
FTIR – Fourier transform infrared spectrometry
Py-GC/MS – analytical pyrolysis-gas chromatography/mass spectrometry
ROS – reactive oxygen species
SPE – solid phase extraction
UPLC – ultra performance liquid chromatography
UPLC–TOF LC/MS – ultra performance liquid chromatography coupled with time-of-flight mass spectrometry

ANNOTATION

Mosses (*Musci*) includes ~18'000 species, have worldwide distribution and significance from environmental and nature conservation perspective, however their studies are not so intensive as of other plant groups. The interest in moss composition and functions in the environment is increasingly growing as the presence of a high number of biologically active compounds in their composition recently has been demonstrated and studies of moss secondary metabolites can help to understand stress reactions in so called resurrection plants.

The aim of the work is to study the composition of mosses, their metabolites and impacts of pollutant stress on it. In the study 16 species of mosses were used. During the study a methodology for a complex characterization of moss composition was elaborated including multi-proxy analysis of mosses and their secondary metabolites. Optimization of extraction conditions enabled to obtain high number of major groups of substances characterizing secondary metabolite pool of studied mosses and scaling-up supported possibilities to obtain quantities of moss secondary metabolite major groups. It has been found that mosses consists of various carbohydrates, but lignin and phenolics are of minor significance. In depth characterization of moss composition can be used to support the taxonomy of mosses. Moss extracts obtained under optimized conditions contain high number of different biologically active substances (lipids, sterols, flavonoids and many others) and biological activity testing of them were done. Moss secondary metabolites demonstrate high antimicrobial activity and ability to inhibit development of cancer cell lines and can be considered as prospective group of new biologically active substances. Environmental stress (droughts/wetness, pollutants) impacts result in a significant changes of moss secondary metabolite pool as well as parameters, characterising their composition and can support identification of environmental stress biomarkers.

Key words: bryophytes, mosses, composition of metabolites, characterization, environmental impacts, lipids

ANOTĀCIJA

Sūnas (*Musci*) ir nozīmīga augu grupa, ņemot vērā to plašo izplatību uz Zemes, nozīmīgumu evolūcijas procesos un no vides aizsardzības viedokļa, tomēr to sastāva un īpašību pētījumu ir ievērojami mazāk nekā par citām augu grupām. Sūnu sastāva pētījumi pēdējā desmitgadē ir kļuvuši aktuāli, ņemot vērā lielo skaitu bioloģiski aktīvu savienojumu, kuri ir atklāti to sastāvā, kā arī sūnu augsto izturību pret vides apstākļu mainības radītā stresa ietekmēm, īpaši saistībā ar klimata pārmaiņām.

Promocijas darba mērķis ir analizēt sūnu sastāvu, to metabolītus un vides stresa ietekmes uz tiem. Pētījumā izmantotas Latvijā izplatītu sūnu sugas un izstrādāta metodoloģija sūnu un to metabolītu sastāva raksturošanai un izpētei, izmantojot daudzparametru fizikāli ķīmiskās analīzes metodes. Veikta sūnu sekundāro metabolītu ekstrakcijas apstākļu izpēte un optimizācija, kas nodrošina liela skaita vielu identifikāciju un kvantifikāciju sūnu sastāvā, kā arī nodrošina iespējas iegūt sūnu metabolītus preparatīvos daudzumos to īpašību padziļinātai izpētei. Pierādīts, ka sūnas sastāv no ogļhidrātiem, bet lignīns un polifenoli, salīdzinot ar augstākajiem augiem, ir atrodami ievērojami mazākos daudzumos. Sūnu lipīdi satur daudzas bioloģiski aktīvas vielas, un veikta to aktivitātes izpēte. Sūnu sekundārie metabolīti uzrāda augstu antimikrobiālo aktivitāti un spējas inhibēt vairāku vēža šūnu līniju attīstību un līdz ar to satur perspektīvas vielu grupas izmantošanai biomedicīnā. Vides stresa ietekmes (sausums/mitrums, piesārņojums) rada būtiskas izmaiņas sūnu sekundāro metabolītu kopā, kā arī ietekmē to metabolismu raksturojošos parametrus un līdz ar to var tikt izmantoti vides stresa biomarķieru identifikācijai.

Atslēgvārdi: sūnaugi, lapu sūnas, metabolītu sastāvs, ķīmiskais sastāvs, vides faktoru ietekmes, lipīdi

INTRODUCTION

Mosses belong to the simplest land plants, but at the same time they are located in the second largest taxonomic group of the plant kingdom as they belong to bryophytes (Goffinet and Shaw, 2008). There are around 25'000 bryophyte species which can be found in most of ecosystems worldwide, including mosses (*Musci* ~18'000 species), liverworts (*Hepaticae* ~6'000 species), and hornworts (*Anthocerotae* ~1'000 species). Despite the major role in the evolution processes, worldwide distribution and significance from environmental and nature conservation perspective, studies of mosses are not so intensive as of other plant groups. The interest in moss composition and functions in the environment is increasingly growing as the presence of a high number of biologically active compounds in their composition recently has been demonstrated (Asakawa *et al.*, 2013). Many compounds isolated from bryophytes have shown high biological activity. Thus, extracts of bryophytes are prospective for search of new pharmaceutically active compounds. Bryophytes might be considered as valuable plants for development of bioeconomy. However, from large amount of bryophytes only negligible number of species has been extensively studied, and most part of recent studies has concentrated on studies of liverwort composition (Asakawa, 2007), especially on substances present in oil bodies of these plants. As the simplest plants bryophytes have long history of evolution and, thus, understanding of their composition and metabolism might shed light on processes of biological and chemical evolution.

The largest taxonomical group of bryophytes is mosses. Mosses are important element of ecosystems, especially in the Northern hemisphere, where they are the main element of bog vegetation and major peat forming plant (*Sphagnum* mosses), but they are of importance also in forest ecosystems. Major structural components of mosses are carbohydrates (Maksimova *et al.*, 2014; Klavina, 2015), but they contain also other secondary metabolites with possibly high biological activity. However, composition of mosses has not been much studied from the perspective of application potential and functions in the environment. One more reason to investigate moss composition is related to the need to understand their metabolism. It is important to study secondary metabolites in mosses, as they are different from those in higher plants, considering environmental changes unavoidable due to climate and global environmental change. Studies of moss secondary metabolites can help to understand stress reactions caused by climate and global environmental change (drought/wetness) as mosses are resistant to rapid and significant changes of the environmental conditions, and they are considered as "resurrection" plants. Mosses are also highly resistant in respect to oxidative stress (including impact of UV radiation) and pollution stress (*e.g.,* impact of heavy metals) indicating the presence of unique functions of their composition and secondary metabolites in their metabolism. Wide application of mosses in pollution biomonitoring programs requires better understanding of processes governing pollutant accumulation in moss bodies and especially changes in their secondary metabolism.

Aim of the work

The aim of the work is to study the composition of mosses, their secondary metabolites and impacts of environmental stress on it.

Hypothesis

Chemical composition of mosses as simpler plants significantly differs from that of higher plants, but its elements (basic composition and secondary metabolites) are of utmost significance to support development of understanding of biological evolution and environmental stress reactions.

Tasks of the work

1. Analysis of moss chemical composition using multiproxy chemical analysis methods to support development of moss chemotaxonomy method.
2. Development of extraction methods for major groups of moss secondary metabolites and study of their fractionation possibilities.
3. Characterization of moss secondary metabolite biological activity to support application possibilities.
4. To study environmental variability and pollution stress impacts on moss chemical composition

Scientific novelty

1. Decisive evidences of the main part of moss biomass being composed of various carbohydrates, but lignin and phenolics being of minor significance.
2. Chemical analysis of moss composition can be used to support the taxonomy of mosses.
3. Optimization of extraction conditions enable to obtain high number of major groups of substances characterizing secondary metabolite pool of studied mosses.
4. Moss secondary metabolites demonstrate high antimicrobial activity and can be considered as a prospective group of new biologically active substances.

Major achievements

1. Methodology for a complex characterization of moss composition was developed.
2. Methods supporting possibilities to obtain quantities of moss secondary metabolite major groups were elaborated, and analytical and biological activity testing of them were done.

Contribution of the author

Laura Kļaviņa has done the sampling of mosses (selection of sampling site, sampling, habitat description *etc.*), pretreatment of the samples (sorting, cleaning *etc.*) and their preparation for analysis. Laura Kļaviņa has elaborated the investigation plan, including

analysis, has participated in the analytical characterization of samples, analysed obtained results, including statistical analysis. Laura Kļaviņa has carried out the preparation of manuscripts for publication.

Approbation of the results

The results of the thesis are published in 8 articles (4 of them included in Scopus database, 3 – Web of Science); in total, the author has 11 publications. The results of the work have been presented in 20 reports in international and local conferences.

Scientific publications (related to the thesis)

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3. G. Tabors, O. Nikodemus, A. Ajanovica, L. Viksna, L. Dobkevica, **L. Klavina**, I. Kruze, K. Viligurs. 2016. Bioindication of atmospheric pollution using mosses: spatial and temporal aspects (Atmosfēras piesārņojuma bioindikācija ar sūnu palīdzību: telpiskie un temporālie aspekti). 74th Annual LU conference, Riga, 496
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1. LITERATURE REVIEW

1.1. Evolution, classification and research limitations of bryophytes

In respect to the evolution theory, bryophytes are the first terrestrial plants directly placed between algae and vascular plants (Goffinet and Shaw, 2008; Glime, 2007). The fossil record of bryophytes dates back to the Upper Carboniferous, however, as many findings are ambiguous as well as possible age estimation differs among samples. On the other hand, molecular phylogeny suggests that the origin of this bryophyte division dates back to the Ordovician, meaning that their evolution has begun at least 400 million years ago (Goffinet and Shaw, 2008; Glime, 2007). Bryophytes play the key role in understanding how the higher plants came to conquer land from fresh water. The main challenge of early land plants was limited water availability. Early land plants were poikilohydric, and that helped to survive drought periods. Nowadays, nearly all bryophytes are poikilohydric, whereas, this water storage strategy has not survived in higher plants. In order to colonize land, desiccation tolerance was crucial step; to maintain mechanisms for tolerance, plants had to lower metabolic rates (Oliver *et al.*, 2000; 2005). While vascular plants became more complex gaining vascular tissue, cuticle, stomata *etc.*, bryophytes evolved their metabolic processes in order to survive. For example, bryophytes have some carbohydrates that can be found only in land plants (xyloglucan) and at the same time they have high concentration of glucuronic acid that is more characteristic for algae (Popper and Fry, 2003). Recent phylogenomic studies show that most of the land plants have retained genetic potential for desiccation tolerance even if it cannot be expressed in their phenotype (Oliver *et al.*, 2004). Studies of bryophyte primary and secondary metabolites could give insight of how evolution of chemical structures supports biological evolution. As an example, importance of lignin in higher plants and lack of it in bryophytes can be mentioned (Popper and Fry, 2003; Popper, 2004). Absence of lignin is also the reason why bryophytes are comparatively small in their size – it is one of their main characteristics (Goffinet and Shaw, 2008; Glime, 2007).

As already mentioned, size of bryophytes is limited due to their anatomical and physiological character as they do not synthesize substances used for building of strong cell walls such as cellulose based polysaccharides and lignin, thus, they lack vascular systems (Goffinet and Shaw, 2008; Popper and Fry, 2003). Due to their small size, it is complicated to harvest big amounts of plant material that is necessary for intensive chemical analysis and identification of individual substances (Goffinet and Shaw, 2008; Zinsmeister and Mues, 1990). Fortunately, in most of the cases bryophytes tend to form tight colonies that slightly eases their gathering. At the same time these colonies often include small amounts of other bryophyte species or vascular plants, as well as small living beings, *e.g.,* bacteria, fungi, ants, spiders and others (Goffinet and Shaw, 2008; Glime, 2007). One of limitations in research of bryophytes is their identification – partly

due to their small size, but also due to the unclear taxonomy (Asakawa *et al.*, 2013; Goffinet and Shaw, 2008; Glime, 2007). There are some bryophyte species which require microscopical check of a leaf cross section (often less than 1 mm in size). This makes such species practically impossible to be collected for analysis of chemical composition (Laine *et al.*, 2011; Zinsmeister and Mues, 1990).

Due to the bryophyte abundance, unique biology, biochemistry and adaptation strategies, they are one of the major plant taxonomical groups in the world. Bryophytes can be found nearly everywhere: globally growing on various substrates and in a variety of growth conditions (Goffinet and Shaw, 2008; Glime, 2007). More than 25'000 bryophyte species have been identified, however, this number is quickly growing due to the increasing application of genetic identification methods and better understanding of taxonomical order and differences of their physiology along with constant discoveries of new species (Asakawa *et al.*, 2013; Goffinet and Shaw, 2008).

As already mentioned, taxonomy of bryophytes is rapidly changing; therefore, in this paper following taxonomy will be used (Figure 1.1.). Bryophytes are mainly divided in 3 phyla – liverworts (*Marchantiophyta*), hornworts (*Anthocerotophyta*) and mosses (*Bryophyta*). In Latvia, bryophytes are represented by at least 550 species, mainly mosses, then liverworts and lastly hornworts. Approximately half of bryophyte species found in Latvia are considered to be rare, 87 species are in the Red Book and 130 species are protected (Strazdina *et al.*, 2012).

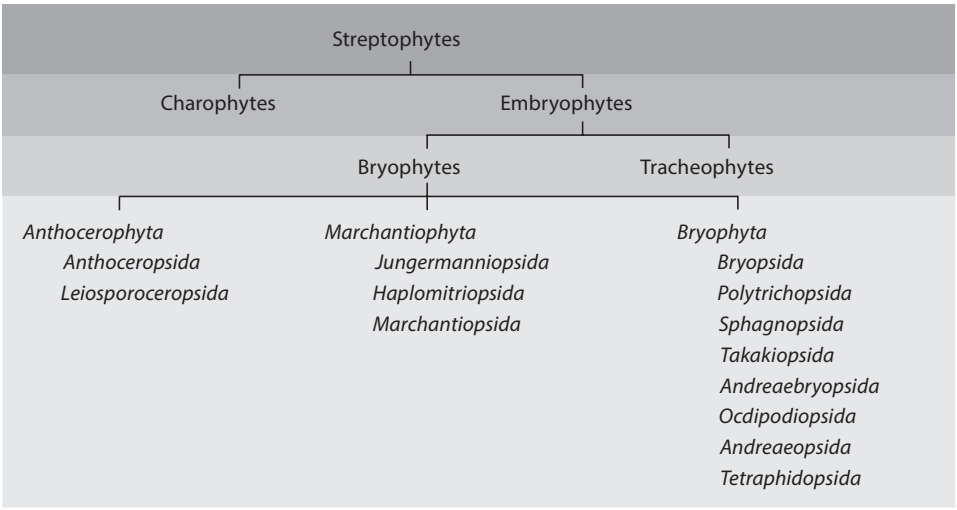

Figure 1.1. **Taxonomical order of bryophytes** (adapted from Asakawa *et al.*, 2013; Glime, 2007)

Some studies suggest that bryophyte phytochemistry can be used as a supportive tool to understand composition of bryophytes, as well as to explain and find new evidence about the genesis of both, higher and lower plants, with bryophytes being the link between them (Asakawa *et al.*, 2013; Goffinet and Shaw, 2007; Glime, 2007). As a consequence

of different evolution pathways of bryophytes, many substances in their composition are unique and many still remain to be discovered. Despite global abundance, high diversity of species and unique chemical composition, bryophyte chemistry has not been extensively studied (Zinsmeister and Mues, 1990; Glime, 2007): it could be estimated that chemical composition of only 2% of known mosses and of 6% of liverworts have been analysed. Liverworts are studied mainly in respect to their chemical composition because of their oil bodies that are not found in mosses or hornworts.

1.2. Functions of bryophytes in environment

Despite the fact that bryophytes are comparatively small plants, their high survival skills and other peculiarities make them to be an important part of many ecosystems. At first glance it is hard to imagine their role in ecosystems, but if looking deeper, it becomes clear that, for example, they are producing large quantities of organic matter (even though their decomposition rate is slower than in higher plants due to low concentration of nitrogen). That is especially important in bog and tundra ecosystems, by formation of peat (Turetsky, 2003). Important bryophyte functions also are stabilization of soil or debris and capturing and filtration of sediments and water. Due to captured sediments and other organic matter as well as stable moisture and temperature levels (bryophytes have low thermal conductivity), they also are a perfect habitat for algae, fungi, small invertebrates and amphibians (Turetsky, 2003; Glime, 2007).

One of the first association that people have about mosses are bogs and peat. Other bryophyte groups also can be found in bogs, but bog ecosystems mainly are overruled by different moss species of which main percentage is *Sphagnum* species (Goffinet and Shaw, 2009; Glime, 2007). Not only mosses are responsible for unique acidic and anaerobic environment in bog ponds and surface, they also are the main material which decomposition product is peat. One of the main uses of peat nowadays is gardening substrate and material for other gardening supplies. In past peat has been used as fuel. At this point peat is considered to be partially renewable resource due to the slow formation process. Studies of moss chemical composition give interesting and valuable perspective on peat chemical composition and peat formation. Better understanding of moss chemistry allows better analysis and description of evolution processes of bogs (Goffinet and Shaw, 2009).

Due to specific physiology of bryophytes, they have larger surface area for water evaporation in proportion to plant mass than higher plants, and this factor is important when it comes to questions related to their functions in environment. Structure of bryophyte habitat is regulated by hydrological requirements of species as well as requirements in respect to growth substrate. Each bryophyte species has its own hydrological requirements that are partly connected with their stress tolerance. The most important bryophyte characteristics are that they are poikilohydric, do not have root systems and have very well developed desiccation tolerance which slows down photosynthesis, metabolism and growth processes during drought periods. This feature supports survival in most unfriendly environments and their colonization. Some species are more adapted to cold or extremely cold climates such as Arctic and some are able to

survive in heat and long-term drought. These unique adaptation skills are the reason why bryophytes are so widespread in all ecosystems (Glime, 2007), have wide ecological niche and higher stress tolerance than higher vegetation.

Even though bryophytes are extraordinary, just based on their adaptation possibilities, they are also essential in many processes in environment. One of the most important but often neglected role of bryophytes is their photosynthetic potential. In difference from higher plants, where roots and stems do not have photosynthetic capacity, bryophytes can photosynthesize with all their plant body. As the most of the soil surface in forests and especially bogs are covered with thick layer of bryophytes, it yields up to quite impressive amount of oxygen production *via* photosynthesis. Even if bryophytes cannot compete with higher plants in this process, in environments such as tundra and bogs they are the main oxygen producing plants (Glime, 2007; Hanson and Rice, 2014) and might play a significant role in global oxygen and carbon biogeochemical cycle.

Bryophytes play the major role in carbon and nitrogen cycles. It has been evaluated that a net primary production of carbon on land is 6×10^{16} g C/yr (Turetsky, 2003). Depending on region of bryophyte growth, carbon production rates can vary from 24 to 350 g/m^2/yr reaching maximum of 450 g/m^2/yr, thus, giving important contribution in global C production. Bryophytes cannot fix N, but they are forming facultative symbiosis with cyanobacteria *Nostoc* spp. which easily can be located anywhere on the large surface area of bryophytes (Turetsky, 2003).

1.3. Bryophyte chemical composition

Most of studies related to bryophyte chemical composition concentrate on their secondary metabolites. Many secondary metabolites in bryophytes have shown high biological activity thus only fortifying this area of research (Asakawa *et al.*, 2013; Zinsmeister and Mues, 1991). Nevertheless some attention has been dedicated to primary metabolite research in context of carbohydrates and creation of "bog bodies". Studies show that carbohydrates from mosses in bogs undergo acidic and anaerobic reactions and *via* Mailard reaction create antimicrobial conditions which leads to "conservation" of organic, fat and aminoacids containing materials (Balance *et al.*, 2007; Painter, 1991; Painter, 1983).

Chemistry of bryophytes has been analyzed in several studies but many articles concentrate on liverworts as they have oil bodies in which high amount of interesting and unique substance can be found easier than in other bryophyte groups (Asakawa *et al.,* 2013; Glime, 2007).

As discussed previously, studies of bryophyte chemical composition are challenging due to several limitations. However in recent decades development of many analytical techniques has also led to increased interest in bryophyte chemistry. Number of studies that concentrate on bryophyte chemistry has grown as well as the collected data amount. Especially influencial in bryophyte chemistry research are techniques such as, 2D NMR and NMR in high fields (500 MHz and above), several detection methods coupled with liquid chromatography such as, time-of-flight and, tandem quadrupole mass spectrometry, as well as many other techniques (Asakawa *et al.*, 2013). Only some studies concentrate

on methods used for structural characterization of organic materials for example Fourier transform infrared spectrometry (FTIR) and analytical pyrolysis – gas chromatography (Py-GC/MS) (Kracht and Gleixner, 2000).

Important part in moss phytochemistry is not only chemical composition analysis methods but also extraction methods. Using different extraction methods different compounds can be detected, therefore extraction optimization is important part of studies. Currently many extraction methods can be used and there are many studies looking for best conditions for extraction of higher plants. Only few studies have investigated best extraction conditions for bryophytes and their secondary metabolites (Klavina *et al.*, 2015; Irudayaray *et al.*, 2010). While some extraction principles apply for both higher plants and bryophytes, some remain different, *e.g.* optimal extrahent concentrations and treatment conditions (Klavina *et al.*, 2015). In order to analyse secondary metabolite profile, several extraction methods have to be used, because of different compound polarities. As already mentioned, main interest in bryophyte chemical research is secondary metabolites with biological activity, extrahents mainly used in order to extract are such substances are methanol, ethanol, water and chloroform (Basile *et al.*, 1999; Sing *et al.*, 2006; Saboljevic *et al.*, 2010; Fu *et al.,* 2012).

1.3.1. Bryophyte basic composition

Bryophytes consists of the same main organic substance groups as higher plants, however proportions of these substances are different. Main mass of bryophytes consist of hemicellulose and pectin (30–60%) following cellulose (15–25%), proteins (5–10%), polyphenols (5–10%) and lastly inorganic substances (3–10%) (Orlov *et al.*, 2005). Basic chemical elements in bryophytes are the same as in higher plants – C, H, N, O and S and others in low concentrations. There are not many organic compounds in bryophytes containing nitrogen or sulfur. Inorganic substances such as nitrogen, potassium, calcium and phosphorus concentrations are changing based on plants age. As a plant gets older, nitrogen, potassium and phosphorus concentrations decrease, but calcium concentration increases. This phenomena might be connected with stimulation of production of protein calmodulin which increases ion flow in plant that helps to provide more nutrients (Goffinet and Shaw, 2009).

1.3.2. Bryophyte primary metabolites – carbohydrates and polysaccharides

Some substance groups in bryophytes are more interesting than others in respect to understanding their metabolism or practical application possibilities. Even though the majority of biologically active substances present are secondary metabolites, there is significant measure of primary metabolites and carbohydrates. Carbohydrate content plays major role in bryophyte stress tolerance and is quite different compared to higher plants. Both water soluble and insoluble polysaharides are formed from many small monosacharide molecules. Usually carbohydrates are used in plant as energy source or as a building material for cell walls. Carbohydrates in bryophytes play two major roles: 1) building blocks for cell walls; 2) functional substances that help in interaction with surrounding environments.

First studies of bryophyte carbohydrate content started using *Sphagnum* mosses that are represented by more than 120 different species and are one of the major plant species forming Northern bogs and wetlands and one of the main precursors of raised bog peat (Glime, 2007; Judina *et al.*, 1999). In early studies of *Sphagnum,* water extracts showed presence of glucose (7%), sucrose, pentoses (6%), fructose and fructosides as well as some short chain fructans. In later studies also D-galacturonic acid (25%), D-galactose (10%), L-rhamnose (19%) and glycuronoglycan were identified as main polysaccharides (Maass and Craigie, 1964; Painter, 1983; Judina *et al.*, 1999; Graham *et al.*, 2010). Bryophyte cell walls consists of cross – linked hemicellulosic, cellulosic and pectin – like chains (up to 37,000 Da) which may form up to 20% of a moss dry weight (Kosonogova *et al.*, 1994). Also in mosses can be found acid – insoluble carbohydrates that have polymer – like structure and behaves similar to holocellulose found in higher plants, for example D-glucose, D-galactose, D-mannose, D-xylose and L-fucose as well as some identified carbohydrates (Painter, 1990; Painter, 1983; Hajek *et al.*, 2011). Main portion of carbohydrate research concentrate on *Sphagnum* species mosses but there are also some studies on other bryophytes. Liverworts show different carbohydrate composition also including volemitol, L-bornesitol, D-manitol and others which have not been found in mosses. Thus, to some extent carbohydrate composition could be used for taxonomic characterisation (Suleiman *et al.*, 1978; Marschall *et al.*, 1997; Marschall *et al.*, 1998; Nagao *et al.*, 2006). Additionally other carbohydrates have been identified as materials of primary cell walls such as xyloglucans and uronic acids (Popper and Fry, 2003; Popper *et al.*, 2003; Thomas *et al.*, 1984). Galacturonic acid in high concentrations has been found especially in leafy and thalloid liwervorts. Some carbohydrates are specific to bryophytes, such as: a) mixed – linkage glucan; b) mannose; c) uronic acid; d) xyloglucans and e) 3-O-methylrhamnose (Popper *et al.*, 2003).

Sphagnan is complex, pectin – like substance which can be found in *Sphagnum* mosses and has showed biological activity especially regarding food – born bacteria. Properties of mosses, more specific sphagnan, within the mosses, was first noticed in bog bodies: Lindow (2000 years old) and Tollund (1400 years old), as well as in some animal tissues or wooden artifacts that have been well preserved in bogs (Stalheim *et al.*, 2009; Borsheim *et al.*, 2001; Painter, 1998; Painter, 1991). Sphagnan in combination with reactive carbonyl groups go trough Mailard reaction (brownification reaction). This reaction can be easily spotted due to the tanning that it causes, thus improving artefact preservation. Tanning reaction is a result of collagen reaction with sphagnan causing the specific tan-like colour change. Because of this reaction peculiar outer layer on the body is formed, keeping its insides unaccessible to microorganisms (Stalheim *et al.*, 2009; Painter, 1991). Good preservation ability could also be caused by decay of *Sphagnum* moss cell walls, which causes hyaline and galacturonic acid synthesis and results in formation of acidic pH which could also show antimicrobial effect (Balance *et al.*, 2012). Some studies have show that antimicrobial activity in peat bogs is partially caused by humic substances that were formed due to moss decay, especially in anoxic environment, and that still contain reactive carbonyl groups (Stalheim *et al.*, 2009; Painter, 1991).

Still to this day it is not clear from what are *Sphagnum* moss cell walls formed, but there are some studies on it. Main possibility is that cell walls are formed from pectin – like polysaccharide sphagnan which is located in hyaline cell walls, papillae, fibrils and

chlorophyllous cell walls (Balance *et al.*, 2012; Stahlheim *et al.*, 2009; Painter, 2003). This idea has been proven by using staining with ruthenium red. The presence of reactive pectin in cell walls makes *Sphagnum* moss especially interesting for food preservation (Balance *et al.*, 2012).

Isolation of polysaccharides from bryophytes usually is done by extensive removal of secondary metabolites by means of exhaustive extraction with low-polar solvents (hexane, chloroform, dichloromethane etc), followed with more polar solvent extractions (methanol, ethanol, acetone) and extractions with water, acid hydrolysis or autohydrolysis (Balance *et al.*, 2007; Painter, 1991). The extraction process of bryophyte polysaccharides is well elaborated in respect to *Sphagnum* mosses, however, suggested approaches includes as an intermediate step, oxidation with $NaClO_2$ possibly resulting in oxidation of carbohydrate functional groups (Balance *et al.,* 2007; Painter, 1991; Painter, 1983) thus possibly giving false results.

1.3.3. Bryophyte secondary metabolites

While primary metabolites are directly involved in growth and plant metabolism, secondary metabolites are not. Secondary metabolites largely are synthesized from primary metabolites as well as through diverse biosynthetic pathways.

The most interesting and well studied secondary metabolite groups are lipophilic substances: terpenoids, steroids, bis-bibenzyls, unsaturated fatty acids, flavonoids and flavonoid glycosides as well as many others (Table 1.1.) (Asakawa *et al.,* 2013).

Table 1.1. **Major chemical constituent groups of bryophytes** (Adapted from Asakawa *et al.*, 2013; Ignat *et al.*, 2011; Iwashina, 2003; Dembitsky *et al.*, 1993; Tutschek, 1982)

Chemical constituents	Bryophyta	Marchantiophyta	Anthocerotophyta	Reference
Acetogenins	x		x	Asakawa *et al.,* 2013
Lipids	x	x	x	Asakawa *et al.,* 2013; Dembitsky *et al.,* 1993
Benzoic acid derivatives	x	x		Zinsmeister and Mues, 1990
Bibenzyl derivatives		x		Asakawa *et al.,* 2013
Cinnamic acid derivatives	x	x	x	Asakawa *et al.,* 2013
Flavonoids	x	x		Ignat *et al.,* 2011; Iwashina, 2003
Sphagnorubin	x			Tutschek, 1982
Steroids	x	x	x	Zinsmeister and Mues, 1990
Sitosterol	x		x	Asakawa *et al.,* 2013
Fitosterol			x	Asakawa *et al.,* 2013
Monoterpenes	x	x		Dembitsky *et al.,* 1993
Sesquiterpenes		x	x	Dembitsky *et al.,* 1993
Diterpenoids	x	x	x	Asakawa *et al.,* 2013
Tetraterpenes	x	x	x	Dembitsky *et al.,* 1993

There are also some pigments identified in bryophytes, for example, pigments from *Sphagnum* moss (Zinsmeister and Mues, 1990). Many secondary metabolites are unique to bryophytes, for example, almost all bisflavonoids identified are exclusively found only in bryophytes.

Range of compounds in bryophytes is remarkable, and many compounds are found only in few species, for example aurones that have been identified only in 3 liverwort species and in 1 moss (Asakawa *et al.,* 2013; Zinsmeister and Mues, 1990).

Bryophyte chemical composition not only determines their unique survival skills, but also such factors as taste and smell (Asakawa *et al.,* 2013; Beike *et al.,* 2010; Zinsmeister and Mues, 1991). Some bryophytes have pleasant smell such as carrot – like or even mint – like, but mainly smells are unpleasant: turpentine; fungal, sea weed, stink bug etc. (Asakawa *et al.,* 2013; Asakawa, 2007).

1.3.4. Lipids in bryophytes

One of the most important groups of secondary metabolites in bryophytes are lipids. Lipids are better characterised by their physical properties (poor solubility in water and hydrophobic properties) than by chemical properties. According to their physical properties, polarity of molecules and functions in organism, lipids can be classified into neutral lipids (triglycerides), polar lipids (phospholipids), glycolipids, as well as fatty acids, fatty alcohols, waxes, sterols, terpenoids and other substances (Gunstone, 1996). Despite the fact that the content of lipids in bryophytes seems to be low, there are several studies indicating that different low-polarity substance groups are present there (Dembitsky, 1993; Asakawa *et al.,* 2013) and their functions are significant for functioning of plants. Lipid content was studied in relatively many species of mosses and liverworts sampled in different regions of the world and is from 1 to 9.1% of dry mass (Dembitsky, 1993), at the same time studies indicated significant changes of lipid concentrations depending on seasonal processes (Lehtonen and Ketola, 1990), thus variability might be higher.

Lipids in bryophytes have a significant function to protect them from outside impacts, such as pathogens and environmental stresses and they participate in the regulatory processes of the plants (Gunstone, 1996; Asakawa *et al.,* 2013). Lipids in bryophytes could be localised not only in the cytoplasm or bound to cellular membranes but also in oil bodies, common for liverworts. Composition of lipids demonstrates the significant differences between three major classes of bryophytes: *Hepaticea*, *Musci* and *Anthocerotae*. To the development of studies of lipids, advancement of analytical methods, such as GC-MS and NMR as well as others in combination with preparative chromatography have significantly contributed. During last decades major research on bryophyte lipids has concentrated on studies of liverwort oil bodies and essential oils, which are composed of lipophilic terpenoids and aromatic compounds and at present more than 700 terpenoids and 220 aromatic compounds have been isolated and characterised from *Hepaticea* and book (Asakawa *et al.,* 2013) as well as several review articles are dedicated to these studies (Asakawa, 2001, Asakawa *et al.,* 2013). Many of isolated compounds demonstrate differences between higher plants and bryophytes as in higher vegetation have not been found or only their enantiomers were isolated. Most of substances isolated from liverworts have remarkable biological activity, for example, antimicrobial, antifungal and antiviral,

cytotoxic, insecticidal, insect antifeedant, antioxidant, muscle relaxing activities and others (Asakawa *et al.,* 2013). Thus the recent studies have demonstrated the significance of the bryophyte lipid studies. Further, differences amongst species of liverworts have been demonstrated, which is valuable for their chemotaxonomic studies. However much less attention, especially during last decades, has been paid to studies of lipids in other groups of bryophytes, at first in mosses, as lipid concentrations in their bodies is much lower and the treatment of the whole plant is needed to obtain quantities of material needed for studies.

Studies of lipids in the largest group of bryophytes – *Musci* (mosses) are of key importance as they: a) are essential to understand the evolutionary process from lower plants to higher; b) to compare stress (drought, pollution, light and other) resistance mechanisms in lower plants; c) to identify new, potentially biologically active substances for applications in biopharmacy, but in wider sense in bioeconomy. Another aspect of the moss lipid study actuality is related to their significance (especially of *Sphagnum* species) of the peat formation, humification and genesis of the peat. First studies of lipids in mosses were related to studies of humification processes and development of peat composition. In the first studies long-chain hydroxyl acids have been isolated from *Sphagnum fuscum* as well as presence of insoluble polymeric lipid esters in moss have been demonstrated, possibly contributing to formation of highly stable structures (Ekman and Karunen, 1982). Recent studies confirm these findings (You *et al.,* 2007). Another group of substances, specific for *Sphagnum* species were long-chain acyclic methyl ketones (Lehtonen and Ketola, 1990). Long chain, odd carbon-numbered n-alkan-2-ones are absent in other bog plants and moss groups and thus might be considered as viable biomarkers for *Sphagnum* in ombrotrophic bogs (Nichols and Huang, 2007). For most of moss species a common feature is presence of polyunsaturated, sometimes, long-chain fatty acids (Dembitsky *et al.,* 1993) and fatty acid composition has been relatively well studied (Dembitsky, 1993). For majority of mosses presence of several dominant acids is common: 18:1; 18:2; 18:3; 20:4; 20:5; 22:5, however very long-chain fatty acids also have been found up to C_{30} (Dembitsky, 1993). As an interesting feature of the moss lipid pool composition can be considered presence of acetylenic acids, for example cis-9,12,15-octadecatrien-6-ynoic acid, 9,12,15-octadecatrien-6-ynoic acid and others (Pejin *et al.,* 2012). Another group of lipids presenting high interest from perspective of pharmacologically potent agents, produced by plants are epoxy acetylenic lipids, found in mosses (Kuklev and Dembitsky, 2014). The fatty acid composition differs in different parts of the studied mosses and varied throughout the annual cycle and was found to be highest during the period of rapid development of the reproductive parts (Martinez-Abaigar *et al.*, 1994). Monoglycosyldiacylglycerides and diglycosyldiacylglycerides are important polar lipids and in mosses *Mnium punctatum* and *Hygrohypnum luridum,* they vary from 47 to 73% of total lipids, respectively (Dembitsky, 1993). Neutral lipids make up the highest percentage of the total lipid fraction (Dembitsky, 1993). To pool of moss lipids belong hydrocarbons (Corrigan *et al.,* 1973). Chain length distributions of alkanes have significant differences depending on the species, studied and sampling conditions (season) and vary between C_{13} and C_{34}. Also fatty alcohols with chain length from C_{20} up to C_{32} have been found with an odd/even ratio of C atoms from 5:1 till 9:1 (Dembitsky, 1993).

From functional perspective of major importance are sterols considering their regulatory functions in plants (Chiu *et al.,* 1985). In mosses sterols have not been much studied, however a number of sterols have been found and amongst them: phytolesterol, campesterol, stigmasterol, sitosterol and others (Chiu *et al.,* 1985). The sterol composition in six moss species varied from 0.04 to 0.21% of the dry tissue. Sterols of the mosses have been found in four types: a) the free form, b) fatty acid esters, c) glycosides and d) esterified glycosides. Carotenoids have been studied in a large number of mosses and they are one of the most important of the naturally occurring pigments. Studies of a large number of mosses demonstrated presence of α- and β-carotene, lutein and few others (Dembitsky, 1993). Composition of number of mosses demonstrated that the carotenoid distribution seems to be fairly uniform in mosses, the difference being mainly in quantities in each species.

Terpenoids are a very important group of natural products isolated from bryophytes and presently ~300 novel compounds have been isolated and their structures identified, however most of them has been found in liverworts (Asakawa *et al.,* 2013). Still also in mosses a number of terpenoids has been isolated (Saritas *et al.,* 2001), however mono- and sesquiterpenes have not been found in mosses.

During recent decades significant attention was paid to studies of lipids in *Sphagnum* species (Baas *et al.,* 2000; Huang *et al.,* 2012; Bingham *et al.,* 2010; Ronkainen *et al.,* 2013). Complex mixtures of lipids in 12 *Sphagnum* species, comprised of C_{28}±C_{29} sterols, C_{30} triterpenoids, C_{16}±C_{30} fatty acids, C_{22}±C_{30} fatty alcohols, C_{21}±C_{33} n-alkanes and isoprenoid and straight-chain wax esters, were identified and quantified. Sterols are dominated by the C_{29} sterols, 24-ethylcholesta-5,22-dien-3b-ol and 24-ethylcholest-5-en-3b-ol, whilst in some species C_{28} sterols are also abundant. So, the analysis of lipid pool, but especially amount of C_{23} and C_{25} n-alkanes, can be used for chemotaxonomic fingerprint for *Sphagnum* species and in studies of peat bogs (Baas *et al.,* 2000). Substances of lipid pool (alkanes, sterols, fatty acid esters and others) are considered to be refractory, but at the same time their amount is highly dependent on the environmental conditions and thus lipid analysis in peat can be used as a reliable tool for past climate reconstruction. Application of n-alkane biomarkers from *Sphagnum* species has been used for tracking of past climate change signals (Bingham *et al.,* 2010). Further, in these studies organic geochemistry techniques have been successfully applied to identify historical plant communities contributing at formation of bog peat. Organic geochemistry methods have been used to determine the composition of the neutral lipid fractions of raised bog mosses and fen plants, to investigate the potential for the distributions to characterize and separate different fen plants and plant groups (Ronkainen *et al.,* 2013). *Sphagnum* alkane composition (alkane concentration ratios $C_{25}/(C_{25}+C_{29})$ has been suggested as proxies for terrestrial organic matter source apportionment to Arctic waters and it has been found that 68–100% of the terrestrial organic matter fraction is derived from *Sphagnum*-rich peatlands (Vonk and Gustafsson, 2009). The lipids composition of *Sphagnum* mosses much depend also on hydrologic conditions of their growth and samples collected from different hydrological settings show clear variation along the hydrological gradient, with higher n-C_{23}/n-C_{25} alkane ratio values and lower average chain length values of long chain n-alkanes, n-fatty alcohols and n-fatty acids (Huang *et al.,* 2012). Thus analysis of lipid composition might be used for reconstruction of past hydrological conditions. Studies of

moss lipids are of importance as they might be used as a tool to study the character of the metabolism – lipidomics (Carrasco-Pancorbo *et al.,* 2009).

1.4. Application potential of bryophytes and their secondary metabolites

Use of biological materials (land, plants, etc.) as source material for high value products and materials is a growing trend that is supporting EU development (Sillanpaa and Ncibi, 2017). Bioeconomy is declared as one of smart specialization directions also in Latvia (Latvijas bioekonomikas stratēģija, 2030).

Advancement of biotechnology largerly depends also on diversification of biological materials and identification of their new application fields. From perspective of new solutions in bioeconomy, bryophytes can be considered as previously underestimated material but in future as a prospective plant group in many fields starting as a raw material and ending with possible use in pharmacy. Possible application in biopharmacy doubles interest in bryophytes from perspective of economical development based on smart specialization directions as it falls also in the field of biomedicine, biopharmacy an biotechnology.

Mosses can be found everywhere where human beings are living and it has influenced their use historically for different purposes. Mosses have been used for animal feeding hovewer their calorific value is low and so commonly they have been used as raindeer forage in areas beyond Polar circle (Glime, 2007; Prins, 1981). Despite low calorific value of mosses, their use as supplement in bread baking or as soup additive in Native Indians culture, especially in case of hunger has been documented (Bland, 1971; Hart, 1992). Use of mosses as food additive is mainly based on their neutral taste and high fiber content (Hart, 1992). More common and widely distributed have been use of mosses for wound dressing as well as for hygenic purposes all over the world and not only for humans, but animals as well (Hotson, 1918). Only in extreme situation moss applications became actual, for example, as surgical dressing material in Franco-Prussian and Russo-Japanese war, nevertheless more extensively used this type of dressing became in I and II World War, when large amount of this type material was prepared and improved by adding different chemical substances (Hotson, 1918). These application fields have been supported by moss (especially *Sphagnum* species) high water absorbtion capacity as well as antimicrobial activity (as found nowadays). Different mosses have found their use in different ethnomedical aplications in many traditional cultures. For example by Native American and Alaskan indians, inuits, tibetans and in Chinese medicine (Hotson, 1918; Glime, 2007). High water and air holding capacity of mosses has supported their application for thermal insulation, for example in buildings in Latvian, Native Indian and other traditional cultures, as mosses was considered to be more effective in heat preservation than sawdust or cork (Bozsaky, 2011). *Sphagnum* mosses also have been and still are used as packing material for food transportation, benefits for their use are their high water absorbtion capacity as well as antimicrobial properties against food – born bacteria (Hotson, 1918; Mellegard *et al.,* 2009). It is considered that one of the main reasons for moss antimicrobial activity against food – born bacteria is sphagnan found in their composition. Some argues that mosses are not good of a packing material because

the requirements for it to have maximum effect are high – no inner spoilage of product and undamaged outer layer of product (Borsheim *et al.,* 2001; Stalheim *et al.,* 2009). Historically mosses have been relatively widely used, however, most of these application fields have not found their place in contemporary society as many other more effective solutions were offered. The major reasons why bryophyte applications nowadays are so limited is their small size, relatively low efficiency of their application in comparison to synthetic chemicals and materials. At the same time recent movement of people who prefer natural, organic, environmentally friendly and free from chemical food and other items, moss usage could find its comeback.

Nowadays situation has changed as far as recognisition of bioeconomy and it's importance in future society and need to look for new types of biological materials, posibilities of large scale cultivation of bryophytes in natural environments (Wichtmann *et al.,* 2016) and using biotechnological aproaches in bioreactors (bryotechnology). Therefore use of bryophytes can be reasessed to gain new value. There are also studies which show that *Sphagnum* mosses can be relatively easily cultivated in greenhouses using peat or water as a substrate (Gaudig *et al.,* 2014). The use of bryophytes in bioeconomy can support basic properties of bryophytes: their water and air retention capacity. Possibly one of large scale application fields of bryophytes could be their use as alternative for peat as a growing media. This approach has been tested by many researchers and its effiency has been proved by many studies (Glatzel and Rochefort, 2017; Kumar, 2017; Gaudig *et al.,* 2014). Likely it might seem as a new idea to use *Sphagnum* moss as a substrate, however actually many people are already doing in their homes. Mosses are the most widespread substrate for growing orchids as it provides the nesecary moisture levels and also some warmth that helps development of the plant. At the same time there are not many studies that take in consideration biological activity of bryophytes and their possible reaction with other plant cultures due to their secondary metabolite excretion in surrounding environment. For example compounds, 3-hydroxy-β-ionone and momilactone A and B, that are found in bryophytes, have shown inhibitor activity on growth of higher plants as well as other bryophytes (Kato-Noguchi *et al.,* 2010).

Bryophyte main potential for use in bioeconomy can be related to biological activity of their secondary metabolites. Large proportion of pharmaceuticals worldwide are either genuine or modified naturaly occuring compounds. One of the main challenges that modern world is facing is bacteria resistance to antibiotics, as the number of this type of bacteria as well as their resistance is growing, more new substances that could be used to fight them are required. Biologically active substances can be extracted both from animal kingdom and plant kingdom. Very rarely lower plants are used for extraction of pharmaceuticals and often only higher plants are considered to be of a value. This assumption has left bryophytes neglected for many years, but in last 20 years many have started to investigate chemical compostion in connection with their possible biological activity (Harris, 2009; Zinsmeister *et al.,* 1991). There are two main chemical substance groups that are assumed to have biological activity – polyphenols and lipids. More commonly bryophyte extracts are tested for their antimicrobial activity but there are also some proof of other types of biological activity. There has not been yet identified one specific substance that could be used as antibiotic, but the research continues and many moss extracts show this activity.

As already mentioned antimicrobial activity is the most tested parameter and it shows great promise. Moss lipid fraction has shown both antimicrobial an antifungal activities against – *Y. pseudotuberculosis, E. coli, S. aureus, E. faecalis, B. cereus, M. smegmatis, C. albicans* etc (Cansu *et al.,* 2012; Mellegard *et al.,* 2009). There is also evidence for bryophytes that their extracts help against wide range off illnesses such as – bruises, burns, snake bites, neurasthenia, convulsions, pneumonia, tuberculosis, scald and others (Asakawa *et al.,* 2013; Saboljevic *et al.,* 2010; Sing *et al.,* 2006; Spjut *et al.,* 1986; Hotson, 1921). Mostly studies show that biological activity such as: cytotoxicity, anti–HIV, DNA polymerase β inhibitory activity, antimicrobial, antifungal, insect antifeedant activity as well as nematocidal activity, is due to the terpenoids and aromatic compounds found in bryophytes. *Polytrichum* moss species also present diuretic activity and can be used to promote hair growth, *Polytrichum commune* have antipyretic and antitodal activity. Some mosses can be used as sedatives and for different heart problems (Cansu *et al.,* 2012; Asakawa, 2007; Zinsmeister *et al.,* 1991).

1.5. Environmental pollution impact on bryophyte composition

Different environmental factors can significantly influence bryophyte composition and their metabolism. Commonly as indicators to environmental (pollution) stress are used higher plants, considering their abundance and significance, hovewer in bryophytes as in lower plants the stress reaction could be significantly different (Kaiser, 2001). Major differences between bioindication processes if bryophytes are used instead of higher plants could be related to more simple biochemical reactions in their bodies on one hand, but mechanisms ensuring higher stress resistence on the other hand. Bryophytes as environmental stress indicators can be considered interesting as they are widely abundant and can be found in any ecosystem. Just this last aspect is a major reason why bryophytes and especially mosses have been widely used to study different pollutant impacts.

Early studies of metal accumulation in mosses and possibilities to use them for metal pollution indication were convincing in respect to high application possibilities, possibilities to cover large territories and to correlate air and water pollution with metal accumulation in mosses (Steinnes, 1995; Berg and Steinnes, 1997; Rühling and Steinnes, 1998). Metal uptake happens through whole moss surface area from atmospheric precipitation, both from particulate particles, both from precipitation water. Metal uptake is furthermore improved due to the high surface-to-volume ratio of mosses (Sun *et al.,* 2007) as well as absence of cuticles and root system. Recent studies on moss bioindication mechanisms provided controversial results, nevertheless value of bryophytes and especially mosses for biomonitoring purposes is high (Aceto *et al.,* 2003).

Abilities of mosses to absorb metals is a reason why they are widely used for monitoring of environmental pollution with metals, particulate matter and organic substances as well as biosorbents in environmental technologies to remove pollutants, for example, from waters. Mosses are also used for pollution analysis in so called "moss bag" method when alive or dead plants are packed in different material bags and afterwards exposed to air or water in order to provide contact with pollutants and then analyzed to estimate pollution level (Robalds un Kļaviņš, 2011; Ares *et al.,* 2012).

Several techniques are based on moss ability to absorb metals, however commonly mosses are considered as an inert matrix not much taking into consideration changes happening within their plant bodies (Gonzalez and Pokrovsky, 2014). The statement, "…that mosses are among the most efficient natural adsorbents of heavy metals" was based on assumption that adsorption of metal ions onto mosses takes place onto surface of non-living plants (Gonzalez and Pokrovsky, 2014). Amongst factors influencing adsorption capacity can be presence of acidic groups containing carbohydrates, for example, polygalacturonic acid on the external part of cellular wall and proteins in plasmatic membrane (Aceto *et al.,* 2003). Nonetheless it is important not only to study moss ability to absorb metals, but also to understand impacts of pollutants on biological and physiological processes of mosses. High metal concentration under experimental conditions can result in foliar injury (Taylor, 1984) and significant changes in moss chemistry due to yet unknown stress responses. Changes of metabolism in mosses can serve as suitable indicator of pollution impacts in sublethal concentrations and might find application to study pollutant impacts on their secondary metabolite synthesis. In higher plants number of biomarkers have been identified and they are widely used to study pollutant impacts. Most commonly studied biomarker is chlorophyll changes as well as oxidative stress in higher plants (Appalasamy *et al.,* 2017).

Reactive oxygen species (ROS) is a common product of plants reaction to pollution stress with metals. ROS includes hydroxyl radical, superoxide radical, hydrogen peroxide and others. ROS in plant can react with lipids, nucleic acids, proteins and their action can result in membrane damage, peroxidation of unsaturated compounds and other impacts. Metal ion impacts on plant bodies has been widely studied and metabolic changes has been detected, however in case of mosses only some aspects have been studied. One example are studies done on moss *Hypnum plumeaforme* (Sun *et al.,* 2009; Sun *et al.,* 2011). The stress reaction of single or combined Pb and/or Ni has been studied, and the dose dependant production of ROS as well as subsequent lipid peroxidation has been observed. Metal stress is resulting in increased activity of some enzymes, for example, of peroxidase. However enzyme activities as biomarkers have only limited value, considering their low stability and high costs of enzymatic activity analysis.

Metal ion impact on plant bodies has been widely studied and metabolic changes have been detected (Hall, 2002; Bhaduri and Fulekar, 2012) however in case of mosses only some aspects have been studied. As a major factor of metal pollution stress impacts, antioxidant enzyme responses have been identified (Bhaduri and Fulekar, 2012).

Also biomolecules, responsible about metal binding might play a significant role in metal detoxification as it has been demonstrated on example of functions of glutathione in water moss *Fontinalis antipyretica* (Bruns *et al.,* 2001). Exposure of the moss to the elevated concentrations of Cd resulted in a significant increase in glutathione pool and chelation of metals with –SH groups (Bruns *et al.,* 2001).

Another study on lead (Pb) and arsenic (As) impact on moss *Taxithelium nepalense* demonstrated decrease of dry matter and total chlorophyll as well as increase in lipid peroxidation, hydrogen peroxide and superoxide anion radical production as a result of the pollutant stress (Choudhury and Panda, 2004). An increase in superoxide dismutase activity with a simultaneous decrease in catalase, peroxidase and glutathione reductase activity was recorded as a result of metal impact stress (Choudhury and Panda, 2004).

In case of *Rhytadiadelphus squarrosus* moss it has been showed that induced stress using Cu and Pb cause qualitative changes in moss chemical composition (Guschina and Harwood, 2002). Pollution with led showed increased quantities of wax esters as well as decreased amounts of triacylglycerols. Increased amounts of Cu showed opposite results – decreased amounts of wax esters and increased of triacylglycerols. Results from studies suggest that heavy metal pollution causes changes in carbon flux in oxylation reactions that are associated with Kennedy pathway. In case of led pollution not only quantitative changes were observed but also qualitative – polar lipid diversity was influenced, range of phosphatidylcholine and unsaturated fatty acids containing 3 or more double bonds were decreased, while variation of zviterionic lipids and chloroplast glycerolipids was increased (Guschina and Harwood, 2002).

However the analysis of the amount of studies dedicated to stress impact on moss (bryophyte) secondary metabolism indicate only few studies, where the composition of metabolites has been studied. Further, more attention has been paid to studies of physiological stress impacts, especially drought stress impacts on the metabolite composition, for example, on lipid metabolism. It has been demonstrated that under desiccation conditions most vascular plants decrease the content in glycol- and phospholipids, while the content of neutral lipids is increased, but the degree of unsaturation of polyunsaturated fatty acids is decreased (Phan Ti *et al.,* 1987). The same result of drought stress reaction was found to be common for mosses on example of moss *Atrichum androgynum* and *Dicranum scoparia* (Guschina *et al.,* 2002). It has been demonstrated that the production of polyunsaturated fatty acids is regulated by presence of ROS and combination of light stress and ROS can initiate accumulation of acetylenic fatty acids (Guschina *et al.,* 2002). As a stress factor can be considered increased nutrient concentrations for moss species growing in nutrient poor environment. It has been found that increased nutrient (biologically available nitrogen compounds) load to *Sphagnum* species is accompanied by a decreasing concentration of polyphenols. This inverse relationship is consistent with reports that in *Sphagnum* mosses, polyphenol and protein biosynthesis compete for the same precursor (Bragazza and Freeman, 2007). For studies of abiotic stress, at first considering drought and salt stress, proteomic studies of moss species *Physcomitrella patens* has been suggested (Wang *et al.,* 2012). Important part in bryophyte cold/ heat and drought tolerance is their cell wall structure that regulates cell expansion and also has crucial role in plant development as well as in defence against microorganisms (Matsunaga *et al.,* 2004). Vascular plant cell walls are usually composed of cellulose, hemicellulose and pectin, together with enzymes, lignin and other functioning molecules. Bryophyte cell wall structure is much simpler in comparison to vascular plants and the structure of cell walls and existence of cytoplasmic monosaccharides influence resistance to many environmental stress factors (Proctor, 2000; Oldenhof *et al.,* 2006; Carpita and Gibeaut, 1993). This factor helps bryophytes to withstand many environmental stress factors and to adapt. One of the ways how bryophytes accomplish cold resistance has been tested on mosses *Hookeria lucens, Dicranum majus, Racomitrum lanuginosum* and *Polytrichum commune* were during cold weather as well as aging process levels of glucose and fructose decresed while level of sucrose didn't change (Melick and Seppelt, 1992; Smirnoff, 1991). Also due to the freezing bryophyte start to develop stress hormone – abscisic acid, which promotes synthesis of theandrose and stachyose, which accumulation helps to survive

cold periods. Usually this type of carbohydrates in bryophytes are located in protonema cells which are located in cell walls (Nagao *et al.,* 2006). In most cases cold an drought tolerance in bryophytes can be explained by alteration of hormone balance and regulation of osmotic pressure by changes of soluble carbohydrate composition, but this is not true in all of the cases as there are evidence of an abcisic acid – independent cold signalling pathway found in *Marchantia polymorpha* that leads to specific gene expression that is related to freezing and dessication tolerance (Minami *et al.,* 2005). Due to different environments in which bryophytes grows, there are different ways how they adapt and achieve tolerance on different stress factors (Nagao *et al.,* 2006; Wasley *et al.,* 2006; Pence *et al.,* 2005; Melick and Seppelt, 1992).

Regarding desiccation tolerance there is one more water source in bryophyte plant in comparison with vascular plants. Bryophytes not only have symplast and apoplast water but also have external capillary water. The importance of specific water source depends on environment where moss grows. In bryophytes with higher desiccation risk, mainly external capillary water dominates as a source of water in plant. Main water movement in bryophytes in comparison to vascular plant happens in the capillary spaces between leaves as well as other external spaces (Proctor, 2000). In moss *Selaginella inaequalifolia* also synthesis of carbohydrate trehalose impacts mosses tolerance from desiccation (Irudayaray *et al.,* 2010). In bryophyte stress tolerance many factors are involved, but one of the repeating and important parts of it is their unique carbohydrate composition which could have mostly supported colonisation of land (Renzaglia *et al.,* 2000).

Considering the complexity of metabolic reactions in plant bodies as a reaction on the stress impacts on mosses and to identify the organism reaction biomarkers and impacts on the metabolism an approach of the metabolomics can be suggested. Metabolomic approach has demonstrated its efficiency in several studies (Asakawa and Ludwiczuk, 2013; Castro *et al.,* 2017) and can be considered as a promising methodology to study pollutant stress on plant metabolism (Petersen *et al.,* 2011; Vecerova *et al.,* 2016). However this approach has not been tested on mosses.

2. MATERIALS AND METHODS

2.1. Sampling sites and sampling program

Moss sampling program was developed: 1) considering the need to use moss species which are common for the moss flora of Latvia (Strazdiņa un citi, 2011), 2) to exclude possibility to damage living habitats for rare species, 3) results of previous studies of bryophytes and possibilities to obtain amounts of secondary metabolites adequate for following studies (Asakawa *et al.,* 2013), 4) lowest possible human impacts (at least 300 m from roads and houses) on the mosses in their living habitats (to exclude impacts of pollution sources onto the moss metabolism), 5) possibilities to obtain bulk samples (> 300 g wet moss mass), 6) distance till sampling site and sampling costs. Considering these criteria sites in relative vicinity of Riga, were selected in ombrotrophic bogs (starting from edges of bogs till the center of bog), deciduous, coniferous and mixed forests (Figure 2.1.).

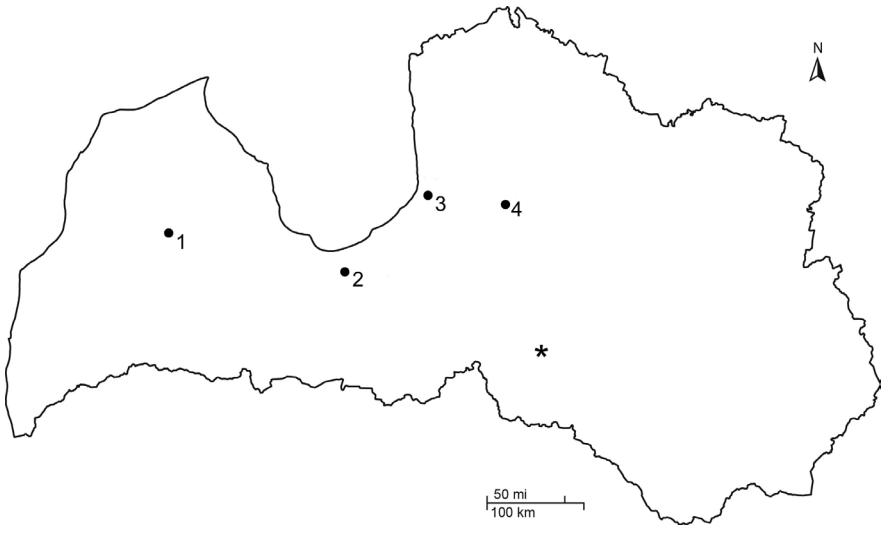

Figure 2.1. **Moss sample sampling sites** (• – sites where bulk samples (>10 g wet mass) of following mosses were sampled: 1, 4 – *Climacium dendroides, Hylocomnium splendens, Schimp Polytrichum commune, Polytrichum juniperinum, Pleurozium schreberi, Ptilium crista-castrensis, Rhytidiadelphus triquetrus*; 2 – *Aulacomnium palustre, Dicranum polysetum, Polytrichum juniperinum, Sphagnum girgensohnii, Sphagnum angustifolium, Sphagnum magellanicum, Sphagnum fallax, Sphagnum tenellum, Sphagnum c apillifolium, Sphagnum rubellum*; 3 – *Hylocomnium splendens, Pleurozium schreberi*. * – site of seasonal moss sampling of species *Sphagnum fallax, Sphagnum magellanicum, Polytrichum juniperinum, Pleurozium schreberi*)

To analyse seasonal samples of the moss composition, a study site in swamp forest, Sērene district, Jaunjelgava region considering mentioned criteria were selected.

Mosses collected and used in this study are epigeic (Strazdiņa u.c., 2011; Glime, 2007). All mosses receive nutritients for both substrate on which they are growing and from atmosphere (Glime, 2007). Some of the mosses might have one of the nutrient consumption types dominant, but it is not distinguished. In this study mosses are separated by their growth habitats – bog (*Sphagnum angustifolium, Sphagnum magellanicum, Sphagnum fallax, Sphagnum tenellum, Sphagnum capillifolium, Sphagnum rubellum*) and forest (*Aulacomnium palustre, Climacium dendroides, Dicranum polysetum, Hylocomnium splendens, Polytrichum commune, Polytrichum juniperinum, Pleurozium schreberi, Ptilium crista-castrensis, Rhytidiadelphus triquetrus, Sphagnum girgensohnii*).

2.1.1. Sampling program

Sampling of moss bulk samples. Moss (list of species and growth conditions are in Table 2.1.) samples were collected in the corresponding sampling site (Figure 2.1.) in the August 2014–2016. Site with the homogeneous coverage of the corresponding moss were selected, identifying them on site. With scissors the top (for example, 3 cm) of the selected mosses were cut off and put into plastic bag. For identification purposes sampled moss specimens were put into paper bags, labelled and put for a storage in Laboratory of Natural material research of University of Latvia. Moss samples were brought to laboratory for analysis as soon as possible (usually within the same day).

Sampling of moss seasonal samples. Moss *(Sphagnum fallax, Sphagnum magellanicum, Polytrichum juniperinum, Pleurozium schreberi*) samples were collected in swamp forest, Sērene district, Jaunjelgava region, south-central part of Latvia in growing seasons from April till October 2014–2015. Sampling sites were selected based on proximity of transport and representativeness close to natural conditions of forest stand. Each moss sample (~10 g) was collected after 2 weeks from April till October in growing season. Moss samples were squeezed to get rid of excess water in the field before collecting. Each collected moss sample was put into plastic bag. Moss samples were brought to laboratory for chemical analysis as soon as possible. In some cases mosses were kept in the refrigerator (not longer than a week) till transportation to laboratory. Samples were collected by Anna Mežaka.

2.1.2. Pre-treatment of samples

After collection, the plants were dry-cleaned of contamination with other mosses, needles, soil, peat, etc. The samples were stored at –20 °C in freezer. Before analysis, the materials were dried at < +40 °C, ground with a grinder and sieved through a 0.25 mm sieve. A voucher specimen has been deposited in the Laboratory of Environmental Quality, University of Latvia, Riga, Latvia.

Table 2.1. **List of the studied moss species with codes and their growth conditions**
(adapted from Strazdiņa u.c., 2014)

Species	Species code	Growth conditions
Aulacomnium palustre (Hedw.) Schwägr.	AP	Edges of bogs and humid places, usually on decaying wood; forms homogenous coverage of moss
Climacium dendroides (Hedw.) F. Weber & D. Mohr	CD	Deciduous forest, humid places, shadow
Dicranum polysetum Swartz	DP	Deciduous forests
Hylocomnium splendens (Hedw.) Schimp	HS	Deciduous forests with poor soil
Polytrichum commune Hedw.	PC	Coniferous forests, humid habitats
Polytrichum juniperinum Hedw.	PJ	Coniferous forests, raised and transitional bogs, near decaying wood or tree base
Pleurozium schreberi (Brid.) Mitt.	PS	Deciduous and coniferous forests, heaths with poor soil
Ptilium crista-castrensis (Hedw.) De Not.	PCC	Mixed forests, sunny, and averagely humid places
Rhytidiadelphus triquetrus (Hedw.) Warnst.	RT	Mixed forests
Sphagnum girgensohnii Russow	SG	Humid coniferous forests with acidic soil
Sphagnum angustifolium (C.E.O. Jensen ex Russow) C.E.O. Jensen	SA	Edge of bog/ bog border
Sphagnum magellanicum Brid.	SM	Bogs with acidic soil, bog pools or near them
Sphagnum fallax (H. Klinggr.) H. Klinggr.	SF	Middle of bogs, bog pools
Sphagnum tenellum (Bridel) Bory, Voy.	ST	Edges of bogs, bogs; humid places
Sphagnum capillifolium (Ehrh.) Hedw.	SS	Edge of bog/ bog border
Sphagnum rubellum Wilson	SR	Middle of bogs, bog pools, and other very humid places

2.2. Characterization of elemental composition of studied mosses

Elemental analysis for determination of moss chemical composition. The elemental analysis (C, H, N) of moss samples was carried out in triplicate using an Elemental Analyzer Model EA-1108 (Carlo Erba Instruments). The determined values were normalized with respect to ash content. Ash content was measured after heating 50 mg of each sample at 750 °C for 8 h. Oxygen was calculated as a difference between 100% and sum of the other elements (C, H, N). Statistical analysis of moss *Pleurozium shreberi* elemental composition variability amongst studied sampling stations was done using ANOVA test. Software used for the analysis was GraphPadPrism 7, confidence interval was 95%, P less than 0.05. N and C data shows significant differences according to ANOVA test amongst studied sampling stations.

Analysis of metals and trace elements in mosses. Wet digestion of organic matter, is widely applied for samples of biological origin, including mosses (Ekholm *et al.,* 2007; Šakalys *et al.*, 2009; Schroder and Pesch, 2010). Samples of moss were mineralized by wet digestion as follows: a) 0.1000±0.0020 g of dry sample was weighed on analytical balance in 50 mL glass beaker; b) 5 mL of concentrated HNO_3 and 2 mL of concentrated H_2O_2 (analytically pure reagents) were added; c) after holding overnight, sample solutions were digested by heating at 160 °C on heating block; d) after complete digestion and cooling sample solutions were filled up to 10 mL with ultra pure deionised water in polypropylene tubes. Each analysis was done in triplicate. Atomic absorption spectrometry was applied for macroelement (Ca, Fe, K, Mg, Na) and some microelement (Cu, Mn, Zn, Pb) quantification. Depending on element to be detected, sample solutions were analysed by flame atomic absorption spectrometry (FAAS), electro-thermal atomic absorption spectrometry (ETAAS). Content of K and Na was detected by FAES; content of Ca, Mg, Fe, Mn, Cu, Co, Cr and Zn – by ETAAS. Measurements were done by atomic absorption spectrometer *AANALYST 200* (Perkin Elmer). Performance of the applied analytical methods (Table 2.2.) was verified by comparative analysis of certified reference samples *IAEA-336 Lichen*.

Table 2.2. **Characteristic values for quantitative metal detection by atomic absorption spectrometer AANALYST 200** (Perkin Elmer)

Element	Limit of detection, mg/kg	Level of quantification, mg/kg	Uncertainty, %
Ca	6.65	21.94	4.42
Cd	0.01	0.04	4.00
Cu	0.15	0.50	4.50
Cr	0.02	0.05	4.00
Fe	0.41	1.35	5.23
K	14.22	46.94	3.01
Mg	3.49	11.55	6.70
Mn	0.51	1.67	4.51
Na	3.29	10.84	3.98
Pb	0.18	0.59	10.53
Zn	0.32	1.06	6.00
V	0.02	0.04	4.20

Use of pyrolysis-gas chromatography with mass-selective detection for study of bryophyte chemical composition. Py-GC/MS analysis was performed using a micro-furnace Frontier Lab Micro Double-shot Pyrolyser (Py-2020iD). The final pyrolysis temperature was 500 °C, heating rate – 600 °C/s. The pyrolyser was directly coupled with a Shimadzu GC/MS-QP 2010 apparatus fitted with a capillary column RTX-1701 (60 m × 0.25 mm) and a 0.25 μm stationary phase film. The injector temperature was set at 250 °C, ion source 250 °C with EI of 70 eV, and MS scan range m/z 15–350. Helium

was used as the carrier gas at a flow rate of 1 mL/min and a split ratio 1:30. The mass of the sample was 1 to 2 mg. The oven was programmed as follows: first 1 min isothermal at 60 °C; then 6 °C 1/min to 270 °C; and finally 10 min at 270 °C. Individual compounds were identified on the basis of a GC/MS chromatogram using Library MS NIST 147.LI13. The total molar areas of the relevant peaks were normalized to 100%, and the data from three repetitive pyrolysis experiments were averaged. Analysis was done in Latvian Institute of wood chemistry by Oskars Bikovens.

2.3. Spectroscopic characterisation of studied mosses

FT-IR spectroscopy for determination of moss chemical composition. Fourier transform infrared spectra were obtained using a FTIR spectrometer Spectrum BX (Perkin-Elmer Instruments) in KBr pellets. The sample pellets used were prepared by pressing 30 mg of mixture. The mixture contained 5 mg of moss sample and 200 mg of infrared-grade KBr.

^{13}C-NMR spectroscopy for determination of moss chemical composition. Solid-state ^{13}C-NMR spectroscopy was carried out using the technique of cross-polarization with magic angle spinning (CP/MAS). The spectra were recorded on a Bruker Avance wide-bore 600 MHz solid state NMR spectrometer equipped with a 4 mm MAS double-resonance probe. A 2 ms contact time and 2 s repetition time were used. The sample magic angle spinning was 10 kHz, and chemical shifts were referenced to adamantane at 38.48 ppm. Analysis was done by Oskars Purmalis.

2.4. Extraction of moss secondary metabolites

2.4.1. Optimization of extraction

Conventional extraction of moss. Dry samples were grinded in a mill, and 0.3 g of moss samples were weighed into 100 mL bottles with screw caps, adding 50 mL of solvent. Solvents such as ethanol (96%, 80%, 60%, 40%, 20%) diluted with demineralized (Millipore) water, acetone, dioxane, 10% hydrochloric acid, 10% formic acid, 5% hydrochloric acid, 5% formic acid, and DMSO (100%, 40%, 20%) were used. The bottles were shaken in a shaker for 24 h at 140 rpm.

Ultrasound-assisted extraction of mosses. Dry samples were grinded in a mill, and 0.3 g of moss samples were weighed into 100 mL bottles with screw caps, adding 50 mL of solvent. Solvents such as ethanol (96%, 80%, 60%, 40%, 20%) diluted with demineralized (Millipore) water, acetone, dioxane, and DMSO (100%, 40%, 20%) were used. Afterwards, the samples were exposed to ultrasound (100 W) in an ultrasound bath (Cole Parmer) for 20 and 40 min. The temperature was kept constant at +40 °C by regularly adding cold water. The bottles were then shaken in a shaker for 24 h at 140 rpm.

Extraction of mosses by microwave treatment. Dry samples were grinded in a mill, and 0.3 g of moss samples were weighed into Teflon extraction vessels, adding 50 mL of solvent (96%, 80%, 60%, 40%, 20% ethanol). The vessels were sealed using a Milestone

Twister. Extraction was performed using a Milestone Ethos One microwave oven, at 120 °C and 150 °C temperatures, with 1500 W power. The extraction took 40 min : 10 min to reach the chosen temperature, 20 minutes for steady extraction at the set temperature, and 10 minutes for the oven to cool down. After the extraction had been completed, the samples in the extraction vessels were placed at room temperature and held still for approximately 1 hour for the extract to cool down.

Supercritical CO_2 extraction. A dry bryophyte sample was grinded in a mill, weighing 15 g of the sample in a metallic column. The column was inserted into a preheated (60 °C) oven, setting the CO_2 flow to 10 mL per minute. The extraction was done using a Separex CO_2 supercritical extractor. After the first trials, it was concluded that coupled extraction was required for best results; therefore, the 96% ethanol flow of 5 mL/min was also set. The extraction experiments were conducted under 20 MPa pressure for 30 minutes and 60 minutes.

Soxlet extraction of mosses. A 20 g dose of dry sample was weighed in a fabric bag, which was then sealed and inserted into an extraction tube. The extraction was done using a Soxlet extractor and 96% ethanol as a solvent. The extraction process was done at 80 °C for 8 h and 24 h time periods.

2.4.2. Lipid extraction

Bryophyte samples (*Polytrichum commune* (PC), *Dicranum polysetum* (DP), were dried at +40 °C in an oven until constant mass. Dry samples were grinded in a mill and 1 g of bryophyte sample was weighed into 100 mL bottles with screw cap: 40 mL of extrahent (Table 3.10.) was added. Samples afterwards were treated for 40 min with ultrasound (100 W) in ultrasound bath (*Cole Parmer*), temperature was regulated with regular adding of cold water to keep constant temperature of +40 °C. The bottles then were shaken in a shaker for 24 h at 140 rpm. Extracts were filtered and stored until analysis at 4 °C up to 1 month. Extraction was done in triplicate and the yield (Table 3.10.) was estimated after removal of solvent at reduced pressure.

2.4.3. Extraction for testing of biological activity

Dry samples were grinded in a mill, and 2 g of moss samples were weighed into 100 mL bottles with screw caps, adding 70 mL of 60% aqeous ethanol. Afterwards, the samples were exposed to ultrasound (100 W) in an ultrasound bath (Cole Parmer) for 40 min. The temperature was kept constant at +40 °C by regularly adding cold water. The bottles were then shaken in a shaker for 24 h at 140 rpm.

2.5. Determination of summary characteristics of moss extracts

Determination of the total concentration of polyphenols in moss extracts. For determination of the total amount of polyphenols, the Folin-Ciocalteu reagent was used (Singleton *et al.,* 1999). After keeping at room temperature for ~1 hour, 1 mL of bryophyte extract was put into a test tube, adding 5 mL of 10% Folin-Ciocalteu reagent (Aldrich). In

5 minutes, 4 mL of 7.5% sodium carbonate (Aldrich) was added. The test tube was shaken thoroughly and kept in a dark place at room temperature for 2 hours. Absorption was then measured in a quartz cuvette (d = 1 cm) on a spectrophotometer (Hach-Lange DR 2800) at 725 nm wavelength. Results were calculated using a standard curve, expressed as gallic acid/100g (GE mg/100 g) dry matter (Singleton *et al.,* 1999; Silverstein *et al.,* 2005).

Analysis of flavonoid content. Before analysis, moss samples were kept in room temperature for ~1 hour. In a test tube 1 mL of moss extract is added and was mixed with 1 mL aluminium hydroxide 20% dispersion (*Aldrich*). Mixture was incubated for 1 h in a dark place in room temperature. Absorption is measured in a quartz cuvette (d = 1 cm) with a spectrophotometer (*Hach-Lange DR 2800*) at 415 nm wavelength. Three parallel measurements were carried out. Flavonoid content was determined with a calibration curve using quercetin dihydrate as a standard (Mitrovič *et al.,* 2011).

Determination of total carbohydrate amount. Before analysis, moss samples were kept in room temperature for ~1 hour. In a test tube 0.1 mL of moss extract was added and was diluted to 1 mL with distilled water. Afterwards 1 mL of 5% phenol (*Aldrich*) solution was added and rapidly 5 mL of concentrated sulfuric acid (*Aldrich*) was added. After 10 minutes the test tubes with the samples were carefully shaken and were left for 20 min in room temperature. Afterwards absorption was measured with a spectrophotometer (*Hach-Lange DR 2800*) at 490 nm. Three paralel measurements were carried out. Carbohydrate amount was determined with a calibration curve using glucose solutions as a standard (Silverstein *et al.,* 2005).

Radical scavenging activity determination in moss extracts using DPPH. After keeping at room temperature for ~1 hour, 0.3 mL of bryophyte extract was put in a test tube and mixed with 3.6 mL of 4% 2-diphenyl-1-picrylhydrazyl (DPPH) solution in 96% ethanol (Aldrich). The mixture was incubated for 20 minutes in a dark place at room temperature. Absorption was measured in a quartz cuvette (d = 1 cm) with a spectrophotometer (Hach-Lange DR 2800) at 517 nm wavelength (Mitrovič *et al.,* 2011). Three paralel measurements were carried out. Radical scavenging activity was expressed as GE mg/100 g.

2.6. Fractionation of moss secondary metabolites

Solid Phase Extraction (SPE) fractionation of lipid mix/ moss lipid extracts. The scheme used to separate the lipid classes is shown in Table 2.3. The SPE columns were placed on a vacuum manifold and conditioned by washing 3 times with a total volume of 9 mL hexane. The vacuum was adjusted to generate a flow rate ~0.5 mL/min. Five replicate samples of bryophyte lipid extract were analysed. The procedures for the fractionation of lipids were as follows: the dried lipid extract was taken up in a minimal volume of (< 0.5 mL) of hexane-chloroform (1:1) and loaded into the cartridge column. The column was eluted with eluents (each eluent 10 mL) in a sequence as indicated in Table 2.3. Eluates were dried under mild stream of nitrogen. First fraction after dissolving in chloroform were directly submitted for analysis with GC-MS, but the remaining 2 after dissolving in acetonitrile and derivatization with BSTFA. Repeatability was evaluated measuring the variability of five consecutive analyses of lipid mixture and moss lipid extract.

Table 2.3. **Elution scheme for separation of lipid classes using bonded phase silica and aminopropylsilica solid phase extraction columns**

Sorbent	Eluent	Groups of substances
Silica	Hexane	Alkanes, sterols, fatty alcohols
	Hexane/chloroform 5:1	Esters, ketones, aromatic substances
	Chloroform	Sterols
Aminopropylsilica	Hexane	Alkanes, sterols, fatty alcohols
	Hexane/chloroform 5:1	Esters, ketones
	Chloroform	Fatty acids, sterols
	Diethylether/acetic acid 98:2	Fatty acids

Eluates were dried under mild stream of nitrogen. First fraction after dissolving in chloroform were directly submitted for analysis with GC-MS, but the remaining 2 after dissolving in acetonitrile and derivatization with BSTFA. Repeatability was evaluated measuring the variability of five consecutive analyses of lipid mixture and moss lipid extract.

2.7. Chromatographic analysis of moss secondary metabolites

UPLC analysis of moss extracts. Chromatography separation was performed with the Waters Acquity ultra-performance liquid chromatography (UPLC) system, equipped with a quaternary solvent manager, UV/Visible detector, thermostated auto sampler and column heater. Data acquisition and analysis were performed using the Empower3 system. The chromatographic separations were carried out using the Acquity UHPLC BEH C8 (2.1 × 50 mm, 1.7 μm) column and solvent gradient elution program. Two solvents – (A) water/acetonitrile (65:35 by volume) with 2% formic acid in water and (B) acetonitrile – were used as mobile phase. Separation was done with the following time-gradient program: initial (100% A), 10.0 min (78.0% A), 16.0 min (65.0% A), 21.0 min, (62.0% A), 30.0 min (50.0% A), 34.0 min (40.0%), 37.0 min (37.0% A), 39.0 min (30.0%), 40.0 min (23.0% A), 53.0 min (20.0%), and 54.0–60.0 min (10.0% A). The elution was performed at a flow rate of 0.25 mL/min, and the detector was set at 270 nm. The column was operated at 30 °C temperature. The sample injection volume was 5 μL, and the total run time was 60 min. The strong wash solvent was 70% acetonitrile in water, and the weak wash solvent – 10% acetonitrile in water.

Analysis of lipid extracts by gas chromatography – mass spectrometry. 20 mg of moss extract dry weight were weighted in glass vessel. Dry residue was dissolved in 1.4 mL of acetonitrile, 0.5 mL of inner standard and 0.1 mL of BSTFA reagent was added. Afterwards vessel was tightly sealed and heated at +60 °C for an hour. In order to ensure that all BSTFA has reacted 0.01 mL of methanol is added to the mixture. After cooling down to room temperature, glass vessel with the mixture is used for further GC/MS analysis.The GC-MS instrumentation consisted of Clarus 680 chromatograph and a Clarus SQ 8 C mass spectrometer. The separations were performed on a Elite-5ms (5% phenyl-95% methyl

polysiloxane) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium (99.9999%) was used as carrier gas at initial flow 2.0 mL/min for 2 min and follow held constant at 1.0 mL with split flow 10.0 mL/min. The column temperature was maintained at 75 °C at 2 minutes, then programmed from 75 to 130 °C at 20 °C 1/min and from 130 to 310 °C at 4 °C 1/min and finally held at 310 °C for 12 min, total run time 59.25 min. The sample of 1.0μL was injected in a split mode injector (4/1) with an autosampler. The mass spectrometer was operated in the electron impact mode at 70 eV ionization energy and scanned from 42 to 650 Da was scanned with a cycle time of 0.5 s. Multiplier was operated at 1700 V. The column injector and the transfer line temperatures were set at 290 and 250 °C, respectively, and the ion source temperature was 230 °C. Retention time (Rt, min) and MS fragmentation patterns of the known compounds were compared with literature and data base NIST. All peaks were quantified by peak area.

Analysis of ethanol extracts by Ultra performance liquid chromatography coupled with time-of-flight mass spectrometry. Chromatographic analyses were performed on a modular UPLC system Agilent *1290 Infinity* series (Agilent Technologies). LC separations were achieved by using an *X Bridge C8 3.5 μm,* 2.1 × 150 mm (Waters) column with a mobile phase composed of 0.1% formic acid (channel A) and acetonitrile (channel B) in a gradient mode at a flow rate of 0.3 mL/min. The injection volume was 10 μL. The UV detection of compounds was monitored using the diode array detector (DAD) at 210, 254, 280 and 330 nm. The high resolution mass spectra (HRMS) were taken on an *Agilent 6230 TOF LC/MS* (Agilent Technologies) with electrospray ionization (ESI). The parameters of the source were: positive ionization mode, drying gas flow 10 L/min and temperature 325 °C, fragmentor ionization 100 V. Internal reference mass 149.02332000 m/z and 922.00979800 m/z for all sample analyses were used. The experimental data were handled using MassHunter version B05.00 software (Agilent Technologies). The ethanol extracts of moss were filtered through 0.45 *μ*m filters (Nonpyrogenic Sterile-R, Sarstedt) to remove solid particles and mechanical admixtures. Filtered samples were diluted with acetonitrile and injected into LC system. The injections were performed in duplicate. The calibration curve of standard solutions was constructed by plotting the ratio of the average chromatographic peak area and mass concentration of the gallic acid. According to reflected data the regression equation of the trend line was calculated. Standard solutions were injected in triplicate and the corresponding peak areas were recorded. The relative standard deviation was determined to be below 1%. The obtained calibration curve showed linearity of correlation coefficient (R^2) in the concentration range 0.993. Analysis was done in LU Faculty of chemistry by Ilva Nakurte.

2.8. Biological activity of moss secondary metabolites

Cytoxicity testing of moss extracts. Following cell lines obtained from ATCC and European ECCC collections were used: Rat glioma cells (C6), Human epidermoid carcinoma (A431), Human lung carcinoma (A549), Mouse melanoma cell lines (B16-F10), Human breast adenocarcinoma (MCF-7), Human colorectal carcinoma (CaCo-2). Cytotoxicity of extracts was tested on monolayer cultures C6, A431, MCF-7 and CaCo-2. Cell lines A431, MCF-7 and CaCo-2 were cultivated in DMEM media

with 10% fetal bovine serum), 2 mM glutamine and 1% amino acids without antibiotics. C6 cells were cultivated in F12HAM media, containing 10% fetal bovine serum and 2 mM glutamine. Cells at concentration 5×10^4 cells/mL (A431, MCF-7, Caco-2 cell lines) and 4×10^4 cells/mL for C6 cell lines, were sown in 96-well plate and cultivated for 24 hours at 37 °C, 5% CO_2 atmosphere. Then cell extracts were added at differing concentrations and cultivated for another 72 hours. Living cells were determined analyzing activity of mitochondrial enzymes after staining with the methylthiazolyldiphenyltetrazolium bromide (Sigma) (MTT). After the cultivation the solution of MTT (final concentration 2 mg/mL in HBSS buffer) were added and cultivated for additional 3 hours at 37 °C, 5% CO_2. Then color was extracted with 0.2 mL DMSO. Optical density of DMSO extract (proportional to amount of living cells) was measured at $\lambda = 540$ nm. IC_{50} was calculated with Graph Pad Prism®. Analysis was done in Institute of organic synthesis.

Antimicrobial activity of moss extracts. Moss ethanol extract antimicrobial activity was determined with the agar well diffusion method (Perez *et al.,* 1990). In experiment used microorganisms were obtained from the Microbial Strain Collection of Latvia. Antimicrobial activity was tested using bacteria: *Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus mirabilis, Escherichia coli.* Antimicrobial activity testing was performed on Muller-Hinton agar. Fresh bacteria material with approximately 10^6 colony forming units/ mL of tested organism was prepared. 70% moss ethanol extracts of 70 µL of each sample and control (70% ethanol) were applied into 7.0 mm diameter wells. Afterwards plates were incubated at 37 °C for 24 h. After incubation inhibition zone around the well was measured in mm and used to express the antimicrobial activity. Each sample was tested in triplicate.

2.9. Accumulation of metals in studied mosses and influence on their composition

Pleurozium schreberi and *Sphagnum magellanicum* are a widespread moss species in Northern Europe and Latvia and they were collected in sampling sites No. 2 (Figure 2.1.) far from local sources of air pollution, cutting the living parts of the mosses off. After collection, samples were transferred to the laboratory in plastic bucket. For toxicity studies of Pb, Zn and Cu in form of their nitrates were used.

Samples were thoroughly washed with deionized water ann put in plastic shales. Each shale were filled with fine granular, washed quartz (20 g), allowing the rhizoids to be directly wetted. On average in each shale were filled in 3 g of mosses. The specimens were cultured with 10 mL modified Mohr medium (Carginale *et al.,* 2004), pH 6.5 (KNO_3 100 mg, $CaCl_2 \times 4H_2O$ 10 mg, $MgSO_4$ 10 mg, KH_2PO_4 136 mg, $FeSO_4$ 0.4 mg to 1000 mL distilled water) used as control and in the same medium with the addition of 0.001; 0.005; 0.01; 0.05; 0.10; 0.25; 0.50, 1, 2, 3, 4 mM $Zn(NO_3)_2 \times 3H_2O$, $Cu(NO_3)_2 \times 5H_2O$ and 0.1; 1;10;100 $Pb(NO_3)_2$ µmol/L. Moss samples were sprayed with part of the solutions and other part of solution portion was poured in plastic shale , to ensure assimilation possibilities through leaves and rhizoids. The cultures were stored for 72 h in a climatic chamber with temperature of 13/20 °C (night/day cycle), 70% constant RH, and 16 h light /8 h dark photoperiod. Each experiment was run in triplicate and repeated three times.

After treatment the samples were removed from the plastic shale, carefully washed with deionised water, dried for metal and oxidative stress parameter analysis.

2.10. Data treatment and analysis

To evaluate and compare the FTIR spectra major sorption line intensity between different functional groups and moss species, the hydroxyl group was adopted as one unit, and another major group's absorption intensity was calculated as a ratio to the hydroxyl group. All CPMAS ^{13}C NMR spectra were integrated by the regions of chemical shift. The sum of positive numbers was adopted as 100% and then the percentage of each region of chemical shift was calculated.

Hierarchical cluster analysis (HCA) was used to identify relatively homogeneous clusters of samples based on their similarity (Levent *et al.,* 2003). The agglomerative procedure starts with each object in a separate cluster and then combines the clusters sequentially, reducing the number of clusters at each step until all objects belong to only one cluster (Nikolova and Jaworska, 2004). We used Ward's method (Ward, 1963) of hierarchical clustering which uses an analysis of variance approach to evaluate the distances between clusters. Beginning with N clusters consisting exactly of one entity, the similarity matrix is searched for the most similar pair of clusters, and the number of clusters is reduced by one by merging the most similar pair of clusters with the minimum increase in the total within-group error sum of squares (Hervada-Sala and Jarauta-Bragulat, 2004). The analysis was performed using IBM SPSS Statistics 19.

Principal Component Analysis (PCA) results were obtained using IBM SPSS by warimax rotation and Kaiser normalization was used as well.

3. RESULTS AND DISCUSSION

3.1. Composition of studied mosses

3.1.1. Elemental composition of studied mosses

Elemental composition of plants characterises their basic composition, can give a hint about major building blocks as well as can characterise growth conditions and even environmental pollution levels. Regarding the studied mosses, relatively low variability in their elemental composition was revealed (Table 3.1.). The concentration ranges of basic elements in the studied mosses were as follows: C 40–43%; H 5.5–6%; N – 0.4–2%; S – ~0%. The O content ranged between 48–53%, (as determined by mass balance). For comparison, the elemental compositions of mosses in another study have been determined following: C – 45–63%; H – 3.6–7.7%; N – 0.4–5.8%; S – 0.5–1.5%; ash content – 0.1–1.2% (Zaccone *et al.,* 2007). Thus, it can be stated that there is high similarity in the basic organic structural molecules of the studied mosses, and C/H as well as C/O ratio indicate that oxygen rich molecules (carbohydrates) are important possible structures of mosses. However, some differences were detected amongst the studied species, such as low concentration of nitrogen in *Sphagnum* species, in comparison with the other mosses.

Table 3.1. **Elemental composition (%) of the studied moss species**

Species	C	H	N	O
Aulacomnium palustre	43.51±0.01	5.72±0.02	0.515±0.015	50.25±0.02
Climacium dendroides	41.32±0.02	5.39±0.02	1.683±0.019	51.61±0.01
Dicranum polysetum	42.15±0.02	5.85±0.03	1.325±0.019	50.67±0.04
Hylocomnium splendens	42.12±0.02	5.43±0.01	1.215±0.007	50.23±0.10
Pleurozium schreberi	43.24±0.01	5.83±0.11	1.230±0.040	49.78±0.10
Polytrichum commune	43.74±0.02	5.95±0.02	1.123±0.015	49.18±0.01
Polytrichum juniperum	41.99±0.02	5.89±0.02	1.988±0.019	50.13±0.01
Ptilium crista-castrensis	42.24±0.02	5.68±0.03	1.219±0.019	50.86±0.04
Plagiochila asplenioides	42.10±0.02	5.80±0.02	1.488±0.003	50.61±0.01
Rhytidiadelphus triquetrus	42.47±0.02	5.52±0.01	1.121±0.007	50.89±0.10
Sphagnum angustifolium	41.75±0.01	5.53±0.11	0.430±0.040	52.28±0.10
Sphagnum capillifolium	40.98±0.01	5.58±0.01	0.416±0.012	53.01±0.01
Sphagnum girgensohnii	42.05±0.02	5.75±0.02	1.050±0.030	51.16±0.04
Sphagnum magellanicum	42.21±0.01	5.54±0.01	0.516±0.013	51.72±0.01
Sphagnum fallax	42.08±0.01	5.32±0.01	0.402±0.012	52.20±0.01
Sphagnum rubellum	42.15±0.02	5.42±0.02	0.850±0.030	51.58±0.04

Growth conditions as well as environmental impacts can influence elemental composition of mosses as demonstrated by a study of spatial distribution of *Pleurozium schreberi* in territory of Latvia in 40 sampling sites regarding composition of N and C (Figure 3.1., 3.2.). Sampling plan and methodology described in Tabors *et al.*, 2017.

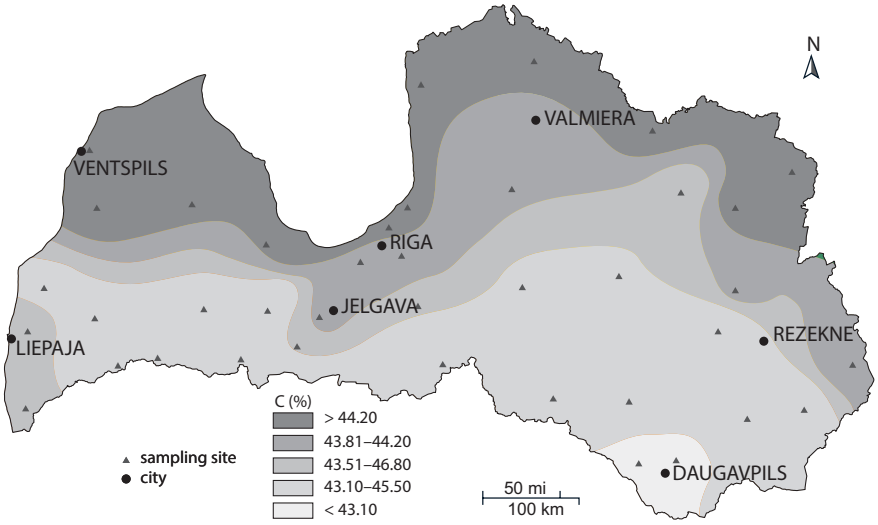

Figure 3.1. **Concentration of nitrogen (%) in moss *Pleurozium schreberi* in Latvia.** Sampling plan and methodology described in Tabors *et al.*, 2017

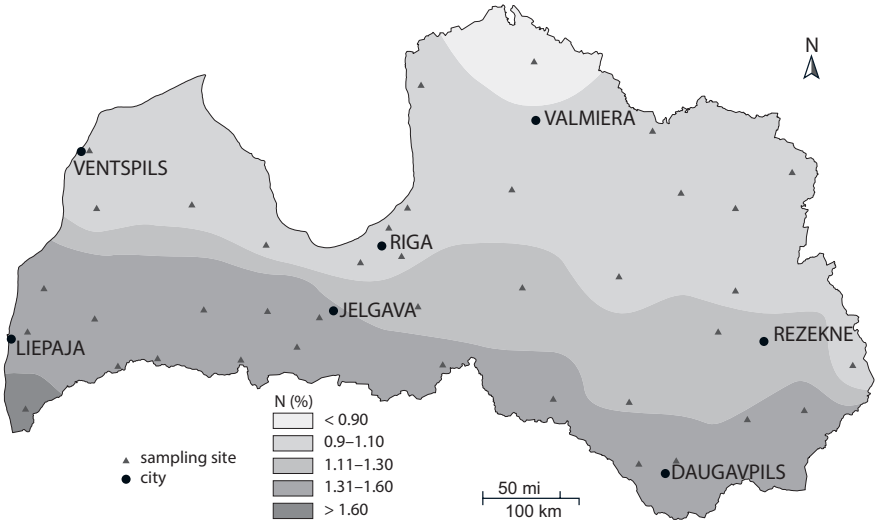

Figure 3.2. **Concentration of carbon (%) in moss *Pleurozium schreberi* in Latvia. S**ampling plan and methodology described in Tabors *et al.*, 2017

Table 3.2. **Major and trace elements in the studied moss samples (mg/kg)** (codes of the studied species as indicated Table 2.1.)

Species	Na	Mg	K	Ca	Fe	Mn	Ni	Cu	Zn	Cd	Pb	Cr	V
AP	178±12	1254±22	4641±30	2662±20	170±5	262±12	8.2±0.1	6.5±0.2	31±2	0.20±0.05	3.2±0.1	0.12±0.03	0.41±0.04
CD	98±6	1839±22	10140±40	9548±40	121±5	77±6	0.5±0.1	5.8±0.2	41±2	0.35±0.05	2.4±0.1	0.28±0.03	0.51±0.04
DP	622±15	2039±22	3060±30	7353±40	206±5	241±12	1.9±0.1	6.3±0.2	204	0.27±0.05	2.9±0.1	0.26±0.03	0.37±0.04
HS	104±6	1620±22	8438±40	3327±20	36±1	51±6	1.5±0.1	5.3±0.2	34±2	0.25±0.05	1.6±0.1	0.22±0.03	0.24±0.04
PC	87±6	944±10	6210±40	2561±20	779±8	126±12	1.7±0.1	8.1±0.2	42±2	0.17±0.05	3.0±0.1	0.18±0.03	0.34±0.04
PJ	405±15	1344±22	3140±30	3390±40	80±1	38±3	0.6±0.1	18.4±0.2	179±8	0.21±0.05	1.7±0.1	0.32±0.03	0.55±0.04
SS	171±12	1238±22	7715±40	2461±20	208±5	175±12	1.6±0.1	11.2±0.2	152±9	0.26±0.05	1.3±0.1	0.38±0.03	0.34±0.04
RT	170±12	1693±22	7311±40	3963±40	64±1	241±12	0.9±0.1	5.5±0.2	87±2	0.23±0.05	1.9±0.1	0.42±0.03	0.54±0.04
PCC	226±12	1287±22	10123±40	4126±40	103±5	256±12	1.8±0.1	14.1±0.2	48±2	0.26±0.05	1.1±0.1	0.28±0.03	0.21±0.04
PS	240±12	1022±22	4779±30	3182±20	157±5	282±12	0.3±0.1	4.2±0.2	34±2	0.33±0.05	1.4±0.1	0.29±0.03	0.30±0.04
SM	643±15	1040±22	2739±20	3695±40	507±8	28±3	1.3±.1	5.0±0.2	42±2	0.10±0.05	1.5±0.1	0.12±0.03	0.23±0.04
SF	615±15	1697±22	3637±30	6341±40	162±8	50±6	1.0±0.1	12.4±0.2	88±5	0.11±0.05	1.6±0.1	0.24±0.03	0.34±0.04
SA	544±15	1185±22	3785±30	4560±40	99±1	85±6	1.2±0.1	11.8±0.2	93±5	0.15±0.05	3.2±0.1	0.18±0.03	0.56±0.04
SG	253±12	1128±22	7535±40	2912±20	83±1	171±12	1.4±0.1	3.5±0.2	190±10	0.22±0.05	2.4±0.1	0.27±0.03	0.43±0.04
SR	572±15	1093±22	5073±30	2801±20	123±5	29±2	0.6±0.1	3.3±0.2	71±5	0.15±0.05	2.3±0.1	0.34±0.03	0.73±0.04

Important groups of elements characterizing composition of mosses are major and trace metallic elements (Table 3.2.). As far as all sampling sites of bulk samples were located in areas remoted from direct pollution sources, it can be supposed that mostly natural processes are the sources of major elements, while presence of many trace elements can occur due to environmental pollution (Berg and Steinnes, 1997). Trace element concentrations in mosses traditionally are used for environmental monitoring purposes (Šakalys *et al.,* 2009; Schroder and Pesch, 2010). However, among the studied metals, those directly associated with pollution (Pb, Cd, Ni) have the lowest variability and low concentrations, but concentrations of major elements (Na, K, Ca, Mg, Fe, Mn) and also essential trace elements with predominant natural origin (Ni, Cu, Zn) have highest variability among the species.

3.1.2. Structural composition of studied mosses

Major structural elements of the studied mosses were characterised using Fourier transform infrared spectroscopy (FTIR), Pyrolysis-gas chromatography/mass spectrometry (PyGC/MS) and Cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance spectroscopy (CP MAS ^{13}C NMR).

The FTIR spectra of the analysed mosses (Figure 3.3.) can be divided into regions (Table 3.3.) depending on the presence of important functional groups. Absorption bands in the 3600–2800 cm^{-1} spectral region were very broad; absorbance in this region is determined by the presence of –OH groups. Sorption at wavelengths 2950 and 2850 cm^{-1} identified the presence of CH_3– and CH_2– groups, respectively. Typical minor sorption lines were common for the region around 1700 cm^{-1} (1725–1700 cm^{-1}), which is characteristic for carbonyl groups in aldehydes, ketones and carbonic acids. The actual sorption maximum greatly depends on the conjugation degree, presence of substituents and hydrogen bonding. In the spectral region 1690–1500 cm^{-1} it was possible to identify the sorption maximum of amide bonds (1640–1620 cm^{-1} and 1550–1540 cm^{-1}). In the region 1625–1610 cm^{-1}, the sorption indicated the presence of aromatic C=C and carbonyl groups, and quinones. At the wavelengths 1470–1370 cm^{-1}, the bands were typical for C–H and O–H bonding and sorption maximums typical for C–O. For the wavelengths < 1000 cm^{-1} fingerprint patterns were evident. Sorption in this spectral region provides information about the possible proportion of carbohydrates. Sorption at 1080 cm^{-1} showed OH deformation or C–O stretch of phenol and alcohol OH groups, and 1040 cm^{-1} indicated C–O stretch of polysaccharide components.

Table 3.3. **Assignment of major spectral bands in the Fourier transform infrared spectra of studied mosses**

Wavenumber, cm^{-1}	FTIR band assignments and main origins.
3460–3380	O-H and N-H stretching vibration. Mainly carbohydrates and protein-originated compounds.
3000–2840	C-H stretch in methyl and methylene groups. Mainly lipids, with a contribution from proteins, carbohydrates, and phenolics.
1735–1710	C=O stretch in unconjugated ketones. Carbonyls in saturated ester groups. Various origins, e.g., carbohydrates.
1650	Amide I and C=O stretch in conjugated aryl ketones. Proteins, with a contribution from phenolic compounds.
1605–1595	Aromatic skeletal vibration plus C=O stretch. Mainly phenolic compounds.
1546	Amide II. Mainly proteins.
1515–1505	Aromatic skeletal vibration. Mainly phenolic compounds.
1430–1420	O-H bending. Cell wall polysaccharides, alcohols.
1380–1370	Aliphatic C-H stretch. Cell wall polysaccharides.
1270–1240	C-C plus C-O. Cell wall polysaccharides.
1160	CH_2, OH and C-O stretch of various groups. Cell wall polysaccharides.
1100–1000	OH and C-O deformation in secondary alcohols and ethers. Cell wall polysaccharides.

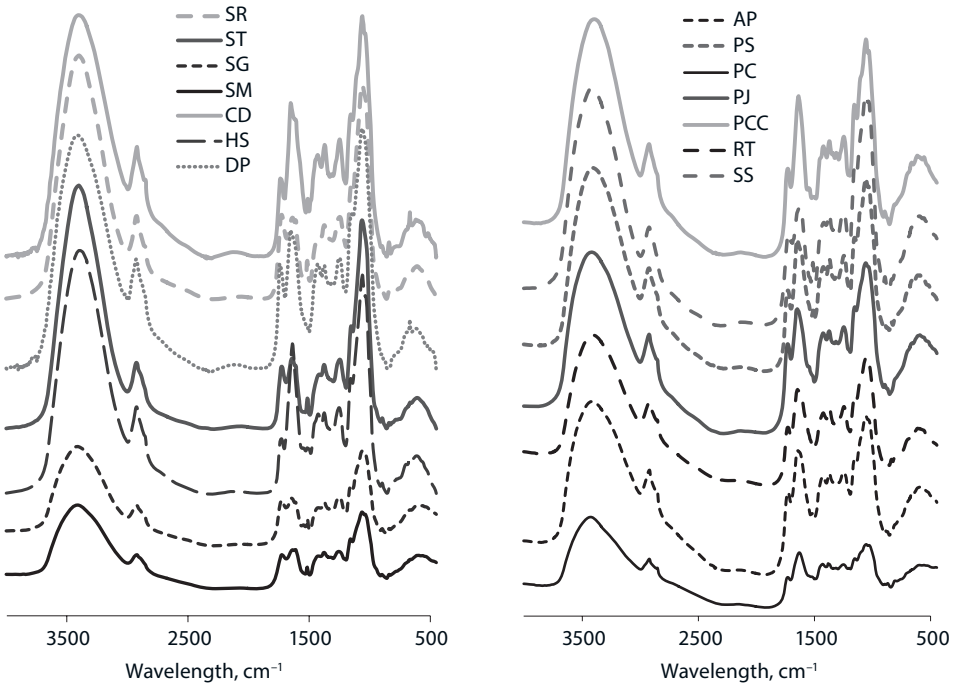

Figure 3.3. **FTIR spectra of mosses** (codes of the studied species as indicated Table 2.1.)

Relative intensities of the main bands and their positions exhibited variations between species (Figure 3.3.). Separate weaker bands could be used as markers for differentiating some species. *Sphagnum* species exhibited a well-resolved band of aromatic skeletal vibrations at 1505–1515 cm^{-1}, which are weak and poorly resolved for other species. In addition, the amide I band intensity in comparison with ester band at 1735 cm^{-1} was lower for *Sphagnum*, and this result coincides with the results of elemental analysis. The previous results indicated that *Sphagnum* species contain more phenolic compounds and less N compounds in comparison with other mosses. *Sphagnum* species could be easily identified on the basis of FTIR spectra. The absence of any absorption band or shoulder at 1470–1460 cm^{-1} typical for C-H deformation of CH_3- in methoxyl groups indicated that the phenolic compounds of *Sphagnum* are mainly non-methoxylated.

FTIR spectra of the studied mosses (Figure 3.3.) demonstrate evident similarities in major sorption lines, but differ in their intensity, as well as in intensity of minor sorption lines. Thus, FT-IR spectra also indicate similarity in the composition of studied mosses. However, the obtained differences do suggest that spectroscopic analysis can be used as a tool to determine differences between species. It is likely that the differences in the composition of studied mosses are determined not so much by their basic structural elements, but rather by the presence of secondary metabolites.

Pyrolysis-gas chromatography/mass spectrometry (Schellekens *et al.,* 2009) was used to characterize the chemical composition of the mosses. Table 3.4. lists the components in pyrolysates of the studied mosses. The presence of dominant product groups from each moss species is illustrated in Table 3.4. The pyrolysates of the studied *Sphagnum* species were dominated by carbohydrate decomposition products and simple phenolic compounds. The dominant compounds were aliphatic compounds of low molecular weight, phenol and 4-ethenylphenol and 1,6-anhydro-β-D-glucopyranose. Their origin is likely carbohydrates and tannin-like polyphenolic biopolymer with 4 – isoprenylphenol,

Table 3.4. **Relative abundance (%) of the main groups of pyrolysis products of mosses**

Species	MA	C	Ar	L	Lp	N
AP	14.30±0.28	10.39±0.23	0.76±0.01	0	1.17±0.03	0
PS	20.68±0.31	5.96±0.18	2.52±0.05	0	1.35±0.03	0
PC	17.03±0.25	6.05±0.11	0.72±0.02	0	1.97±0.04	0.08±0.002
PJ	16.16±0.24	5.75±0.13	0.60±0.02	0	1.23±0.03	0
PCC	19.90±0.39	4.74±0.11	0.79±0.02	0	1.45±0.03	0
RT	20.68±0.31	5.96±0.14	2.52±0.06	0	1.35±0.03	0
SA	17.72±0.26	6.38±0.15	3.78±0.07	0	1.10±0.02	0
SS	15.84±0.42	7.72±0.21	3.06±0.06	0	1.35±0.03	0
SG	16.87±0.21	5.58±0.17	4.26±0.09	0	1.47±0.03	0.02±0.001
SM	14.26±0.21	9.12±0.31	3.48±0.07	0	1.24±0.03	0

MA – multi-origin aliphatic compounds with C<6; C – furan originated from carbohydrates, pyran, and cyclopentene derivatives; Ar – aromatic compounds (except methoxylated phenols); L – methoxylated phenols; Lp – compounds originated from lipids C > 6; N – N-bearing compounds

being at least partially bound to it. The importance of cellulose in *Sphagnum* structures is indicated by the high abundance of polysaccharide products. No lignin markers were detected. The importance of cellulose in *Sphagnum* structures is indicated by the high abundance of polysaccharide products, e.g. methylglyoxal, 2,3–butanedione, 2-hydroxyacetaldehyde, acetic acid and other pyrolysis products of low molecular weight.

Other studied moss species had pyrolysis products similar to the *Sphagnum* species, although with reduced phenol abundance and absence of 4-isoprenylphenol. Abundant polysaccharide products confirm the importance of cellulose in the structural make-up of mosses. No lignin markers were detected, thus indicating the major difference in comparison with the composition of higher plants. The dominant compound from *Polytrichium* species was acetic acid and its methyl ester, derived from xylan (van Smeerdijk and Boon, 1987). In contrast to other studied moss species, *Polytrichium* species contained derivatives of hexadecenes (lipid origin) as well as cyclic compounds indicating presence of a relatively complex lipid fraction. Other studied pyrolysates were dominated by phenols, 2-methoxyphenols (guaiacol units), 2,6 dimethoxyphenols (syringyl units) and polysaccharide pyrolysis products. High abundance of 4-ethenylphenol and 4-ethenyl-2-methoxyphenol indicated the presence of angiosperm ligno-cellulose.

Solid-state cross-polarization with magic angle spinning (CP/MAS) ^{13}C NMR spectra provides direct evidence of structural features of plant material (Spaccini *et al.,* 2006; Francioso *et al.,* 2001). Signals in these NMR spectra represent different types of carbon atoms. In general, the spectra of all studied mosses showed similar characteristics, presenting signals that can be associated with different functional aliphatic (C–H, C–N, O–CH_3 and other groups and carbon in polysaccharides) and aromatic groups. The use of cross-polarization (CP) and magic angle spinning (MAS) techniques resulted in better resolved spectra, thus allowing their division into eight main regions attributed to carbon atoms in major structural units. The cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance spectra (CPMAS ^{13}C NMR) of solid samples of the studied bryophyte species also demonstrated evident similarities in intensive peaks, whereas minor intensity peaks differed depending on a family or species of mosses. The region of chemical shifts from 0 to 50 ppm corresponds to aliphatic carbon. In all spectra, three peaks at 21, 30, and 33 ppm were sharp and well-resolved. In the spectra of *Sphagnum* species, fourth peak at 19 ppm was also well-resolved. These four peaks can be assigned as –CH_3 (19 and 21 ppm) and –$(CH_2)_n$– (30 and 33 ppm) respectively (Nierop *et al.,* 2001; Mao *et al.,* 2011).

The region of chemical shifts from 50 to 92 ppm corresponds mainly to the ring carbon of carbohydrates (Karlström *et al.,* 1995). In all spectra, five peaks at 63, 65, 73, 84, and 89 ppm were sharp. They can be assigned to carbon of methoxyl groups O–CH_3 (63 ppm), HO–CH_2– (65 ppm), –CH(OH)– (73 ppm), and other methoxyl groups (84, 89 ppm) (Karlström *et al.,* 1995). The region of chemical shifts from 92 to 112 ppm corresponds to axial carbon of carbohydrates. The peak at 105 ppm can be assigned to deoxygenated alkyl group, i.e. acetyl and ketal group O–C–O (Karlström *et al.,* 1995).

The region of chemical shifts from 112 to 136 ppm corresponds to aromatic carbon (Karlström *et al.,* 1995). In the spectra of *Sphagnum* species, two peaks at 117 and 130 ppm were well resolved. According to literature, these two peaks can be assigned to protonated (117 ppm) and substituted (130 ppm) aryl carbon. In the spectra of other mosses, peaks with low intensities or poorly-resolved were observed. The region of chemical shifts

from 136 to 159 ppm corresponds to phenolic and N-substituted aromatic carbon. In the spectra of *Sphagnum* species, one peak at 158 ppm was well resolved, and it can be assigned to O-substituted aryl carbon, i.e., C–O group carbon in aromatics. In the spectra of other mosses, at 158 ppm no peak was observed, even though poorly-resolved peaks in this region were observed. The presence of aromatic structures generally and lignin in mosses has been an object of discussions and controversies for a long time (Farmer and Morrison, 1964; Bland *et al.,* 1968; Miksche and Yasuda, 1978), as many authors have claimed to found lignin and other phenolic substances in mosses in significant amounts. Also in recent studies (Hajek *et al.,* 2011; Ballance *et al.,* 2012), discussions on "lignin-like phenolics" and "Klason lignin" are going on, and the arguments are supported with findings obtained using the classical methods of lignin analysis common in wood chemistry. Clear answer to the issue of the presence of lignin in mosses is of importance not only for understanding the chemical evolution process from simpler plants to more complex ones but also for bioprospecting, since the process of lignin synthesis goes through shikimic acid pathway and is a source of phenolics in higher plants. Our study by using the cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance spectrometry gives a definite answer to the issue of the presence of lignin, and this answer is negative: only minor quantities of phenolic substances were found in the studied mosses, and in general lignin does not play a significant role in their chemical composition.

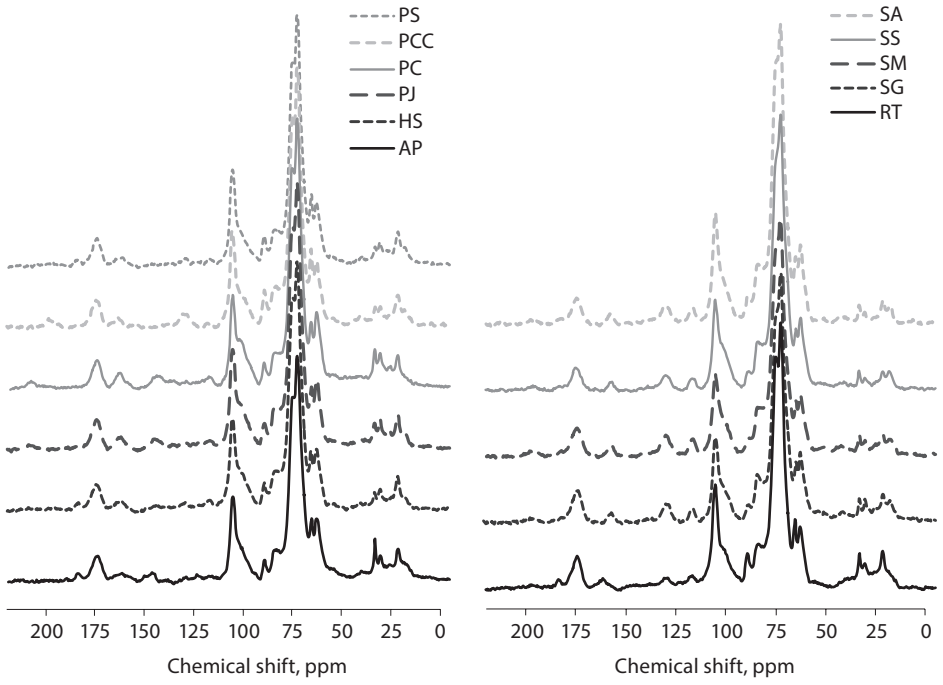

Figure 3.4. **Cross-polarization magic angle spinning ^{13}C NMR spectra for the studied mosses** (codes of the studied species as indicated Table 2.1.)

The region of chemical shifts from 159 to 190 ppm corresponds to carbon in fatty acids, including free carboxylic acids, their esters, and also amides. In all spectra, one well-resolved peak at 175 ppm could be observed, and it can be assigned to fatty acid carboxyl group carbon. In some spectra, there was a peak observed at 163 ppm, which can be assigned to carboxyl group in amides, and a peak at 183 ppm, which can be assigned to carbon of carboxyl anion.

Table 3.5. **Intervals of chemical shift regions (ppm) in cross-polarization magic angle spinning ^{13}C NMR spectra (%) of the studied mosses**

Species	212–190	190–159	159–136	136–112	112–92	92–50	50–0
Aulacomnium palustre	nd*	5.49	0.71	0.72	14.57	64.96	13.56
Pleurozium schreberi	0.03	5.29	nd*	1.66	15.46	66.45	11.12
Polytrichum commune	0.43	4.75	1.95	2.62	14.80	62.33	13.11
Polytrichum juniperum	0.13	5.02	1.22	2.08	16.07	64.88	10.59
Ptilium crista-castrensis	0.66	4.44	0.07	1.82	15.06	67.83	10.12
Rhytidiadelphus triquetrus	0.14	5.54	0.49	3.42	16.19	63.88	10.35
Sphagnum angustifolium	0.28	3.67	1.05	4.18	15.40	69.51	5.91
Sphagnum capillifolium	0.23	3.24	0.44	3.10	14.58	71.14	7.27
Sphagnum girgensohnii	0.22	5.05	0.53	3.40	13.29	66.28	11.23
Sphagnum magellanicum	0.53	4.72	0.93	5.05	14.03	65.44	9.31

*nd – not detected

The region of chemical shifts from 190 to 212 ppm corresponds to carbonyl group carbon. In some spectra, poorly-resolved peaks were observed (Table 3.5.), while in some species (*A. palustre*), carboxylic carbon was not detected. Aliphatic carbon signals can be mainly attributed to lipids.

Cluster analysis of the percentage of structural groups showed by ^{13}C NMR spectra indicated the similarity degree among bryophyte species (Figure 3.5.). Strong differences between *Sphagnum* and other bryophyte species were established. It is possible that the differences are determined by the presence of secondary metabolites, because the major peaks of ^{13}C NMR spectra were similar in all species. The studied bryophyte species formed two groups with the lowest similarity. All *Sphagnum* species formed one group, but there were differences between them. Other bryophyte species also formed a few groups, and only two or three species were similar to each other. *A. palustre* had the lowest similarity with other non-*Sphagnum* species. Cluster analysis showed that bryophyte species differ by percentages of structural elements, and the ^{13}C NMR spectra can be used for their grouping.

Cluster analysis of pyrolysis products indicates similarities of the major structural elements among the groups of the studied mosses (Figure 3.6.). For example, despite the evident similarity in the elemental composition, *Sphagnum* species were grouped together.

However, minor structural elements are also important in chemical taxonomy of mosses, particularly regarding substances belonging to lipid class.

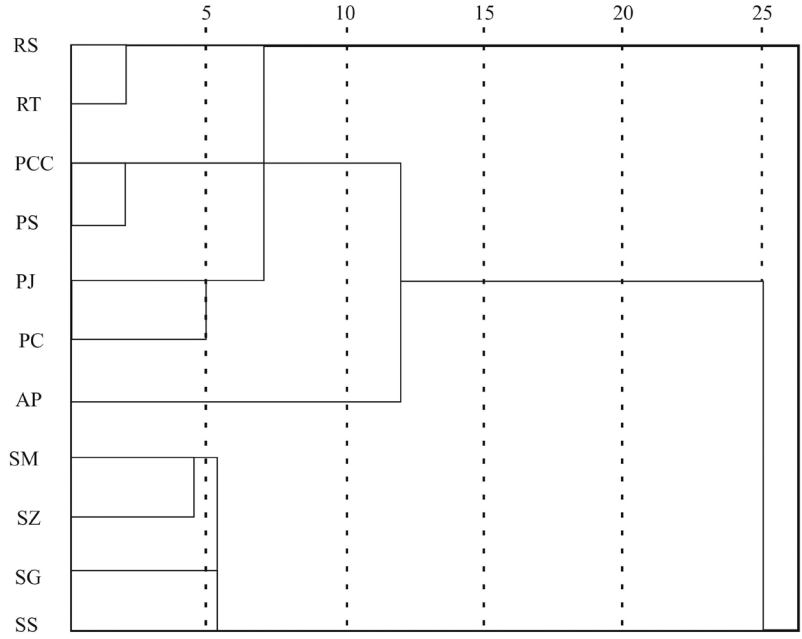

Figure 3.5. **Cluster analysis of the percentage of the structural groups showed by solid state CPMAS ^{13}C NMR spectra** (codes of the studied species as indicated Table 2.1.)

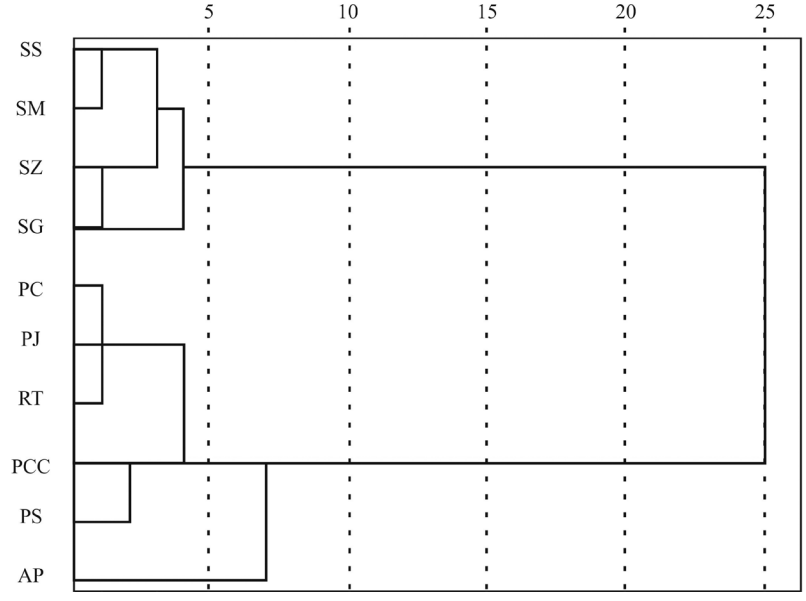

Figure 3.6. **Cluster analysis of the percentage of pyrolysis products of mosses** (codes of the studied species as indicated Table 2.1.)

3.2. Secondary metabolites of mosses and their characterisation

3.2.1. Extraction of moss secondary metabolites

For further studies of moss composition, extracts of their secondary metabolites were obtained. Historically mosses and their extracts have found application in ethnopharmacology as medical plants for treatment of wounds and burns, also for curing tuberculosis, pneumonia, neurasthenia and other illnesses (Hotson, 1921; Spjut *et al.,* 1986; Sing *et al.,* 2006; Saboljevic *et al.,* 2010) and a presence of a high number of biologically active compounds in their composition has been demonstrated (Asakawa *et al.,* 2013; Fu *et al.,* 2012; Basile *et al.,* 1999). Many compounds isolated from mosses have shown high biological activity (Krzaczkowski *et al.,* 2009; Üçüncü *et al.,* 2010). Likewise, analysis of extracts of mosses have shown multiple kinds of biological activity. For this reason, they are prospective in search of new pharmaceutically active compounds (Asakawa, 2007; Wang *et al.,* 2005). Another reason to study moss composition is related to the need to understand their metabolism. It is important to study secondary metabolites in mosses, as the metabolic processes are quite different from those in higher plants (Goffinet and Shaw, 2008). Studies of secondary metabolites can help understand stress reactions (drought/wetness), oxidative stress, pollution stress (for example, heavy metal impact), UV radiation impact reaction in mosses, as well as functions of the main secondary metabolites in the overall metabolism (Goffinet and Shaw, 2008; Tutschek, 1979; Xie and Luo, 2009; Huang *et al.,* 2012). Due to different reasons, studies of bryophyte biological activity have been concentrated on liverworts rather than mosses and relatively low-polar compounds amongst their secondary metabolites, considering the presence of oil bodies in the plants. Traditionally, low-polarity extrahents have been used in studying the presence of biologically active compounds in mosses (especially liverworts) (Singh *et al.,* 2006; Huang *et al.,* 2012; Üçüncü *et al.,* 2010), while the extraction process itself has not been specifically studied. However, to further advance the research of moss secondary metabolites, it is important to study the possibilities of improving the process of their extraction by looking not only for some specific group of compounds but rather for opportunities to obtain a maximally high number of secondary metabolites.

Two well-characterized moss species (Maksimova *et al.,* 2013) common in Northern Europe were used in the study to develop extraction procedure for mosses. Moss extraction efficiency were compared using following criteria: yield of extracts (dry residue and D_{280}), total polyphenolic content, antiradical activity, the number of individual compounds determined by means of UPLC analysis. Considering the interest in studies of biologically active compounds of mosses and, more broadly, in the composition of moss secondary metabolites used for the extraction of low-cost, low-toxicity, volatile solvents with differing polarity, their mixtures were selected with regard to the ability of extracting substances with a possibly wider range of properties: water, methanol, ethanol, acetone, dioxane, dimethylsulphoxide and several extraction methods were compared: a) conventional extraction (shaking at room temperature); b) Soxlet extraction; c) ultrasound-assisted extraction; d) extraction using treatment with microwaves; and e) extraction with supercritical CO_2, conditions of the extraction (time and temperature) were changed to compare the efficiency of each selected method (Table 3.6.). The Folin-Ciocalteu assay has

been used to estimate total phenolics in natural products (Cheynier *et al.,* 2013), although the basic mechanism of this assay is oxidation/reduction reaction, which itself can be considered as another method of antioxidant determination. Amongst these criteria, the major stress was put on the yield of extracted substances and the antioxidant activities of the extracts, considering recent interest in this kind of activity of natural compounds (Cheynier *et al.,* 2013). For this purpose, the DPPH radical scavenging activity analysis was used. Following parameters were used as criteria for evaluating the extraction efficiency: yield of extracts (dry residue and D_{280}), total polyphenolic content, antiradical activity, and the number of individual compounds under UPLC chromatographic conditions. To study the impact of the extraction procedures, the extraction conditions (time and temperature) were changed to compare the efficiency of each selected method (Table 3.6.). Microwave extraction at 150 °C was proved to be the most efficient extraction method (Table 3.6.), judging by both, total polyphenol content and radical scavenging activity. Conventional and Soxlet extractions provided high yields, but, in comparison with intensive extraction methods, required much more time.

Table 3.6. **Extraction efficiency of different extraction techniques from *R. triquetrus* (solvent: 60% ethanol).** Data were derived in three replicates each ±SE

Extraction method	Extraction conditions		Sample/extrahent ratio		Total polyphenol content, GE mg/100 g	Radical scavenging activity, GE mg/100 g	Extraction yield (dry weight), mg/100 g
	Time, h	T, °C	Volume, mL	Weight, g			
Soxlet	8.0	80	300	11.2	165.5±8.3	116.4±5.8	205.2±10.3
	24.0	80	300	11.2	239.6±11.9	142.6±7.1	231.5±11.6
Microwave	0.5	120	30	0.3	111.2±5.6	167.8±8.4	195.6±9.8
	0.7	150	30	0.3	486.9±24.4	172.9±8.6	150.2±7.5
Ultrasound	0.5	50	40	0.5	243.7±12.2	54.1±2.7	195.5±9.7
	0.7	70	40	0.5	254.6±12.7	63.3±3.2	150.6±7.5
Supercritical CO_2	0.5	102	150	15.0	230.4±11.5	162.4±8.1	125.1±6.3
	1.0	102	150	15.0	274.9±13.5	143.5±7.2	124.8±6.2
Conventional	12.0	24	30	0.4	150.7±7.6	25.8±1.3	99.5±4.9
	24.0	24	30	0.4	194.3±9.7	36.9±1.8	97.2±4.9

Conventional extraction also consumed much more solvent than the other studied methods. Soxlet extraction showed good results when extracts were tested in respect to radical scavenging activity; however, the total polyphenol levels were lower than, for example, in the case of ultrasound extraction. This indicates that polyphenolic compounds in mosses are not the only factor responsible for radical scavenging activity. Supercritical CO_2 extraction provided a good yield of polyphenolics, while the overall yields and yields of radical scavenging substances were relatively low.

For better understanding of the extraction efficiency using ultrasound as well as to understand the impact of the composition differences of moss species, the effect of treatment time as well as composition of extrahents in respect to the application potential of ethanol and the water mixtures ratio were compared (Figure 3.7.). Ultrasound-assisted extraction helped to decrease the extraction time and improve the extraction yield due to mechanical stress which induces cavitation following the cellular breakdown and release of secondary metabolites. As the chemical content of mosses varies from species to species (Asakawa, 2007), two widespread moss species from different growing conditions in forest and mire ecosystems were chosen as the representative samples: *R. triquetrus* and *S. rubellum*. The increase of treatment time with ultrasound helped to increase the yield of polyphenolics significantly (Figure 3.7.). The difference in extraction efficiency between no treatment with ultrasound and 40 min treatment with ultrasound was approximately 20–50% in some cases, while the difference between the treatments for 20 or 40 min was lower than 10%. To achieve a higher optimal outcome in the total polyphenol, radical scavenging and dry matter outcome, various types of solvents were used, ensuring isolation of secondary metabolites, especially, phenolic compounds from mosses (Table 3.7.).

Water, ethanol, methanol, acetone, dioxane, and DMSO were used as solvents. Selection of solvents was based on economic reasons, toxicity of solvents, and polarity of the substances of interest. Microwave extraction was found to be the most effective extraction technique; nevertheless, solvent optimization was done by treating samples with ultrasound and subsequently shaking for 24 h.

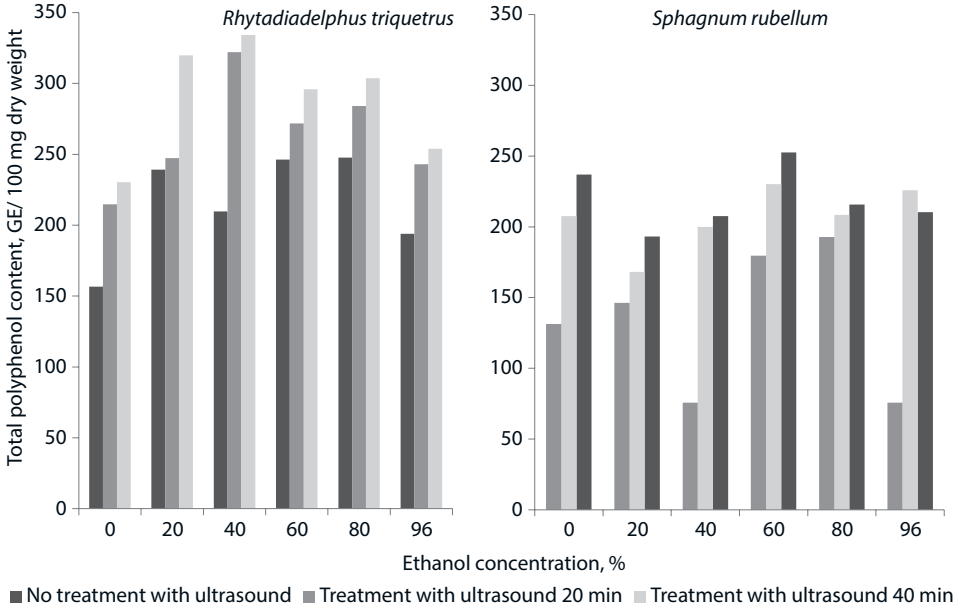

Figure 3.7. **Effect of the ethanol/water ratio and the extraction and ultrasound treatment time on total polyphenol content in extracts from mosses *R. triquetrus* and *S. rubellum***

Table 3.7. **Extraction efficiency from *R. triquetrus* and *S. rubellum* using different solvents and solvent mixtures.** Data were obtained in three replicates ±SE is indicated

Extrahent		Total polyphenol content, GE/100 g		Radical scavenging activity, GE/100 g		Extraction yield (dry weight), mg/100 g	
		R. triquetrus	*S. rubellum*	*R. triquetrus*	*S. rubellum*	*R. triquetrus*	*S. rubellum*
Water		230.0±11.5	237.2±11.9	10.5±0.5	11.9±0.6	36.8±1.8	51.8±2.6
Methanol	100%	183.1±9.2	132.9±6.6	11.6±0.6	45.9±2.3	75±3.8	91.6±4.6
	80%	208.6±10.4	132.9±6.6	32.4±1.6	48.7±2.4	91.7±4.6	91.4±4.6
	60%	189.7±9.5	135.3±6.8	24.1±1.2	42.6±2.1	91.8±4.6	75.1±3.8
	40%	182.8±9.1	120.4±6.0	7.6±0.4	31.2±1.6	108.3±5.4	50.2±2.5
	20%	171.9±8.6	116.2±5.8	6.2±0.3	27.6±1.4	75.0±3.8	50.8±2.5
Ethanol	96%	254.0±12.7	210.4±10.5	11.5±0.6	22.5±1.1	652.5±32.6	345.0±17.3
	80%	304.0±15.2	215.7±10.8	50.4±2.5	26.4±1.3	667.5±33.4	287.5±14.4
	60%	296.0±14.8	252.6±12.6	24.0±1.2	33.5±1.7	195.0±9.8	97.5±4.9
	40%	334.0±16.7	207.5±10.4	7.9±0.4	11.5±0.6	187.5±9.4	179.5±9.0
	20%	320.0±16.0	193.2±9.7	6.3±0.3	1.6±0.1	96.6±4.8	78.0±3.9
Acetone	100%	174.5±8.7	127.9±6.4	9.1±0.5	15±0.8	36.8±1.8	67.5±3.4
	80%	195.7±9.8	178.1±8.9	47.2±2.4	52.6±2.6	91.5±4.6	58.3±2.9
	60%	238.1±11.9	177.6±8.9	46.2±2.3	52.1±2.6	116.3±5.8	50.7±2.5
	40%	214.2±10.7	160.9±8.0	31.2±1.6	49.5±2.5	125.6±6.3	41.6±2.1
	20%	191.1±9.6	128.6±6.4	12.5±0.6	42.6±2.1	50.4±2.5	28.4±1.4
Dioxane		151.2±7.6	138.3±6.9	13.5±0.7	13.4±0.7	15.0±0.8	147.0±7.4
DMSO	100%	363.9±18.2	75.7±3.8	47.9±1.6	31.5±1.6	41.7±2.1	97.5±4.9
	40%	295.1±14.8	75.7±3.8	34.7±2.2	43.6±2.2	25.2±1.3	108.0±5.4
	20%	270.0±13.5	75.7±3.8	19.1±1.0	16.6±0.8	16.9±0.8	68.3±3.4

Higher polyphenolic concentrations were found using such solvents as ethanol and DMSO. Use of acetone and methanol led to lower yields of polyphenolics. Antioxidant capacity (measured with the DPPH method) was also higher when using ethanol, but the differences were not as significant as in the case of total polyphenolics content and total dry extract mass. The optimal yields of polyphenolics and radical scavenging substances, and the total dry extract mass were obtained from *R. triquetrus* and *S. rubellum* using aqueous methanol, ethanol and acetone in the concentration range from 60% to 80%. Polar aprotic solvent DMSO in both pure solvent and aqueous mixture provided higher yields of extracts; however, the application possibilities of this solvent are limited by its costs and difficult removal after extraction. As presented in Table 3.7., the best results were obtained in microwave-assisted extraction, using ethanol as a solvent. For better understanding of the factors controlling the extraction efficiency, an experiment was done to see how ethanol at various concentrations affect the microwave-assisted extraction efficiency (Figure 3.8.).

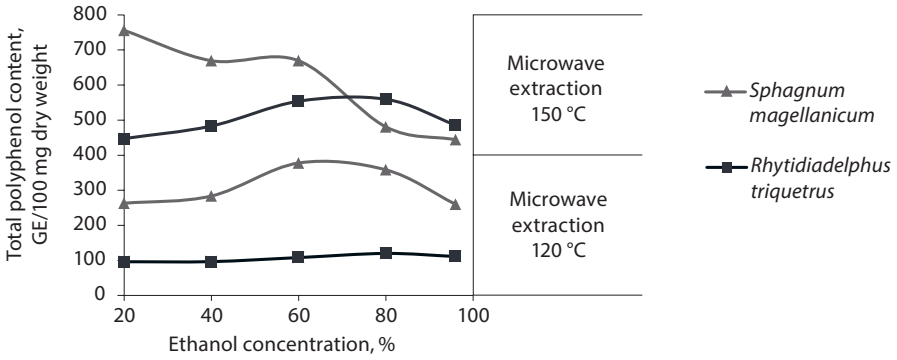

Figure 3.8. **Optimization of microwave-assisted extraction from *R. triquetrus* and *S. rubellum* using aqueous ethanol (20–96%) as a solvent**

Higher polyphenol content was obtained with the microwave treatment at 150 °C in comparison to 120 °C for both studied bryophyte species. Microwave-assisted extraction in comparison with ultrasound-assisted extraction showed similarities in differences between different optimal solvent concentrations. The same moss species also had differences in the optimal solvent concentration under changed extraction conditions. The optimal ethanol concentration for *R. triquetrus* at 120 °C temperature was approximately 60%, while at 150 °C it was 20%; the optimal concentration of ethanol for *S. rubellum* at both tested temperatures was 60%.

Meanwhile, the most optimal ethanol concentrations for *R. triquetrus* and *S. rubellum* in ultrasound-assisted extraction were 40% and 60% respectively. After consideration as optimal extraction conditions was chosen 40 min ultrasound-assisted extraction with 60% ethanol (Table 3.8.).

All of the analyses described above give some understanding about the biologically active substances group count in optimized extract; yet, as there is no strong correlation between the antioxidant activity and other parameters, it is still unclear which substances or groups of substances are responsible for that. Therefore, analysis of some extracts were done using UPLC (Figures 3.9., 3.10.), and the peaks of found substances were compared (Figure 3.11.).

According to Snyder's selectivity range, the extrahents used had well-expressed selectivity in respect to their ability to interact with substances in protonated forms. Addition of water to the extraction system increased its ability to interact with proton-accepting groups.

Table 3.8. **Extraction results in optimal conditions.**
Data were obtained three replicates ±SE is indicated

Species	Total polyphenol content, GE/100 g	Radical scavenging activity, GE/100 g	Total carbohydrate content, GLE/ 100 g	Extraction yield (dry weight), mg/100 g
Aulocomnium palustre	267.5±13.4	39.0±2.0	17.9±0.9	114.2±5.7
Polytrichum juniperum	416.2±20.8	24.1±1.2	18.1±0.9	88.5±4.4
Rhytadiadelphus triquetrus	379.1±19.0	11.3±0.6	18.1±0.9	144.8±7.2
Sphagnum rubellum	345.7±17.3	16.3±0.8	18.3±0.9	65.3±3.3
Climacium dendroides	301.0±15.1	14.9±0.7	18.3±0.9	155.6±7.8
Ptilium crista-castrensis	237.9±11.9	19.6±1.0	18.1±0.9	145.5±7.3
Polytrichum commune	800.7±40.0	12.6±0.6	18.2±0.9	154.8±7.7
Dicranum scoparium	477.3±23.9	11.9±0.6	18.1±0.9	194.6±9.7
Hylocomnium splendens	298.4±14.9	59.3±3.0	18.0±0.9	102.6±5.1
Pleurozium shreberi	275.6±13.8	15.6±0.8	18.1±0.9	145.2±7.3
Rhytadiadelphus squarosum	399.2±20.0	16.4±0.8	18.1±0.9	133.5±6.7
Sphagnum squarosum	280.1±14.0	14.9±0.7	18.2±0.9	149.3±7.5
Sphagnum girgensonii	783.2±39.2	17.6±0.9	18.1±0.9	142.5±7.1
Sphagnum magelanicum	370.6±18.5	18.2±0.9	18.1±0.9	140.6±7.0

UPLC analysis of moss extracts (Figure 3.9., 3.10.) demonstrated the presence of high number of individual substances and the impact of conditions of extraction on the composition of extracts. The extraction ability under elaborated extraction conditions was high, as indicated by the presence of individual components of moss SR and RT in chromatograms, with the retention times 12.19; 18.50; 20.59; 26.57; 33.15; 37.24; 38.90; and 51.23 (Figure 3.9., 3.10.). As it is evident from Figure 3.11., the increase of water content in the composition of extrahent helps to increase yields of a definite number of compounds in the obtained UPLC chromatograms, supporting isolation of a larger number of secondary metabolites from moss. The extraction method can be considered as an additional factor influencing the effect of water addition. Dioxane and acetone have medium selectivity in respect to their ability to interact with low-polarity compounds, and, as it can be seen from Figure 3.11., use of dioxane does not ensure high yields of secondary metabolites. However, water addition to the extraction mixtures increase the proton-donating ability of these solvents, just as in the case of methanol and ethanol, although to a lesser extent. As it can be seen from Figure 3.10, the composition of isolated compounds for 80% acetone-water mixture did not differ much from the qualitative composition resulting from the use of 60% ethanol-water. Considering that the chromatographic separation was achieved using an apolar column, the suggested extrahent systems helped to isolate a high number of compounds with relatively high polarity, despite differences in the qualitative composition of isolated compounds.

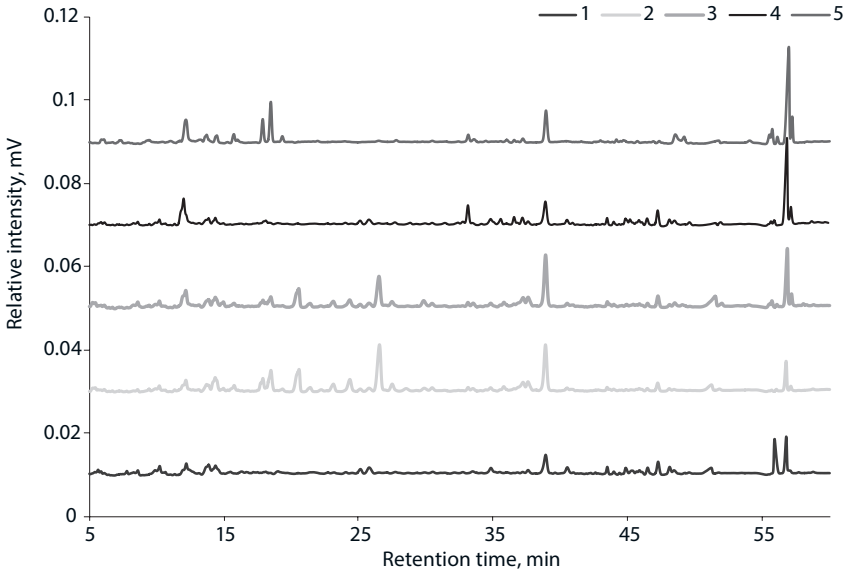

Figure 3.9. **UPLC chromatograms of optimized extracts of *S. rubellum* using following solvents and 20 min ultrasound treatment*: 1. dioxane; 2. 60% ethanol (24 h shaking* no ultrasound treatment); 3. 60% ethanol; 4. 100% methanol; 5. 80% acetone**

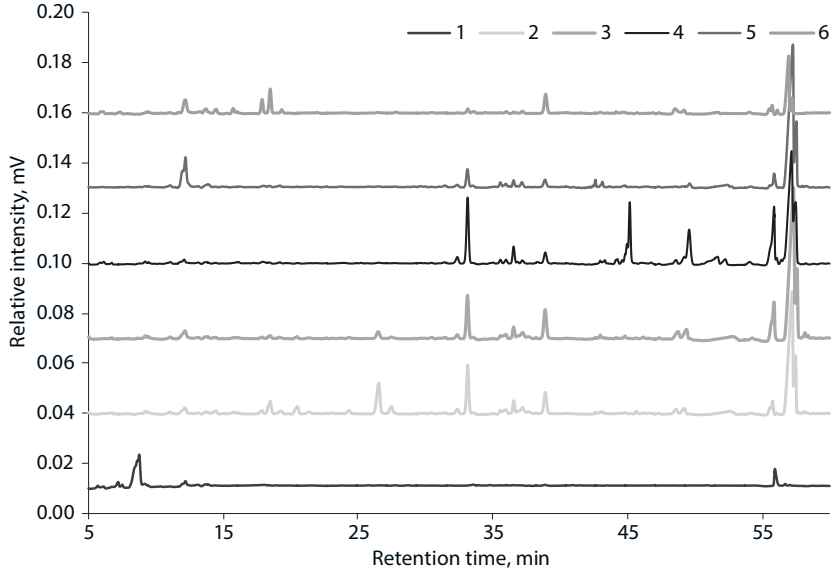

Figure 3.10. **UPLC chromatograms of optimized extracts of *R. triquetrus* using following solvents and 20 min ultrasound treatment*: 1. dioxane; 2. 60% ethanol (24 h shaking* no ultrasound treatment); 3. 60% ethanol; 4. 100% methanol; 5. 80% acetone; 6. *Supercritical CO_2 extraction coupled with 96% ethanol treatment for 30 min**

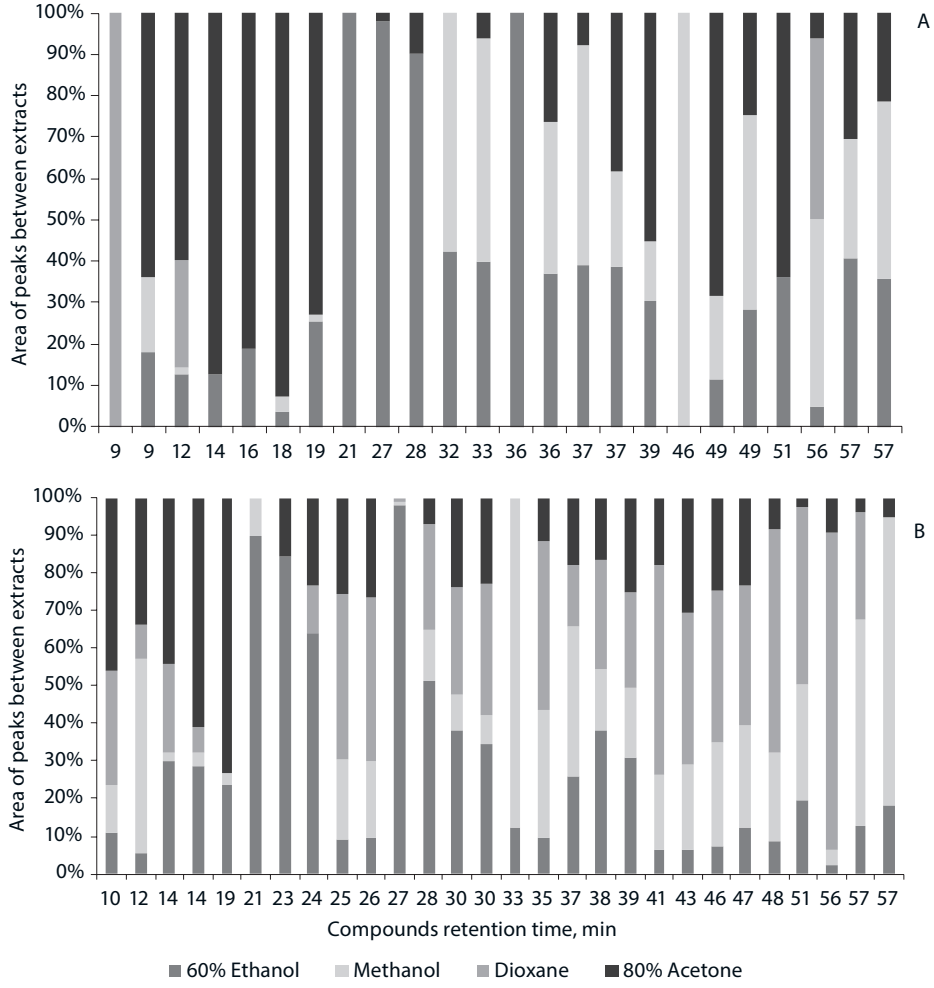

Figure 3.11. **Comparison of peak areas of substances detected using UPLC of different moss extraction methods (A – *Sphagnum rubellum*; B – *Rhytadiadelphus triquetrus*)**

It may be concluded that ultrasound is effective in increasing the extraction efficiency, while the sonification duration does not have major impact if it exceeds 20 min. Each bryophyte species has different optimal extraction conditions in respect to the composition of solvent mixture (ethanol and water). The highest yield of polyphenolics in the case of *R. triquetrus* was ensured by the use of 20–60% ethanol, while in the case of *S. rubellum* 20% ethanol also gave a relatively high yield. The differences found in the extraction yield of total polyphenolics indicated differences in the moss composition and the need to estimate the optimal solvent concentration for each moss species individually. It can be stated that 60% ethanol is the suggested concentration for the screening of moss secondary metabolite composition and, thus, this kind of solvent was further used in this study.

For extracts from higher vegetation, a high correlation between the total phenolic content and antioxidant activity has been observed (Tabart *et al.,* 2006; Ehala *et al.,* 2005). However, for the extracts of mosses, the correlation was not so well expressed, indicating that the antioxidant activity could be determined not only by phenolics but also by other groups of substances.

3.2.2. Characterization of moss secondary metabolites

Major components of mosses are composed from polysaccharides of different composition (Asakawa, 2007; Klavina, 2015), however secondary metabolites consists of lower polarity substances (lipids) and polar substances (carbohydrates, amino acids, phenolics and others). According to their physical properties, polarity of molecules and functions in organism (Gunstone, 1996), fatty acids, fatty alcohols, waxes, sterols, terpenoids are considered as lipids as well as other substances. Such classification concept is widely applied and is also supported by similarity of substances during the extraction process with low polarity solvents. For isolation of major groups of secondary metabolites sequential extraction approach has been used at first isolating lipids and afterwards – more polar substances (Dean, 2009). Considering experience in studies of higher plants (Samuelson and Bohlin, 2009), for extraction of secondary metabolites from mosses two solvents were selected: 1) chloroform (lipids); 2) ethanol (polar metabolites) (Table 3.9.). Studied mosses contain relatively high concentrations of secondary metabolites (Table 3.9.) and amount of more polar substances (in ethanol extracts) is lower than lipids (chloroform extracts).

Table 3.9. **Total polyphenolics, carbohydrates, radical scavenging activity of ethanol extracts* of studied mosses and yields of chloroform extracts** expressed on 100 g dry moss weight**

Species code	Total polyphenol content*, GE	Total carbohydrates*, GLE	Radical scavenging activity*, GE	Extraction yield*, mg	Extraction yield**, mg
AP	267.5±13.1	10.6±0.4	39.0±1.9	114.2±5.7	259±12.8
CD	272.1±14.3	7.6±0.3	42.3±2.8	98.6±4.7	412±22.7
DP	399.2±19.5	8.4±0.3	16.4±0.8	133.5±6.7	482±24.2
HS	298.4±14.6	6.9±0.3	59.3±3.1	102.6±5.1	443±23.2
PS	275.6±13.4	11.4±0.5	15.6±0.8	145.2±7.3	561±27.9
PC	800.7±39.1	12.1±0.5	12.6±0.6	154.8±7.7	513±26.1
PJ	416.2±20.2	5.6±0.2	24.1±1.2	88.5±4.4	510±26.1
PCC	237.9±11.7	9.7±0.3	19.6±1.0	145.5±7.3	464±23.9
RT	379.1±18.5	15.3±0.7	11.3±0.5	144.8±7.2	451±23.5
SF	783.2±38.1	8.9±0.4	17.6±0.9	142.5±7.1	213±10.1
SM	370.6±18.1	6.6±0.3	18.2±0.9	140.6±7.0	378±18.4
SR	345.7±16.7	10.3±0.4	16.3±0.8	65.3±3.3	181±9.8
ST	280.1±13.7	7.9±0.3	14.9±0.7	149.3±7.4	240±11.6

Mosses contain polyphenols (from 200 up to 800 mg gallic acid equivalent/100g of dry weight), but the radical scavenging activity (estimated as the inhibition of 2,2-diphenyl-1-picrylhydrazyl radical) is much less expressed than in higher plants (Sun *et al.,* 2009). If in higher plants polyphenolic substances determine radical scavenging activity, then in moss extracts there are no statistically significant correlation (correlation coefficient < 0.3) between total polyphenol concentration and radical scavenging activity (Table 3.9.) as it is common for higher plants.

3.2.2.1. Lipid composition of mosses

Lipid composition of mosses has been an object of pioneering studies (Dembitsky, 1993) of moss secondary metabolites and from perspective of biogeochemistry to support paleoclimatologic studies (Baas *et al.,* 2000), however recently not much attention has been dedicated to this group of substances in mosses. For extraction of lipids from bryophytes different solvents have been used, usually chloroform, however the extraction conditions and solvent selection has not been much studied. Brief comparison (Table 3.10.) of different solvents demonstrates that chloroform and diethyl ether can be considered as the most preferable. As a significant tool to increase extraction yield, a treatment with ultrasound can be applied. Studied leaf moss species (*Polytrichum commune, Dicranum polysetum*) contained < 12 mg/g of lipids. Exhaustive extraction (repeated treatment with fresh solvent) might yield up to 21–60 mg/g depending on the moss species (however such yield requires intensive and repeated treatment).

Table 3.10. **Yield of moss lipid extracts (mg/g)**

Moss species	C_6H_{12}	CH_2Cl_2	$CHCl_3$	$C_2H_5OOCCH_3$	$(C_2H_5)_2O$
Polytrichum commune	7.2	6.8	10.8	8.8	8.0
Dicranum polysetum	4.0	11.7	12.4	9.2	12.0

Chromatographic analysis of freely available lipids (Table 3.11., 3.12.) reveal a high number of different groups of substances (in total 88 different substances has been identified and quantified in the studied mosses) playing significant functions in metabolism of mosses and possibly affecting the biological activity of their extracts. Significant differences are remarkable amongst the studied moss species: many substances have been quantified only in some moss species and this, at first, is relevant in respect to biologically most active substances like sterols and terpenoids. Thus, in *Ptilium crista-castrensis* in high concentration ergost-7-en-3-ol and pimaric acid was found, while in *Polytrichum commune* other sterols were found at comparatively higher concentrations than in other mosses. Highest number of substances was found in *Pleurozium shreberi*, but lowest – in *Sphagnum magellanicum*. For the first time following substances has been identified in mosses: diploptene, α-amyrin, oleanolic acid, uvaol, cycloartenol, ursolic acid, neophytadiene and others. Studied mosses contain a significant number of alkanes and fatty acids, including many unsaturated fatty acids and fatty alcohols. In respect to functions of secondary metabolites, presence of sterols, terpenoids can be considered as important.

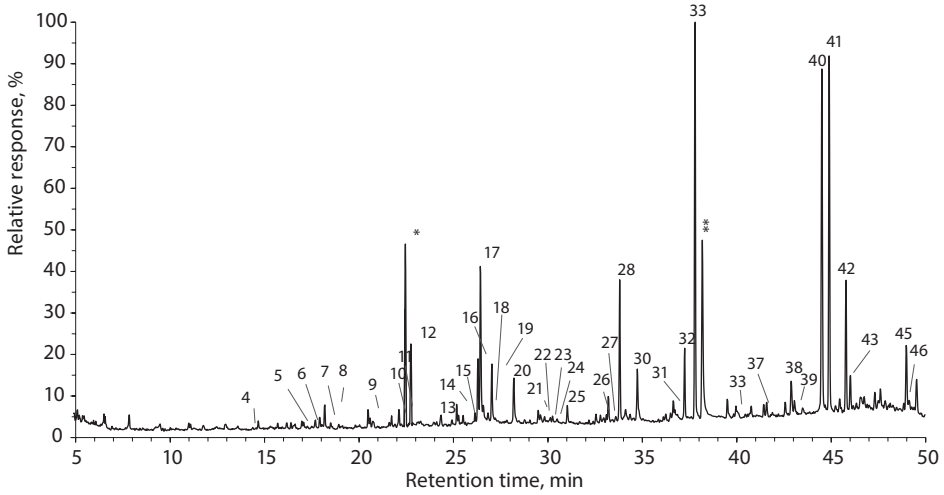

Figure 3.12. **Gas chromatogram with mass-spectrometric detection of the total lipid extract of *Polytrichum commune* (PC).** IS – internal standard (* – methylheptadecanoic acid; ** – progesterone); numbers refer to compounds listed in Table 3.11. Acid and alcohol groups were derivatised prior to the GC/MS analysis

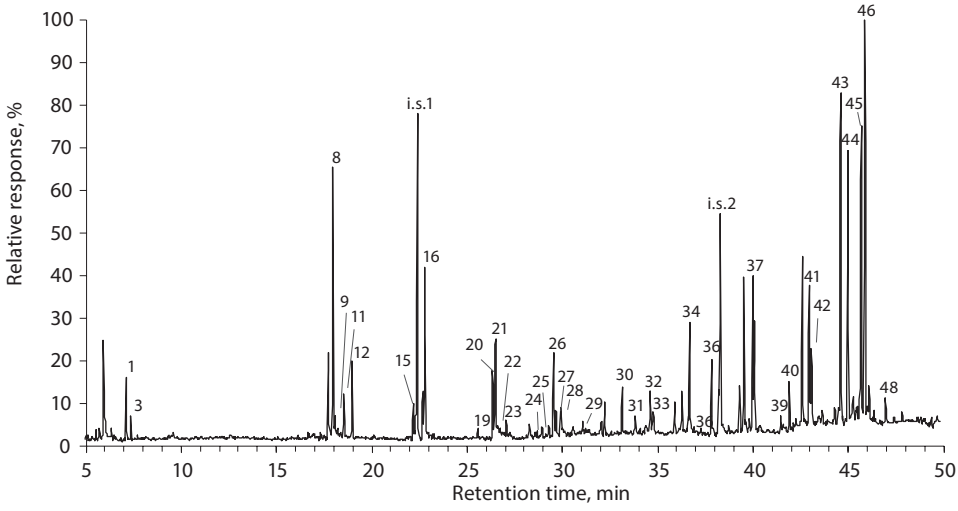

Figure 3.13. **Gas chromatogram with mass-spectrometric detection of the total lipid extract of *Rhytidiadelphus triquetrus*.** IS – internal standard (* – methylheptadecanoic acid; ** – progesterone); numbers refer to compounds listed in Table 3.11. Acid and alcohol groups were derivatised prior to the GC/MS analysis

In both Figure 3.12. and 3.13., the differences in extract GC/MS chromatograms depending on moss species can be seen. Not only the substances but also the quantities of identified substances are evidently different. Some substances can be found only in one moss extract such as succinic, malic and isopimaric acid as well as diploptene, cycloartenol and others. Major part of substances has been identified and quantified, however, it was not possible to identify several substances by their mass spectra. Identification of unknown substances can be achieved by fractionation, therefore, concentration and purification of a substance could support identification.

In the studied mosses, hydrocarbons occur in relatively low concentrations (100±290 μg/g dry weight; Table 3.11.). Distribution of alkanes dominates by C_{21+} odd-carbon numbered homologues. These results are in agreement with the literature data for other moss species, and in peat and soil studies it can help to differentiate lower plants (n-alkanes with carbon numbers C_{21} – C_{25}) from higher plants (alkane distribution is dominated by $C_{29,}$ C_{31}, C_{33}) (Baas *et al.,* 2000; Ronkainen *et al.,* 2013). Sterols are the most abundant lipids in the extracts of studied mosses and their concentrations vary from 450 to 1600 μg/g dry weight (Table 3.12.). For the studied samples common is the dominance of C_{29} sterols in respect to C_{28} sterols, and 24-methyl-cholest-5-en-3b-ol, 24-ethylcholesta-5,22-dien-3b-ol, 24-ethylcholest-5-en-3b-ol, 24-ethylcholest-5-en-3b-ol, 24-methylcholest-5-en-3b-ol, 24-methylcholesta-5,22-dien-3b-ol, and 24-methylcholesta-5,7,22-trien-3b-ol can be found in the highest concentrations. Sterols are an important component of moss secondary metabolites. Concentrations of terpenoids are much more variable than those of sterols (Table 3.12.). Amongst terpenoids dominate ursolic acid, oleanoic acid, amyrin and lupeol. Concentrations of fatty acids also much differ amongst the studied bryophytes and range from 200 to 1050 μg/g dry weight. As a bacterial contribution can be considered C_{16}±C_{18} fatty acids (and especially unsaturated compounds). Only higher fatty acids (C_{24} or C_{26} members) are common for bryophytes (Karunen and Salin, 1980; Ficken *et al.,* 1998; Marsili *et al.,* 1972). Total concentrations of fatty alcohols are much lower than those of fatty acids and vary from 80 to 340 μg/g dry weight. Fatty alcohols are dominated by even-carbon numbered compounds in the range C_{22}±C_{30}. Significant group of moss lipids are waxes and they are comprised of $C_{16:0}$ fatty acids esterified with $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$ fatty alcohols. Lipid extracts of mosses contain also α-tocopherol as well as C_{25} and C_{27} methyl ketones.

Table 3.11. **Concentrations (mg/100 g dry weight) of substances in *Polytrichum commune* and *Rhytadiadelphus triquetrus* lipid extracts accordingly to the GC/MS analysis**

No.*	RT**	RIc***	Components	*Rhytidiadelphus triquetrus*	*Polytrichum commune*
1.	7.11	1289	**2,4-Decadienal**	19.0±0.5	2.5±0.5
2.	7.14	1306	**Succinic acid**	0.8 ±0.1	ND
3.	7.37	1351	**Nonanoic acid**	7.4±0.2	1.2±0.2
5.	10.03	1481	**Malic acid**	0.6±0.2	ND
6.	13.59	1645	**Dodecanoic acid**	0.8±0.1	3.3±0.1
7.	16.42	1784	**2-Methylfructofuranoside**	0.6±0.1	2.8±0.1
8.	16.97	1811	**Azelaic acid**	0.3±0.1	3.5±0.1
9.	17.96	1844	**Neophytadiene**	123.9±1.5	5.6±0.2
10.	18.09	1846	**Hexahydrofarnesyl acetone**	5.3±0.2	1.3±0.1
11.	18.20	1852	**Tetradecanoic acid**	2.7±0.2	11.1±0.2
12.	18.53	1862	**Phyta-1,3(Z)-diene**	16.0±0.5	1.7±0.1
13.	18.97	1877	**Phyta-1,3(E)-diene**	34.4±0.5	1.2±0.1
14.	20.47	1940	**Pentadecanoic acid**	0.3±0.1	8.7±0.5
15.	21.73	2000	**Eicosane**	0.5±0.1	5.6±0.2
16.	22.20	2009	**9-hexadecenoate + 7,10,13-hexadecatrienoic acid**	4.6±0.3	8.9±0.3
17.	22.77	2040	**Hexadecanoic acid**	68.3±1.7	2.6±0.2
18.	24.38	2109	**9-Heptadecenoic acid**	0.4±0.1	6.4±0.2
19.	25.17	2149	**1-Octadecanol**	0.4±0.1	8.6±0.7
20.	25.56	2165	**Phytol**	12.6±0.6	5.3±0.3
21.	26.33	2201	**9,12-Octadecadienoic acid (Z,Z)-,**	28.3±1.6	31.2±1.6
22.	26.49	2207–2209	**9-Octadecenoic acid + (3Z,6Z,9Z)-3,6,9-octadecatrienoate**	46.7±2.7	74.2±3.2
23.	26.62	2214	**11-Octadecenoic acid**	3.2±0.4	11.1±0.5
24.	27.06	2235	**Octadecanoic acid**	6.6±0.3	25.5±0.8
25.	28.56	2295	**Pimaric acid + C23**	3.3±0.2	27.8±1.6
26.	28.92	2326	**Isopimaric acid**	6.2±1.1	ND
27.	29.54	2355	**cis-5,8,11,14-Eicosatetraenoic acid**	30.6±1.3	7.5±0.5
28.	29.64	2361	**cis-5,8,11,14,17-Eicosapentaenoic acid**	8.8±0.8	5.5±0.3
29.	29.93	2369	**Dehydroabietic acid**	18.4±0.2	4.8±0.4
31.	31.06	2432	**Eicosanoic acid**	6.0±0.3	7.9±1.2
32.	33.13	2546	**1-Docosanol**	19.9±1.3	28.7±2.5
33.	33.83	2580	**Hexadecanoic acid, 2,3-dihydroxypropyl ester**	12.2±0.5	58.1±3.4
34.	34.60	2634	**Sucrose**	19.9±1.6	1.1±0.2
35.	34.76	2635	**Docosanoic acid**	11.0±0.7	28.7±3.6
37.	36.67	2746	**Tetracosan-1-ol**	62.8±2.8	1.6±0.2

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No.*	RT**	RI_C***	Components	*Rhytidiadelphus triquetrus*	*Polytrichum commune*
38.	37.26	2781	**Octadecanoic acid, 2,3-dihydroxypropyl ester**	3.3±0.3	31.4±3.1
39.	37.83	2811	**Squalene**	39.7±1.5	247.6±5.3
40.	39.98	2939	**Hexacosanol**	72.6±2.3	8.4±0.2
41.	40.63	2989	**γ-Tocopherol**	1.4±0.1	ND
42.	41.45	3040	**Hexacosanoic acid**	6.8±0.3	6.2±0.9
43.	41.91	3061	**Decanyl tetradecanoate**	24.4±1.2	4.6±0.6
44.	42.95	3119	**(+)-α-Tocopherol + Cholesterol**	58.8±4.2	55.8±4.8
45.	43.08	3131	**Octacosanol**	28.0±2.2	10.2±0.7
46.	44.60	3229	**Campesterol**	195.2±6.8	453.7±4.3
47.	44.99	3261	**Stigmasterol**	128.0±7.2	408.8±6.7
48.	45.69	3321	**Diploptene**	147.3±6.3	ND
49.	45.87	3324	**β-Sitosterol**	180.7±2.8	153.8±5.3
50.	46.02	3339	**Isofucosterol**	0.8±0.1	44.6±2.1
51.	46.93	3386	**Cycloartenol**	11.4±2.1	ND
53.	49.06	3545	**Phytylhexadecanoate**	ND	6.8±0.2

*Peak number as indicated in Figure 3.12., 3.13.; **RT – retention time; ***RI_C – retention index calculated for temperature programmed GC analysis, constant heating rate by mean values indices relativity to a series of n alkanes (C_8 – C_{40}). Values are expressed as mean ±SD of three separate analyses. ND: not detected

In order to better understand chemical composition of mosses in total 13 moss samples were analysed. GC/MS data of analysed mosses revealed high variability of substances and their quantities (Table 3.12.).

Analysis of moss extracts using GC/MS method showed that there are some substances that can be found only in some moss species, and more scrutinized research with more moss species should be used to identify genus characteristic substances such as sphagnic acid, 9-hexadecenoic acid. Most of the triterpene group substances are specific only for some moss species, thus, possibly could be used for chemotaxonomic purposes but also could influence biological activity of moss extracts.

3.2.2.2. Moss lipid fractionation

Traditionally lipids might be fractionated as neutral lipids (triglycerides), phospholipids, glycolipids etc. (Dembitsky, 1993). Fatty acids and sterols of bound lipids can be analysed using transesterification. However from a perspective of search of biologically active compounds and study of moss metabolism most interesting are freely available substances, as far as biological activity of glycolipids and others usually is lower than for freely available substances. For separation of individual lipid classes silica modified with aminopropylgroups has been suggested (Kaluzny *et al.,* 1985) and later this approach has been extended for lipids from different sources.

Table 3.12. **Concentrations (mg/ 100 g dry weight) of substances from lipid extracts of studied mosses accordingly to GC/MS analysis**

Identified substance	AP	CD	DP	HS	PCC	PJ	PS	SF	SM	SR	ST
Tetradecanoic acid	14.9	4.9	16.9	14.7	13.5	0.4	28.6	4.2	3.1	63.2	12.8
Pentadecanoic acid	0.1	1.2	1.5	11.3	8.3	0.4	11.9	4.2	2.9	14.5	17.3
9-Hexadecenoic acid	ND	ND	ND	ND	ND	ND	11.7	ND	ND	ND	ND
n-Hexadecanoic acid	103.0	127.3	93.1	224.4	104.0	109.8	148.5	176.8	122.3	91.6	272.5
(Z)-3,7,11,15-Tetramethylhexadec-2-enoic acid	0.6	1.9	0.4	1.0	ND	ND	58.0	12.3	2.0	ND	4.3
Heptadecanoic acid	0.3	1.1	13.4	3.3	9.8	ND	148.5	11.4	0.7	4.0	12.0
9,12-Octadecadienoic acid (Z,Z)-	20.0	187.5	34.4	169.6	33.6	86.4	49.7	191.1	57.9	50.5	174.2
9,12,15-Octadecatrienoic acid	104.4	223.0	70.8	416.2	75.4	230.9	108.9	241.1	85.0	80.5	219.9
11-Octadecenoic acid	0.4	2.3	14.5	48.7	15.3	16.5	19.5	21.3	14.2	22.5	29.3
Octadecanoic acid	21.3	11.4	24.9	59.9	29.2	34.5	24.9	25.6	17.9	27.3	38.0
5,8,11,14-Eicosatetraenoic acid, (all-Z)-	1.8	89.0	36.6	238.3	22.2	12.9	65.9	44.1	17.7	31.6	23.9
5,8,11,14,17-Eicosapentaenoic acid	10.3	9.6	24.1	176.1	16.1	0.3	64.9	11.9	0.1	40.1	11.1
8,11,14-Eicosatrienoic acid	ND	24.9	15.1	42.2	17.0	0.2	18.2	2.7	0.5	ND	10.4
11,14-Eicosadienoic acid	ND	1.6	ND	21.1	ND	13.1	3.8	0.9	0.2	1.9	9.2
11-Eicosenoic acid	ND	ND	14.1	ND	11.3	23.1	10.5	4.7	0.3	ND	20.1
Eicosanoic acid	16.2	7.3	24.1	43.9	15.9	50.1	18.2	4.7	1.5	10.5	33.2
Docosanoic acid	17.7	18.0	21.9	49.7	26.4	57.4	23.1	27.1	23.0	36.8	32.6
Tricosanoic acid	16.8	ND	ND	ND	ND	14.2	ND	11.6	14.9	12.1	15.2
Pentacosanoic acid	0.4	0.3	ND	55.6	ND	30.2	26.1	10.3	13.4	39.4	15.5
Hexacosanoic acid	1.0	5.5	58.7	69.7	ND	40.0	23.6	28.1	47.7	81.0	48.6
Octacosanoic acid	0.5	3.4	7.1	61.6	398.2	50.7	13.4	2.1	21.1	6.3	17.3
1-Monopalmitoylglycerol	11.4	2.4	3.0	63.6	26.4	19.6	87.2	77.9	41.3	35.7	27.1
Octadecanoic acid 2,3-dihydroxypropyl ester	14.3	0.4	20.7	19.4	38.2	27.5	65.9	53.6	24.8	28.8	18.5
Neophytadiene	20.2	84.8	65.6	190.3	113.8	47.3	137.0	49.3	36.3	48.2	38.4
Phyta-1,3(Z)diene	1.0	10.1	5.0	20.4	ND	1.9	33.7	12.4	4.3	9.0	8.7
Phyta-1,3(E)diene	1.2	16.5	14.8	38.6	ND	1.3	54.5	18.4	7.5	12.7	11.1
Phytol	1.5	4.9	18.3	28.7	19.1	12.2	20.4	21.4	14.6	17.5	49.9
Squalene	174.5	42.9	64.7	117.6	43.6	206.3	111.3	14.1	19.7	37.3	25.7
1-Hexadecanol	ND	ND	11.0	ND	12.0	ND	ND	ND	0.2	ND	1.0
Hexadecane-1,2-diol	ND	ND	ND	22.2	ND	ND	ND	2.5	0.8	ND	ND
1-Octadecanol	0.2	1.4	2.1	ND	18.1	0.6	13.1	4.9	0.4	ND	ND

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Identified substance	AP	CD	DP	HS	PCC	PJ	PS	SF	SM	SR	ST
1-Docosanol	0.3	10.0	27.7	22.9	23.9	14.0	10.8	12.8	0.9	18.7	ND
1-Tricosanol	ND	ND	ND	19.4	ND	0.1	0.9	ND	ND	1.7	ND
1-Tetracosanol	1.5	16.6	21.7	38.0	30.7	28.2	25.6	25.0	19.0	76.9	24.1
1-Hexacosanol	20.4	28.1	49.8	55.6	83.0	3.6	26.1	34.0	30.2	117.0	44.1
1-Octacosanol	20.9	37.6	54.0	57.2	ND	5.2	98.1	50.2	22.4	4.4	49.0
1-Triacontanol	37.2	ND	ND	ND	ND	22.5	3.3	ND	ND	7.0	2.3
Cholesterol+ α-Tocopherol	64.0	99.2	168.3	189.5	107.7	80.5	231.9	64.3	18.2	57.2	42.0
Desmosterol	1.0	ND	117.9	49.3	59.0	ND	102.3	ND	ND	63.2	ND
Ergosterol	ND	0.7	5.0	91.0	49.6	27.8	144.7	189.5	82.9	81.1	91.8
Campesterol	256.8	187.6	476.2	348.1	452.3	238.9	554.0	374.5	94.5	96.4	152.4
Stigmasterol	273.3	157.7	495.1	282.4	506.6	206.0	362.0	387.8	122.2	189.1	152.4
Ergosta-5,8-dien-3β-ol	ND	ND	ND	282.4	ND	ND	183.6	157.8	53.4	ND	ND
Ergost-7-en-3-ol	ND	ND	ND	ND	179.4	ND	ND	2.8	29.7	ND	18.3
β-Sitosterol	140.6	299.4	356.3	430.7	361.1	161.4	552.2	518.8	231.9	199.1	176.4
Pimaric acid	11.4	ND	ND	ND	26.6	ND	ND	12.2	0.3	ND	4.2
Isopimaric acid	ND	ND	16.3	22.9	ND	ND	24.8	15.7	ND	37.6	10.1
Dehydroabietic acid	12.5	14.6	16.9	61.5	20.0	13.0	71.0	11.9	ND	43.8	41.2
Abietic acid	ND	1.2	ND	25.9	5.1	ND	10.9	ND	ND	ND	3.8
Heneicosane	0.1	3.5	ND	19.3	ND	ND	ND	19.9	1.3	11.2	5.3
Tricosane	1.1	22.6	2.9	85.5	26.6	ND	ND	25.1	1.6	22.6	40.9
Pentacosane	11.1	20.9	13.4	53.7	29.7	13.9	23.0	22.4	13.8	19.9	17.9
Heptacosane	1.0	8.9	28.9	42.8	96.2	26.4	30.3	23.5	1.3	26.6	1.5
Nonacosane	20.8	9.7	60.4	77.9	21.3	16.2	39.3	11.5	0.7	35.3	14.5
Decanyl tetradecanoate	0.5	5.4	23.6	16.2	3.2	5.5	78.5	1.7	2.7	13.9	15.1
Phytylhexadecanoate	ND	ND	147.8	ND	ND	ND	83.2	38.0	ND	333.3	40.1
Cycloartenol	33.5	28.3	202.5	92.9	177.2	55.6	211.4	3.6	55.3	77.7	85.4
Ursolic acid	1.6	ND	ND	ND	ND	13.6	ND	3.5	598.2	143.7	188.9
Diploptene	ND	109.7	ND	280.3	86.1	ND	275.8	2.5	0.3	ND	ND
α-Amyrin	ND	18.5	ND	ND	ND	ND	ND	ND	ND	53.0	ND
Oleanolic acid	2.6	ND	ND	ND	ND	ND	ND	ND	ND	165.3	ND
Uvaol	ND	ND	ND	ND	ND	ND	ND	ND	ND	99.4	ND
Stigmasta-3,5-diene	2.5	5.2	11.2	22.5	ND	1.0	47.4	11.8	4.2	8.5	5.5

For fractionation of moss lipids aminopropyl and silica SPE were selected. Considering the interest in specific groups of substances found in bryophytes, the elution system was selected based onto polarity of the eluents and fractions, as a separate class identifying acids (Table 3.13.).

Table 3.13. **Concentrations (µg/g dry weight) of lipids in moss *Polytrichum commune* in the fraction eluted with hexane from silica SPE and SPE-NH_2**

Compounds	Silica SPE Conc., µg/g	SPE-NH_2 Conc., µg/g
Decane	2.3	0.0
Tetradecane	3.2	1.1
Hexadecane	4.9	1.5
Heptadecane	4.7	0.9
Octadecane	6.2	2.1
Neophytadiene	38.9	5.4
(2E)-3,7,11,15-Tetramethyl-2-hexadecene	3.0	0.8
Phyta-1,3(Z)-diene	5.7	1.3
Phyta-1,3(E)-diene	9.9	1.8
Nonadecane	0.3	0.1
Eicosane	6.8	2.1
Octadeca-3,6,9,12-tetraene	17.1	5.7
Phytol	1.2	-
Heneicosane	2.7	1.3
Docosane	6.0	2.6
Tricosane	13.7	1.2
Tetracosane	7.0	1.5
Pentacosane	0.5	0.3
Hexacosane	6.8	0.8
Tetracosanal	4.0	-
Heptacosane	4.5	0.2
Octacosane	5.0	0.5
Squalene	232.3	3.4
Nonacosane	4.2	0.4
Triacontane	5.6	0.4
Octacosanal	17.8	0.9
Hentriacontane	1.5	-
α-Tocopherol	27.9	9.2
Dotriacontane	3.9	-
Campesterol	53.3	9.4
Stigmasterol	55.1	9.6
γ-Sitosterol	15.2	-
Fucosterol	2.5	2.3
Cycloartenol	-	3.5
Phytylhexadecanoate	9.5	19.6

The fraction eluted with hexane contained relatively most low-polar compounds. Remarkable is presence of a high number of hydrocarbons, starting from C_{10} up to C_{30} compounds. Amongst other substances also phytol, phytadiene were identified, some aldehydes, α-tocopherol as well as campesterol, stigmasterol, γ-sitosterol and cycloartenol were found. Still significant differences between fractions obtained from silica SPE and SPE-NH_2 were found and silica SPE can be recommended as the most appropriate for the fractionation supporting identification of hydrocarbons as biomarkers of importance in biogeochemical studies (Baas *et al.,* 2000; Ronkainen *et al.,* 2013). Elution using eluents with an increased polarity – hexane/chloroform (5:1) yielded (Table 3.14.) relatively few compounds, including several dicarboxylic acids and their esters, phytol, squalene, α-tocopherol, cycloartenol and others. Also for this eluent system preferable is the use of silica SPE columns.

Table 3.14. **Concentrations (μg/g dry weight) of lipids in moss *Polytrichum commune* in the fraction eluted with hexane/chloroform (5:1) from silica SPE and SPE-NH_2**

Compounds	Silica SPE Conc., μg/g	SPE-NH_2 Conc., μg/g
Succinic acid	-	-
Hexanedioic acid	-	-
Malic acid	-	-
2-Methyl fructofuranoside	-	-
Hexadecanoic acid methyl ester	1.3	0.1
Octadeca 3,6,9,12 tetraene	0.3	-
9,12-Octadecadienoic acid, methyl ester	0.3	-
Linolenic acid, methyl ester	0.6	-
Phytol	0.8	0.8
Tetracosanal	1.2	-
Squalene	5.4	0.2
Octacosanal	2.8	-
Decanyl tetradecanoate	0.9	0.5
α-Tocopherol	18.9	7.8
Triacontanal	0.7	-
Cycloartenol	-	-
Phytylhexadecanoate	1.9	-

Elution using eluents with an increasing polarity support possibilities to get analytical characterisation of lipid ingredients characterising different groups of moss metabolites. In this respect of interest there are possibilities to quantify substances like tocoferol, squalene, phytol and others. However several of these substances can be found in all fractions, thus, the aim of their isolation in one fraction using the suggested system was not achieved. If eluent polarity change principle is used to achieve acceptable separation efficiency, silica (more polar column) has evident preferences on comparison with aminopropyl bonded phase columns SPE-NH_2.

Elution of both silica SPE and SPE-NH_2 with chloroform (Table 3.15.) yielded relatively high number of substances with evidently high polarity.

Table 3.15. **Concentrations (µg/g dry weight) of lipids in moss *Polytrichum commune* in the fraction eluted with chloroform from silica SPE and SPE-NH_2**

Compounds	Silica SPE conc., µg/g	SPE-NH_2 conc., µg/g
Glycerol	13.2	12.6
Decanoic acid,	0.9	-
Dodecanoic acid	1.4	-
Neophytadiene	62.8	165.7
(2E)-3,7,11,15-Tetramethyl-2-hexadecene	3.1	6.9
Tetradecanoic acid,	4.6	-
Phyta-1,3(Z)-diene	10.9	21.8
Phyta-1,3(E)-diene	15.3	41.9
n-Pentadecanoic acid	2.1	-
7,10-Hexadecadienoic acid	3.5	-
7-Hexadecenoic acid	8.8	-
Hexadecanoic acid	109.2	8.4
Phytol	52.6	49.4
9,12-Octadecadienoic acid (Z,Z)	81.3	-
9-Octadecenoic acid	190.6	-
11-Octadecenoic acid	3.2	-
Octadecanoic acid	15.7	-
Arachidonic acid	12.0	-
cis-5,8,11,14,17-Eicosapentaenoic acid	1.6	-
Eicosanoic acid	1.9	-
7,10-Hexadecadienoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	5.1	7.0
7,10,13-Hexadecatrienoic acid, 2,3-dihydroxypropyl ester	4.9	8.2
Hexadecanoic acid, 2,3-dihydroxypropyl ester	34.4	32.7
Docosanoic acid	3.6	-
9,12-Octadecadienoic acid, 2,3-dihydroxypropyl ester	4.6	7.1
6,9,12-Octadecatrienoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	7.6	10.3
Octadecanoic acid, 2,3-dihydroxypropyl ester	1.7	-
α-Tocopherol	8.5	16.0
Campesterol	391.9	405.9
Stigmasterol	276.0	383.0
β-Sitosterol	151.1	163.4
Fucosterol	31.6	26.2
Lupa-13(18),22-dien-3-ol	25.8	-

Amongst chloroform fraction many substances with high biological activity were found, for example, neophytadiene, phyta-1,3(Z)-diene, phyta-1,3(E)-diene, phytol, several fatty acids (saturated and unsaturated), campesterol, stigmasterol, β-sitosterol, fucosterol. Still significant differences between studied eluent systems can be observed, resulting in both number and quantities of identified substances. Elution with chloroform ensures removal from silica SPE an absolute majority of extracted substances and further eluent on this SPE were not applied.

Table 3.16. **Concentrations (μg/g dry weight) of lipids in moss *Polytrichum commune* in the fraction eluted with diethylether/acetic acid (98:2) from SPE-NH_2**

Compounds	Conc., μg/g
Neophytadiene	2.5
Tetradecanoic acid,	3.3
7,10-Hexadecadienoic acid	2.9
7,10,13-hexadecatrienoate	6.5
Hexadecanoic acid,	90.5
9,12-Octadecadienoic acid (Z,Z)	65.8
9-Octadecenoic acid	160.6
11-Octadecenoic acid	3.1
Octadecanoic acid	24.3
cis-5,8,11,14-Eicosatetraenoic acid	13.2
cis-5,8,11,14,17-Eicosapentaenoic acid	1.9

On the aminopropylsilica SPE column acids are sorbed most strongly (Table 3.16.), and as the final fraction they might be eluted using diethyl ether/acetic acid (98:2) as suggested by Giacometti and others (2002). Thus, the aim of functional fractionation can be achieved.

3.2.2.3. Composition of polar moss metabolites

In order to better characterize possible sources of moss extract biological activity, chemical composition of ethanol extracts obtained by using microwave extraction was done using LC-TOF-MS (Table 3.18.). Extraction with ethanol could help to isolate substances with higher polarity and groups of substances like phenolics, carbohydrates, amino acids could be identified. Using this extractant and the detection method mainly phenolics, their glycosides and amino acids were analysed in moss extracts. Similar as using the GC/MS analysis, also in ethanol extracts detected variations among moss samples were found to be significant and no similarities that would be characteristic for moss species or genus was not found. Some interesting substances with already in literature described high biological activity were found in moss samples. For the first time in composition of mosses high numbers of different phenolics were identified. Biologically active substances found in moss ethanol samples were matairesinol, apigenin,

atraric acid and others. In some samples also widely known plant hormone abcisic acid was identified. It was identified that substance previously supposed to be characteristic only for *Sphagnum* mosses (sphagnic acid) has been found also in other moss species. Interesting result was gained for *Sphagnum rubellum* moss as some unique substances not characteristic for other tested mosses were found like hydroxyharmane and harmol propionic acid ester which are derivatives of alkaloid harmol and has been found only in water moss by now (Asakawa, 2007).

Total quantities of the main groups of identified substances (Table 3.17.) showed that the most abundant groups of substances in moss chloroform extracts are fatty acids (329–1707 mg / 100 g dry moss) and sterols (632–2130 mg / 100 g dry moss). Overall, the main groups of substituents in moss extracts are amino acids (590–3266 mg / 100 g dry moss) and fatty acids.

Table 3.17. **Major groups of secondary metabolites of the studied mosses, mg / 100 g dry moss** (moss chloroform extract analysis by GC/MS and LC-TOF-MS)

Total substance group amounts	AP	CD	DP	HS	PCC	PJ	PS	SF	SM	SR	ST
Fatty acids	329	720	471	1707	796	771	878	836	447	614	1017
Monoglycerols	46	3	24	167	123	56	284	161	86	107	64
Terpenoids	198	159	168	396	176	269	357	116	82	125	134
Alcohols	80	94	166	215	168	74	178	130	74	226	121
Sterols	735	745	1619	1673	1716	715	2131	1696	633	686	633
Diterpens	23	16	33	110	52	13	107	40	1	81	59
Alkanes	34	66	106	279	174	57	93	102	19	116	80
Wax/Wax esters	01	5	171	16	3	6	162	40	3	347	55
Triterpens	38	156	203	373	263	69	487	10	654	539	274
Steroid hydrocarbons	3	5	11	23	ND	1	47	12	4	9	6
Phenolics	652	711	705	509	252	559	441	522	580	632	504
Amino acids	1440	1866	2810	118	988	2412	3266	2121	2536	590	2621

Total quantities of the main groups of identified substances (Table 3.18.) showed that the most abundant groups of substances in moss chloroform extracts are fatty acids (329–1707 mg / 100 g dry moss) and sterols (632–2130 mg / 100 g dry moss). Overall, the main groups of substituents in moss extracts are amino acids (590–3266 mg / 100 g dry moss) and fatty acids.

Analysis of extracts demonstrates presence of a high number of different substances, and several of them have been identified for the first time in the investigated moss species. High number of polyphenols and amino acids has been identified in moss extracts. Overall, the main groups of substances in moss extracts are amino acids, fatty acids, sterols and amino acids.

Table 3.18. **Composition of polar moss secondary metabolites, mg / 100 g dry moss** (LC-TOF-MS analysis of moss ethanol extracts)

No	Formula	PC	RT	AP	CD	DP	HS	PCC	PJ	PS	SF	SM	SR	ST
v1	Atraric acid	3.0	27.3	14.6	44.8	5.9	4.4	14.9	23.8	74.3	2.0	4.3	5.3	7.9
v2	Benzyl benzoate	1.6	3.4	4.1	4.2	ND	6.9	5.4	8.8	12.6	7.5	13.2	ND	5.1
v3	4-Hydroxybenzoic acid	5.6	17.7	10.1	16.2	11.2	10.1	0.8	ND	9.4	7.4	11.5	11.8	4.7
v4	Methyl 4-hydroxybenzoate	2.5	2.8	7.6	8.8	4.1	6.5	0.5	10.1	7.7	1.5	18.9	2.3	7.2
v5	3-Methoxy-4-hydroxybenzoic acid	7.7	12.5	7.9	13.5	1.2	18.2	0.7	15.4	14.5	18.2	26.6	12.1	13.4
v6	4-*O*-Caffeoylquinic acid	ND	ND	ND	25.7	ND	5.0	ND	ND	5.3	7.9	1.9	15.3	11.6
v7	5-*O*-Caffeoylquinic acid	ND	ND	ND	25.7	ND	5.0	ND	ND	5.3	7.9	1.9	15.3	11.6
v8	Caffeic acid	6.4	3.2	41.8	2.3	ND	4.0	5.8	2.9	4.4	ND	ND	2.1	ND
v9	*p*-Coumaric acid	22.3	6.7	11.4	5.9	1.0	3.5	0.2	12.9	11.2	3.5	7.3	11.5	5.0
v10	Ferulic acid	0.8	2.7	21.3	3.3	1.2	1.1	19.1	2.7	3.0	3.7	5.6	1.5	3.9
v11	7,8-Dihydroxy-5-methoxycoumarin-7-β-sophoroside	63.2	144.1	37.1	25.0	58.0	164.9	95.9	117.3	163.8	65.7	32.8	105.3	106.5
v12	5,6,7,8-Tetrahydroxycoumarin-5-β-glucopyranoside	ND	ND	ND	ND	ND	ND	ND	ND	2.6	ND	ND	ND	ND
v13	Abscisic acid	4.3	5.2	ND	3.9	0.7	5.3	3.9	5.9	6.5	3.7	12.5	2.9	7.3
v14	Sphagnic acid	3.4	5.5	10.0	6.4	3.6	4.4	20.3	4.4	2.1	21.9	109.0	19.0	21.8
v15	5,8-Dihydroxy-7-methoxycoumarin 5-β-glucopyranoside	35.8	1.0	7.2	6.1	12.8	4.3	4.5	4.1	2.6	9.8	6.6	30.6	10.5
v16	5,8-Dihydroxy-7-methoxycoumarin 5-β-glucopyranoside	35.8	1.0	7.2	6.1	12.8	4.3	4.5	4.1	2.6	9.8	6.6	30.6	10.5
v17	Dimethyl phthalide	0.8	2.7	21.3	3.3	1.2	1.1	19.1	2.7	3.0	3.7	5.7	1.5	3.9
v18	Dimethyl terephthalate	0.8	2.7	21.3	3.3	1.2	1.1	19.1	2.7	3.0	3.7	5.7	1.5	3.9
v19	Ohioensin H	208.8	ND	0.8	6.1	5.3	4.1	6.6	5.6	2.5	10.3	11.7	7.4	7.6
v20	Harmol propionic acid ester	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.6	ND
v21	7-Hydroxyharmane	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.6	ND
v22	Apigenin	ND	69.0	69.5	71.6	6.0	36.4	ND	ND	21.7	ND	5.8	ND	ND
v23	2,3-Dihydro-5',3'''-dihydroxyamentoflavone	6.1	25.0	24.7	47.4	ND	9.3	ND	78.8	3.3	ND	ND	ND	ND

Table continued on the next page

Table continued from previous page

No	Formula	PC	RT	AP	CD	DP	HS	PCC	PJ	PS	SF	SM	SR	ST
v24	2,3-Dihydrodicranolomin	6.1	25.0	24.7	47.4	ND	9.3	ND	78.8	3.3	ND	ND	ND	ND
v25	Communin A	20.5	ND	ND	3.5	ND	ND	ND	ND	2.2	ND	1.4	ND	ND
v26	2,3-Dihydro-5'-hydroxyrobustaflavone	ND	0.8	ND	ND	ND	ND	3.7	ND	ND	ND	ND	ND	1.3
v27	3'''-Desoxydicranolomin	ND	114.8	102.7	ND	ND	14.7	ND	ND	17.3	ND	0.7	ND	ND
v28	3-hydroxy-β-ionone	4.9	62.0	91.0	9.7	5.8	66.3	7.7	14.6	21.9	2.1	36.6	5.9	14.5
v29	p-hydroxyacetophenone	1.2	31.2	32.3	16.3	0.0	18.4	12.0	ND	6.2	344.4	63.1	25.2	67.7
v30	3,5-dioxohexanoic acid	179.9	98.3	99.3	43.7	99.0	97.6	162.7	91.4	118.3	64.2	70.4	76.5	103.9
v31	4-hydroxybenzoic acid	5.5	17.7	10.1	17.3	11.2	10.1	0.8	ND	9.4	7.4	11.5	11.8	4.7
v32	A-d-furanallulose	ND	ND	ND	ND	ND	ND	6.3	ND	1.3	0.8	1.6	1.0	ND
v33	Benzoic acid	11.8	5.1	0.0	5.2	ND	8.9	4.1	8.8	15.0	4.9	4.8	11.0	13.1
v34	Protocatechuic acid	6.3	11.3	18.4	18.5	5.2	15.9	12.2	10.6	9.5	8.2	13.0	8.7	10.9
v35	Vanillic acid	6.8	12.5	8.2	13.7	4.2	18.2	10.1	15.4	14.5	11.4	13.6	9.0	13.4
v36	Matairesinol	ND	ND	ND	4.4	0.4	ND	ND	ND	ND	ND	ND	ND	ND
v37	Glycine	7.8	31.2	18.4	20.6	16.2	35.3	31.0	20.5	14.1	12.4	19.0	19.2	23.3
v38	Alanine	200.5	145.4	166.0	84.4	297.0	82.6	154.8	60.0	94.4	78.1	407.8	139.8	137.2
v39	Serine	42.1	77.2	57.4	43.8	72.2	112.5	176.1	144.5	178.6	27.4	46.0	27.1	89.7
v40	Glutamic acid	69.8	765.3	709.9	262.5	75.7	1039.5	890.0	846.4	1134.6	135.6	77.7	10.8	587.8
v41	Proline	21.5	95.0	55.6	105.8	24.6	99.6	61.8	37.2	42.3	17.9	46.6	25.3	73.4
v42	Valine	397.6	253.7	208.4	188.9	290.0	255.1	373.5	443.5	188.6	84.3	176.2	53.4	298.9
v43	Threonine	51.4	51.8	62.3	27.7	43.2	81.7	105.7	72.9	73.0	26.1	98.0	12.1	99.0
v44	Leucine	54.5	45.0	278.4	74.9	47.4	57.2	81.0	68.7	121.0	55.3	448.3	15.1	176.0
v45	Asparagine	147.6	ND	ND	ND	ND	ND	541.1	0.0	ND	ND	589.6	0.9	ND
v46	Aspartic acid	194.0	269.0	276.6	42.3	52.4	419.9	507.9	216.8	427.3	49.1	67.5	15.9	186.7
v47	Glutamine	29.9	9.4	8.3	4.9	5.4	5.6	6.1	17.7	7.2	ND	9.8	ND	1.6
v48	Lysine	3.3	0.8	30.5	30.5	2.7	4.4	5.5	1.2	2.6	0.5	6.2	9.1	3.1
v49	Phenylalanine	103.4	69.1	519.1	124.6	32.6	108.6	171.7	45.4	89.9	22.5	355.7	13.3	56.3
v50	Arginine	5.1	30.4	15.5	11.5	3.7	53.6	25.4	84.3	107.0	59.1	95.9	4.0	9.7
v51	Tyrosine	36.2	2.4	25.1	6.4	13.3	21.2	26.7	16.4	13.3	3.3	15.9	3.6	8.6
v52	Tryptophane	75.4	20.7	378.7	159.6	11.6	35.6	107.4	45.9	42.4	18.5	161.1	27.2	38.3

3.2.3. Biological activity of moss secondary metabolites

Bryophyte secondary metabolites have demonstrated wide range of different types of biological activity making them as attractive object for search of new pharmaceutically active substances (Spjut *et al.,* 1992; Asakawa, 2007; Nikolajeva *et al.,* 2012; Cheng *et al.,* 2012), however majority of these studies has been done on example of liwerworts (Asakawa *et al.,* 2013). In order to evaluate biological activity of moss extract two principal types of analysis were used: evaluation of antiproliferative activity on 6 cancer cell lines and antimicrobial activity on 5 stems.

All extracts demonstrated ability to inhibit development of bacteria (diameter of inhibition zone varied from 9 to 15 mm). In all moss extracts antibacterial activity was found against *Bacillus cereus*. The highest activity (diameter of inhibition zone 12 mm) against *Bacillus cereus* demonstrated extracts from *Climacium dendroide* and *Polytrichum commune*, the lowest activity *Hylocomnium splendens* and *Sphagnum magellanicum* (diameter of inhibition zone 9 mm). The highest activity was observed using extract from *Polytrichum commune* against bacteria *Staphylococcus aureus* (diameter of inhibition zone 15 mm). Some moss species showed antibacterial activity (diameter of inhibition zone 10 mm) against *E.coli*: *Climacium dendroides, Ptilium crista-castrensis, Rhytadiadelphus triquetrus*, as well as *Sphagnum magellanicum. Hylocomnium splendens* and *Pleurozium shreberi* also showed activity (diameter of inhibition zone 12 mm) against *Pseudomonas aeruginosa.* Overall, detected antimicrobial activity could be potentially interesting for further research, especially in the case of *Polytrichum commune* which revealed the highest activity. Unfortunately, no correlation between total polyphenol or radical scavenging activity and antimicrobial activity were observed. Therefore, solely chemical analysis cannot be used as indicator for moss antimicrobial activity.

Biological activity of moss extracts was tested on example of proliferation inhibition of cancer cell lines (Table 3.19.).

Table 3.19. **Antiproliferative activity IC_{50} of moss ethanol extract on 6 cancer cell lines.**
Activity is expressed as μL of moss extract

Species	Rat glioma cells (C6) s1	Human epidermoid carcinoma (A431)	Human lung carcinoma (A549) s3	Mouse melanoma cell lines (B16-F10)	Human breast adenocarcinoma (MCF-7) s5	Human colorectal carcinoma (CaCo-2)
AP	51±8	63±8	100±5	ND	100±4	84±4
CD	43±7	64±8	57±8	98±12	ND	66±6
DP	3±0.5	27±4	57±8	12±2	67±4	45±4
PJ	68±8	84±9	24±5	ND	>100	42±5
PC	53±5	23±3	ND	24±5	47±6	24±3
RT	86±6	40±5	98±9	76±8	ND	49±4
PCC	23±5	77±8	32±8	79±8	>100	42±5
PS	5±0.4	28±4	55±7	39±6	>100	61±5
SF	27±4	57±4	>100	69±9	ND	65±3
ST	26±3	62±7	>100	ND	>100	41±3
SM	0.9±0.1	13±2	44±8	89±9	ND	43±4
SR	53±5	72±8	>100	ND	70±5	42±2

Six cancer cell lines were chosen based on occurrence of cancer types, and the same extracts as for antimicrobial activity were used. Only in some cases no inhibition of cell proliferation was detected, but none of extracts showed cytotoxic effect.

The best results were achieved when the extracts were tested on rat glioma and human epidermoid carcinoma, as well as human colorectal carcinoma cell lines. Some extracts (*Sphagnum magellanicum, Dicranum polysetum, Pleurozium shreberi*) showed especially high inhibitory activity (0.9–5 μL) in case of rat glioma cells. Lower activity was observed on human lung carcinoma, mouse melanoma and human breast adenocarcinoma cell lines; in case of these cell lines some extracts did not show any activity or lower activity than in other cases.

The next step of the research was to try to analyse and understand possible connection between chemical composition and biological activity. Couples of statistical analysis methods were tried, and the chosen ones are linear correlation followed by pair analysis using Pearson correlation. In order to evaluate relations between chemical content and biological activity of moss extract statistical analyses was done. Statistical treatment was done on data of cancer cell proliferation and composition of moss ethanol extracts according to the LC-TOF-MS results. Linear correlation analysis was performed in order to understand variation of individual substances found in cancer cell proliferation inhibition activity of moss ethanol extracts for rat glioma cells, human epidermoid carcinoma, human lung carcinoma, mouse melanoma cell lines, human breast adenocarcinoma and human colorectal carcinoma. Following correlation pairs according to analysis were distinguished (Pearson correlation factors and significance are given in brackets):

- rat glioma cells – 2,3-dihydro-5',3'''-dihydroxyamentoflavone and 2,3-dihydrodicranolomin ($r = 0.587$, $p = 0.05$);
- human lung carcinoma – ohioensin H ($r = –0.579$, $p = 0.05$); communin A ($r = –0.610$, $p = 0.05$);
- human colorectal carcinoma – 5,8-dihydroxy-7-methoxycoumarin 5-β-glucopyranoside and 5,8-dihydroxy-7-methoxycoumarin 5-β-glucopyranoside ($r = –0.599$, $p = 0.05$); apigenin ($r = 0.751$, $p = 0.01$); 3'''-desoxydicranolomin ($r = 0.714$, $p = 0.01$); 3-hydroxy-β-ionone ($r = 0.665$, $p = 0.05$).

While positive Pearson correlation factor indicates decreased proliferation activity, negative values show possible presence of specific substances which could be responsible for this effect. Strong associations with other groups of substances found in moss ethanol extracts were not recognized according to Principal Component Analysis (PCA) (Figure 3.14.) that shows similar results to the above used correlation analysis.

In the case of human epidermoid carcinoma cells no relations with specific substances and components were found. Differently from the correlation analysis, PCA analysis (Figure 3.15.) revealed that human lung carcinoma cells are possibly enclosed together with 4-hydroxybenzoic acid, axis 1 and 2 significantly explains 52% of variation.

Analysis of human breast adenocarcinoma cells differently from previous results showed that cell condition could be estimated together with concentrations of 2,3-dihydro-5'-hydroxyrobustaflavone, A-d-furanallulose, serine, threonine and aspartic acid (Figure 3.16.). Human colorectal carcinoma cells according to the correlation analysis showed high similar variations with wide spectra of substances, and according to PCA situations is quite similar; and they are dislocated in one group with caffeic acid, ferulic acid,

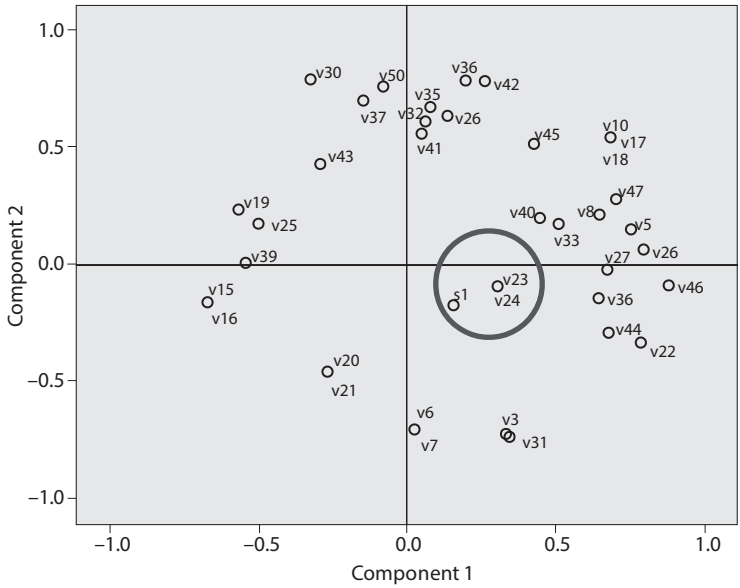

Figure 3.14. **Principal Component Analysis on rat glioma cell line proliferation inhibition and concentration of moss secondary metabolites** (as variables glioma cell proliferation data (S1) and number of substance (v1-v51) according to Table 3.17.; 3.19.were used)

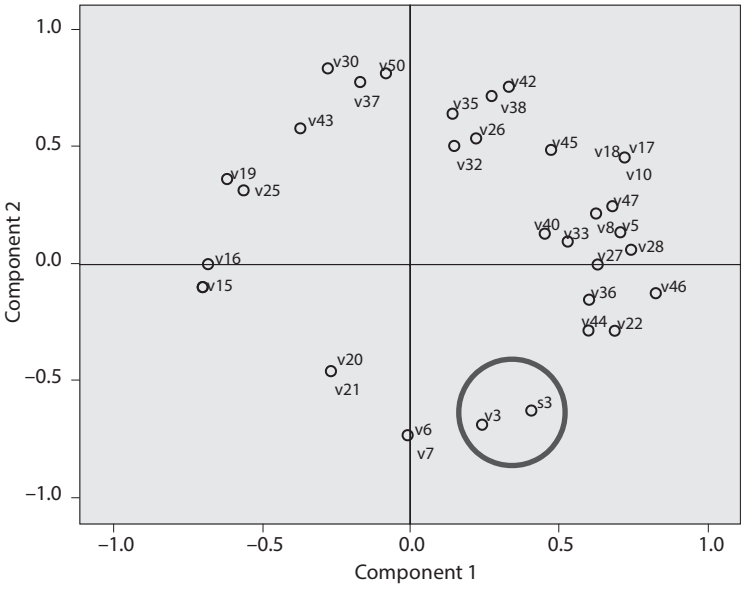

Figure 3.15. **Principal Component Analysis on cell line proliferation inhibition and concentration of moss secondary metabolites** (as variables human lung carcinoma proliferation data (S3) and number of substance (v1-v53) according to Table 3.17.; 3.19. were used)

dimethyl phthalide, dimethyl terephthalate, apigenin, 3'''-desoxydicranolomin, 3-hydroxy-β-ionone, protocatechuic acid. Results of performed study allows to conclude that in order to find biologically active substances PCA analysis as data analytical method can be used. Several studies have indicated antitumor and anti-proapoptic activity of extracts obtained from bryophytes and especially from mosses (Spjut *et al.,* 1992; Asakawa, 2007; Cheng *et al.,* 2012; Asakawa *et al.,* 2013). Also the current study indicates that mosses can be considered as a prospective group of plants for a search of new pharmaceuticals.

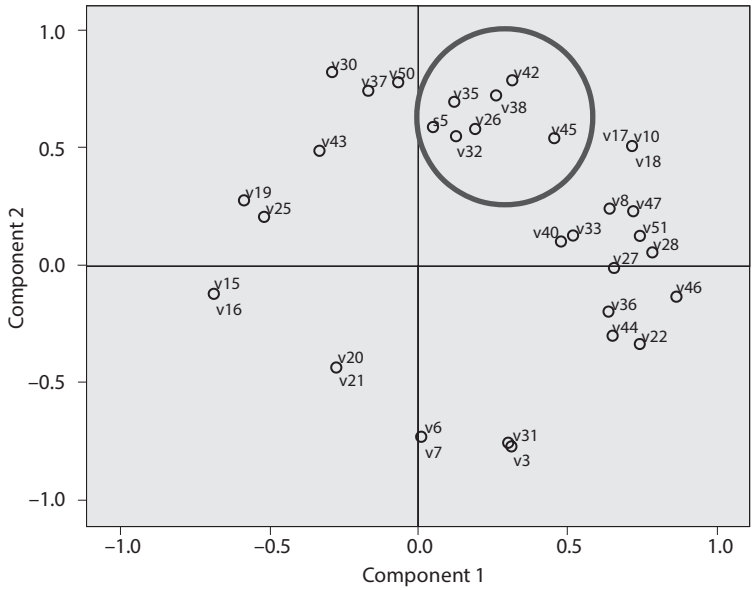

Figure 3.16. **Principal Component Analysis on cell line proliferation inhibition and concentration of moss secondary metabolites** (as variables human breast adenocarcinoma proliferation data (S5) and number of substance (v1-v53) according to Table 3.17.; 3.19. were used)

3.3. Impacts of environmental and pollution stress on moss compostion

3.3.1. Seasonal changes of moss secondary metabolite composition

In respect to better understanding of moss physiology, their reaction on environmental stress, at first drought/wetness stress, oxidative stress, UV radiation impact reaction in mosses, as well as functions of the main secondary metabolites in the overall metabolism it is important to study impacts of seasonal environmental conditions on the secondary metabolites of mosses. Many of mentioned factors have seasonal pattern of change (temperature, amount of precipitations, solar radiation etc.) and, thus, the studies of

seasonal changes of secondary metabolites in mosses can give an insight into regulation pattern and key metabolites controlling moss metabolism. Further, the reaction of moss metabolism on the meteorological conditions (periods with increased solar radiation, prolonged periods with relatively elevated temperatures, reduced amounts of precipitations and other extreme events) might shed a light of possible impacts of the climate change on the moss metabolism. Impact of seasonal changes of vegetation until now has been studied only in very few studies on example of impacts on lipid composition (Karunen and Salin, 1982; Huang *et al.,* 2012) in *Sphagnum* mosses as well as accumulation of some elements (Boquette *et al.,* 2011).

To analyse character of seasonal changes on chemical composition and composition of moss secondary metabolites 4 moss species were selected (*Sphagnum fallax, Sphagnum magellanicum, Polytrichum juniperinum, Pleurozium schreberi*). Samples of these mosses were collected in swamp forest for a two vegetation seasons, 2014–2015.

The study period can be characterised by weather conditions common for Northern Europe/Baltic region with relatively rapid changes. For 2014, more characteristic were periods up to 2 weeks of dry weather conditions with intensive solar radiation changing to periods of intensive precipitation (Figure 3.17.). For 2015, pattern of weather conditions was more even, and dry and wet periods changed more frequently, thus, impacts of heat and wetness were less probable.

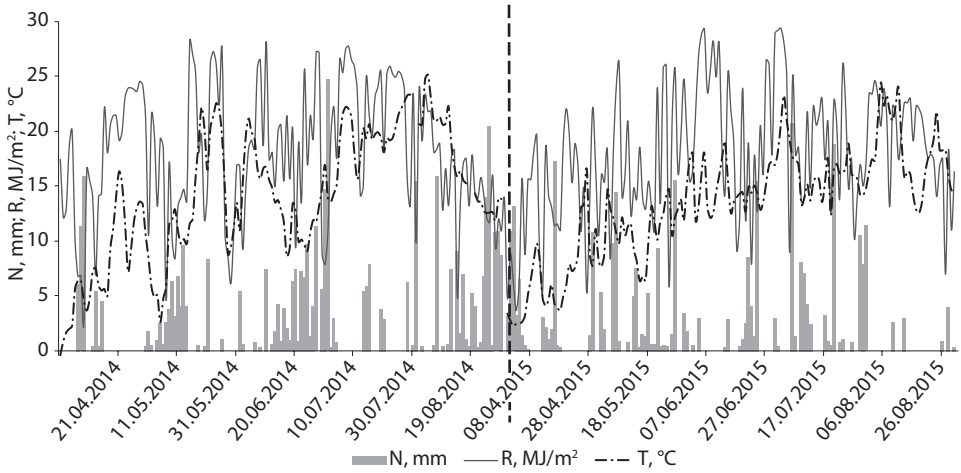

Figure 3.17. **Changes of air temperature (T, °C), amount of precipitations (N, mm) and intensity of solar radiation (R, MJ/m²) during the sampling of studied mosses**

Considering the significance for in-cell processes, total concentration of carbohydrates and polyphenols were analysed. During the study period significant changes in the basic composition of the studied 4 mosses (Figure 3.18.) were observed. During 2014, for a period of increased amount of precipitations, the amount of carbohydrates in all studied mosses increased, but amount of polyphenols decreased. For 2015 when the meteorological conditions were smoother, also the content of polyphenols and

carbohydrates did not change so significantly. Correlation between the summary parameters characterising meteorological conditions (sampling time, temperature and amount of precipitation) during the study period and summary characteristics of the moss extracts indicated strong correlation in several cases. The total yield of extracted

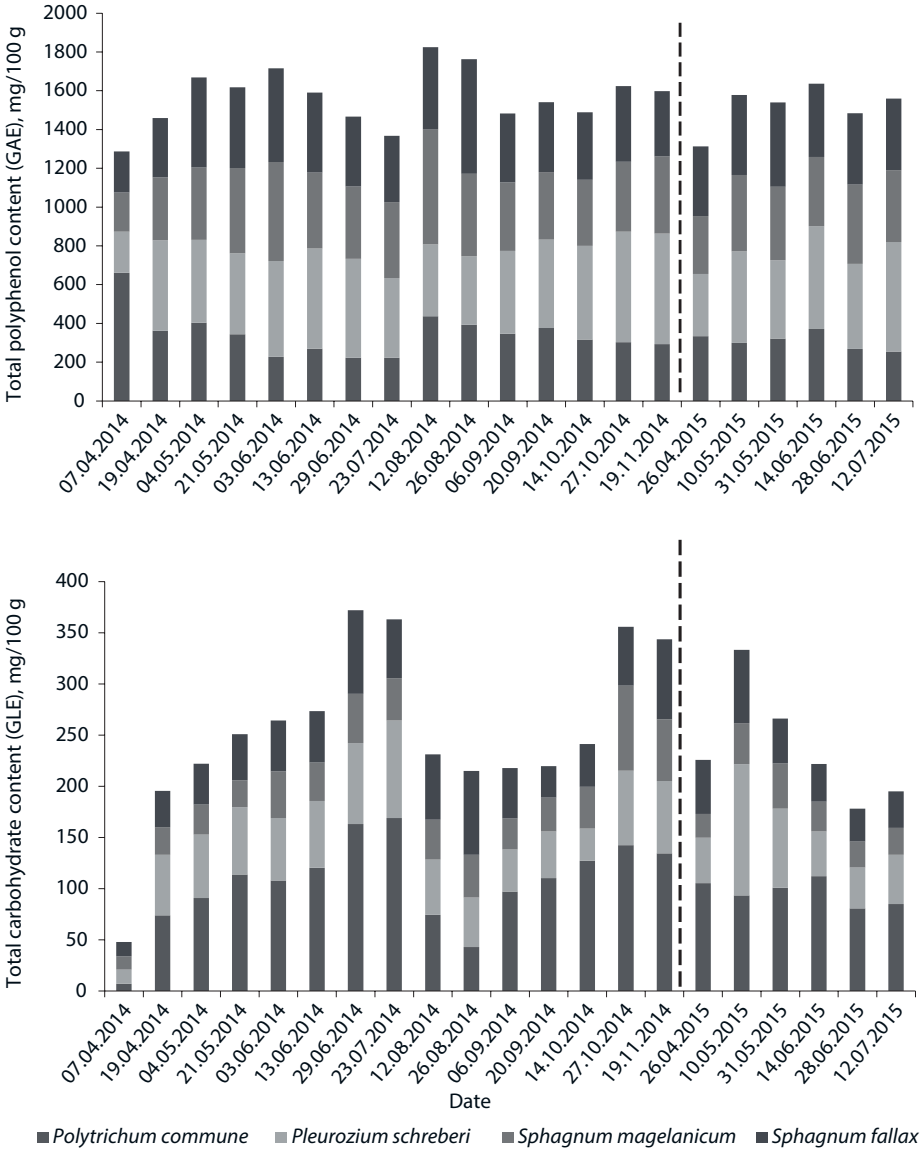

Figure 3.18. **Changes of total content of polyphenols and carbohydrates content in the studied mosses during the study period**

substances correlated with the amount of precipitation. UV sorption of the extracts correlated with the moss species, but the correlation with the radical scavenging activity was observed opposite. Radical scavenging activity strongly correlated with the type of moss species and sampling season, and is the highest at the beginning of the vegetation season, afterwards gradually decreasing, however, trend of changes for carbohydrates is just opposite – it is highest at the end of vegetation season. Concentration of flavonoids correlated with total polyphenolics.

The influence of seasonal processes on composition of secondary metabolites further were studied on example of lipids (as lipids are considered not only triglycerides, but also waxes, fatty acids and alcohols, sterols, terpenes and other substances, considering their polarity) and pattern of their changes becomes evident from the results of chromatographic analysis of lipids in *Sphagnum fallax* (Figure 3.19., Table 3.20.). Both, the number of individual substances and their concentration, significantly changed during the vegetation season, and the highest number of secondary metabolites can be found in autumn season.

Table 3.20. **Correlation between changes of meteorological parameters and composition indicators of studied mosses during the study period** (Kendall tau correlation)

	Temp	Precip	Month	Type	AA	PD	OD	FD	SI
Precip	–0.257**								
Month	–0.050	–0.050							
AA	–0.131	0.018	–0.217*	0.693**					
PD	0.136	–0.016	–0.011	0.042	0.017				
OD	0.120	–0.011	0.155*	–0.387**	–0.546**	–0.102			
FD	0.009	0.035	0.086	–0.043	–0.035	0.273**	–0.077		
SI	–0.097	0.211*	0.136	0.278**	0.140	0.021	0.085	0.122	
UV	–0.011	0.064	0.008	–0.649**	–0.682**	–0.017	0.463**	0.018	–0.141

** – Correlation is significant at the 0.01 level (1-tailed)
* – Correlation is significant at the 0.05 level (1-tailed)
Precip – amount of precipitations; Temp – air temperature, Month – sampling month;
AA – antioxidant activity; PD – total polyphenolics, FD – total flavonoids, SI – dry residue of extracts; UV – sorption of extracts at 280 nm

Chromatographic analysis revealed significant differences in the lipid fraction composition depending on the moss species (Figure 3.20.) and sampling season as it is demonstrated on example of *Sphagnum fallax*.

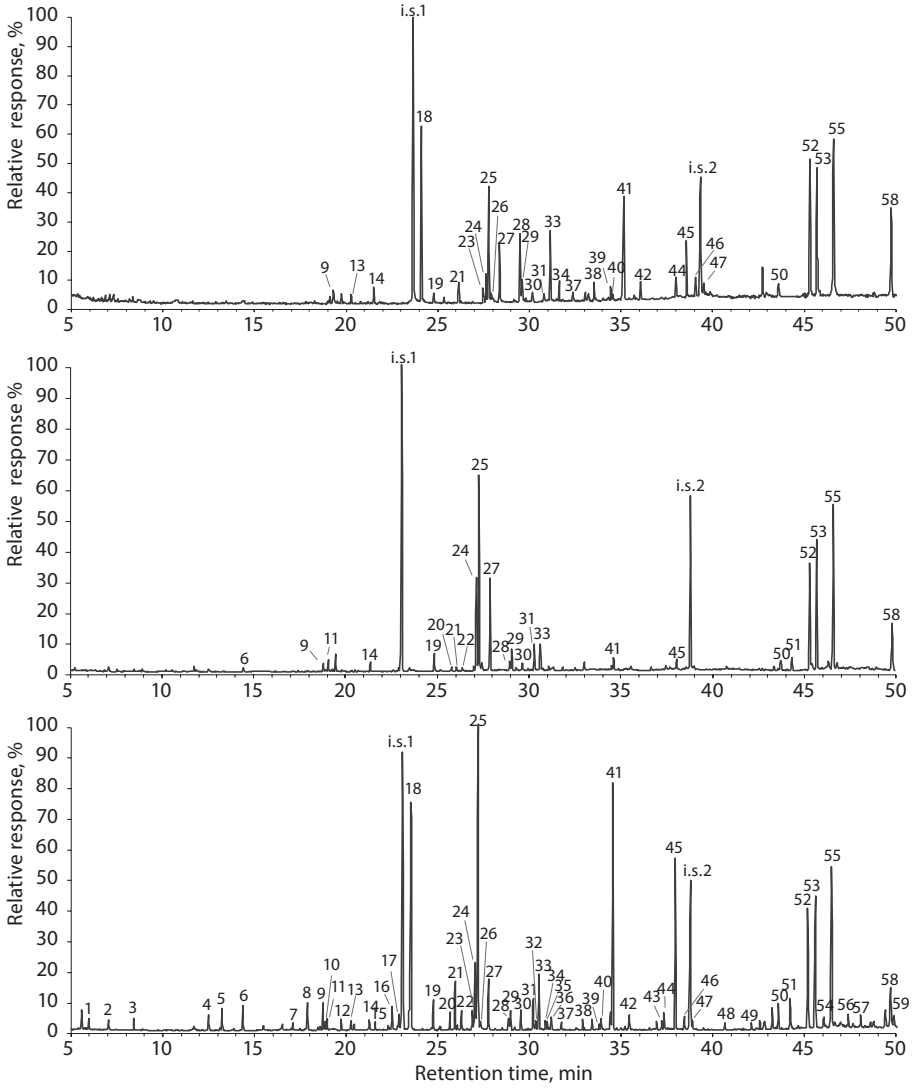

Figure 3.19. **Gas chromatogram with mass-spectrometric detection of lipid extract of *Sphagnum fallax* A – spring (07.04.2014); B summer (23.07.2014); C – autumn (25.10.2014)** (i.s.1 internal standard methyl hexadecanoate; i.s.2 internal standard progesterone). Acid and alcohol groups were derivatised prior to the GC/MS analysis. Peak numbers refer to compounds: 1 – 1-Octanol; 2 – Glycerol; 3 – Nonanoic acid; 4 – 1-Dodecanol; 5 – Hexadecane; 6 – Dodecanoic acid; 7 – 1-Tetradecanol; 8 – Octadecane; 9 – Neophytadiene; 10 – Hexahydrofarnesyl acetone; 11 – Tetradecanoic acid (myristic acid); 12 – Phyta-1,3(E)-diene; 13 – 7,9-Ditertbutyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione; 14 – Pentadecanoic acid; 15 – 1-Hexadecanol; 16 – Eicosane; 17 – 9-Hexadecenoic acid; 18 – Hexadecanoic acid (palmitic acid); 19 – Heneicosane; 20 – Heptadecanoic acid (margaric acid); 21 – 1-Octadecanol; 22 – Phytol; 23 – Docosane; 24 – (Z,Z) 9,12-Octadecadienoic acid; 25 – (Z,Z,Z) 9,12,15-Octadecatrienoic acid; 26 – (E) 9-Octadecenoic acid (elaidic acid); 27 – Octadecanoic acid (stearic acid);

28 – Pimaric acid; 29 – Tricosane; 30 – Isopimaric acid; 31 – (all-Z) 5,8,11,14-Eicosatetraenoic acid; 32 – (all-Z) 8,11,14-Eicosatrienoic acid; 33 – Dehydroabietic acid; 34 – 2,3-Dihydroxypropyl tetradecanoate; 35 – Tetracosane; 36 – Abietic acid; 37 – Eicosanoic acid (arachidic acid); 38 – Pentacosane; 39 – Docosanol; 40 – 2-Hydroxy-1-(hydroxymethyl)ethyl hexadecanoate; 41 – 2,3-Dihydroxypropyl hexadecanoate; 42 – Docosanoic acid (behenic acid); 43 – Tricosanoic acid; 44 – 2-Hydroxy-1-(hydroxymethyl)ethyl octadecanoate; 45 – 2,3-Dihydroxypropyl octadecanoate; 46 – Squalene; 47 – Tetracosanoic acid (lignoceric acid); 48 – 1-Hexacosanol; 49 – Hexacosanoic acid (ceratinic acid); 50 – α-Tocopherol + Cholesterol; 51 – (22E)-Campesta-5,22-dien-3β-ol; 52 – Campesterol; 53 – Stigmasterol; 54 – Ergost-7-en-3-ol (γ-ergostenol); 55 – β-Sitosterol; 56 – Cycloartenol; 57 – γ-Sitostenone; 58 – Betulin; 59 – Phytylhexadecanoate

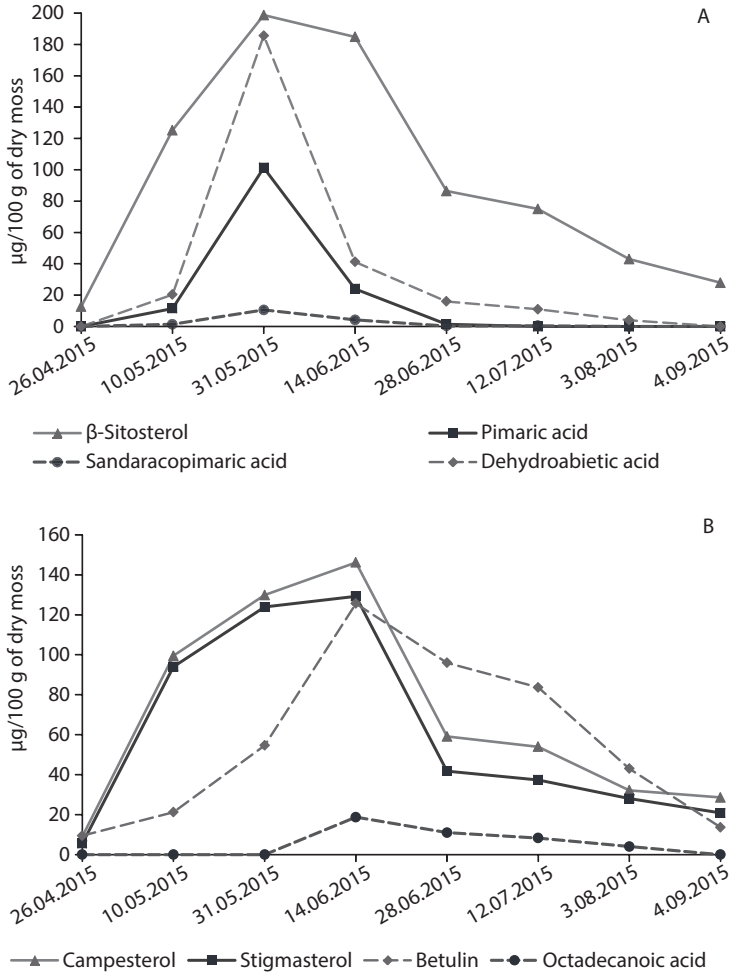

Figure 3.20. **Changes of lipid concentration in the extract of *Sphagnum fallax*.**
Lipid analysis done by GC/MS

However concentrations of moss lipids have major dependence on the season, thus reflecting pattern of their biosynthesis and decomposition during the vegetation period (Figures 3.20., 3.21.). All selected representatives of lipid class (campesterol, stigmasterol, β-sitosterol, betulin, octadecanoic acid, pimaric acid, sandaracopimaric acid, dehydroabietic acid) have well expressed pattern of seasonal dependence on their concentration with a maximum around the time of moss most intensive growth (June, July), gradually decreasing towards autumn. For different mosses at the same time maximum concentrations has been reached at different time periods indicating differences in the biosynthetic processes (Figure 3.21.).

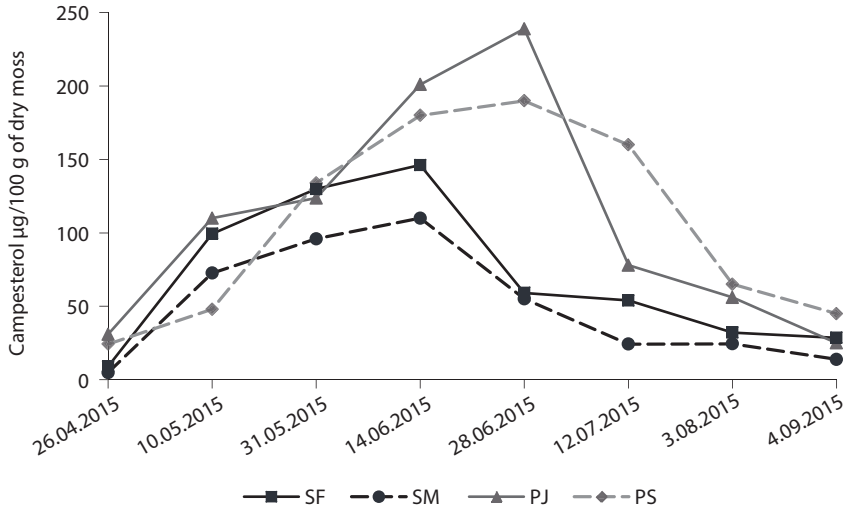

Figure 3.21. **Changes of campesterol concentration in *Sphagnum fallax* (SF), *Sphagnum magellanicum* (SM), *Polytrichum juniperinum* (PJ), *Pleurozium schreberi* (PS).** Campesterol analysis done by GC/MS

The seasonal pattern of concentration changes of moss lipids is of importance to understand character of the regulation processes in mosses and bryophytes in general as well as to select sampling seasons for bioprospecting studies: to study and isolate biologically active substances present in mosses.

3.3.2. Effects of metal pollution stress on moss elemental and secondary metabolite composition

Use of living organisms to study environmental quality and indicate environmental pollution is widely applied, including also everyday praxis of environmental monitoring (Harmens *et al.,* 2010); however, the data from biomonitoring studies should be supported with physical-chemical measurements of model systems. Mosses are widely used for air pollution biomonitoring, considering character of pollutant accumulation and their

ability to take up contaminants from air (Zechmeistar *et al.,* 2005). Another possibility of moss application in environmental quality control is related to the use of so called moss bags – samples of commonly devitalised mosses packed in bags and being able to absorb pollutants from air or water (Basile *et al.,* 2009; Capozzi, 2017). Using devitalised mosses the physical adsorption and chemisorption are the key processes governing both particulate and ionic metal species retention in the moss mass. Ionic metal species mostly are bound to the extracellular anionic exchange sites located in cell walls and plasma membrane surfaces (Brown and Brumelis, 1996). However to support further advancement of the biomonitoring methods and to understand metal stress reactions it is needed to study moss reaction on metal pollution.

As the first step to study metal stress impacts, cultivation of several studied mosses with metal solutions were done on example of species *Pleurozium schreberi* and *Sphagnum magellanicum* considering their abundance and perspective to use for biomonitoring. Stress reaction of mosses to metal ions were studied after incubation for 72 h cultivating the selected mosses under experimental conditions as suggested by several studies (Sun *et al.,* 2011; Basile *et al.*, 2012). The experimental study demonstrated ability of the studied mosses to accumulate metals (Figure 3.22.). Concentrations of Cu and Pb in metal treated samples were statistically higher ($P<0.05$) than in control samples. However evident differences between the studied mosses were detected as the metal accumulation capacity of *Sphagnum magellanicum* species are higher than in *Pleurozium schreberi* species, indicating their different metal tolerance and accumulation processes. Differences amongst the mosses have been found also in previous studies (Chen *et al.,* 2015).

The mechanisms governing metal accumulation in mosses include metal cation binding to extracellular anionic exchange sites located in the cell wall and plasma membrane surface (Basile *et al.,* 2012), thus immobilising metal ions and reducing their biological accessibility. It is known that anionic exchange sites can be characterised by cation exchange capacity (CEC) and CEC values are remarkably higher in *Sphagnum* mosses in comparison to other moss species (Gonzalez and Pokrovsky, 2014; Gonzalez *et al.,* 2016), thus explaining high metal absorption capacity also in the current experiments. High sorption capacity at the anionic exchange sites explains also high sorption capacity of devitalised *Sphagnum* mosses used in moss bags (Ares *et al.,* 2015).

However physical sorption and chemisorption by far is not the only one factor governing the retention of metal ions in mosses and plant reaction. Heavy metals are able to induce production of reactive oxygen species (ROS) in plant cells, such as hydrogen peroxide and free oxygen containing radicals (Reddy *et al.,* 2005) and this stress reaction mechanism has been demonstrated also on moss species (Sun *et al.,* 2009; Sun *et al.,* 2011).

On several moss species it has been demonstrated that treatment of mosses with metal ions is resulting in overproduction of ROS which could react with lipids, proteins, carbohydrates causing membrane damage, lipid peroxidation and other harmful effects (Sun *et al.,* 2009; Sun *et al.,* 2011). Further reaction on oxidative stress is induction of production of enzymes like superoxide dismutase, peroxidase and other enzymes as well as other biomolecules (ascorbic acid, glutathione and others), responsible for scavenging of reactive oxygen species (Bhaduri and Fulekar, 2012).

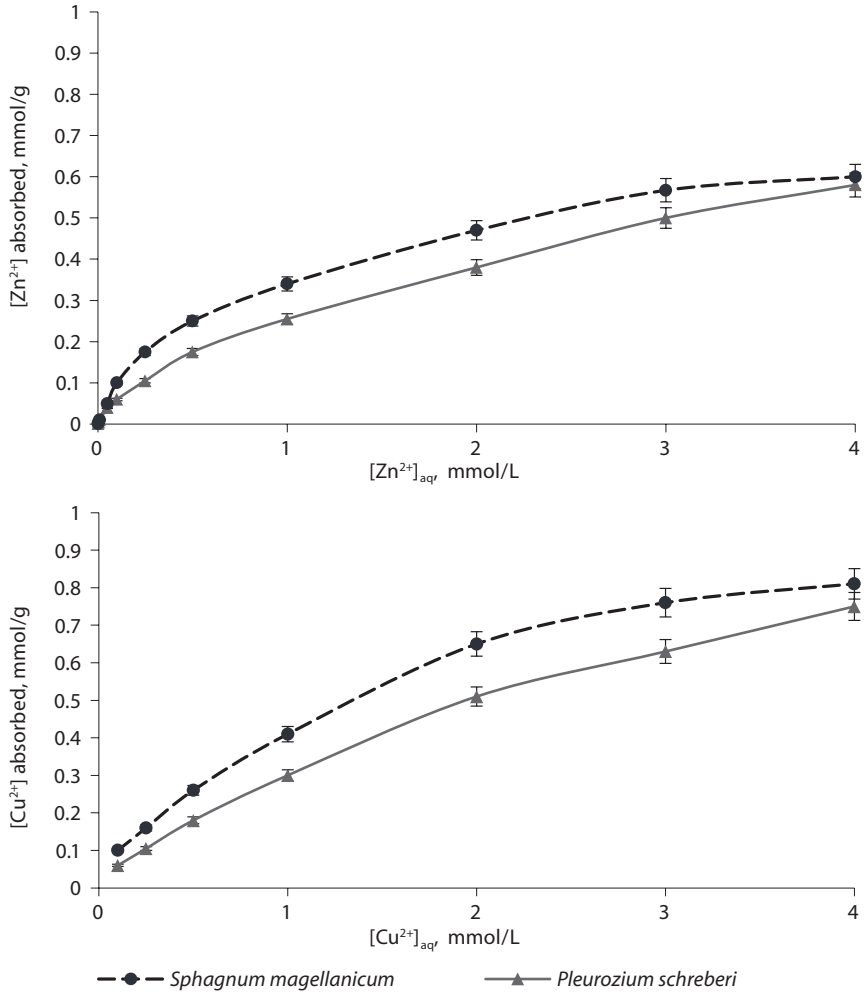

Figure 3.22. **Accumulation of Zn and Cu in mosses *Pleurozium schreberi* and *Sphagnum magellanicum* after treatment for 72 h with varying concentrations of $Zn(NO_3)_2 \times 3H_2O$, $Cu(NO_3)_2 \times 5H_2O$ solutions**

Current study of metal ion (Zn^{2+} and Cu^{2+}) impacts on metabolism of moss *Pleurozium schreberi* and *Sphagnum magellanicum* (Figure 3.23.) indicates a steady increase of radical scavenging activity in respect to Zn^{2+} however for Cu^{2+} it take place until concentration 4 mmol/L, indicating higher toxicity of Cu^{2+} ions (lower concentrations at which compensating mechanisms are exhausted) in comparison with Zn^{2+} ions. On the other hand, remarkable are changes in total polyphenol concentrations. In higher vegetation phenolics are a common factor significantly contributing to pollution stress mitigation. However, as previously stated in this work (Chapter 3.1.) aromatic compounds and thus lignin in studied mosses is at minor quantities.

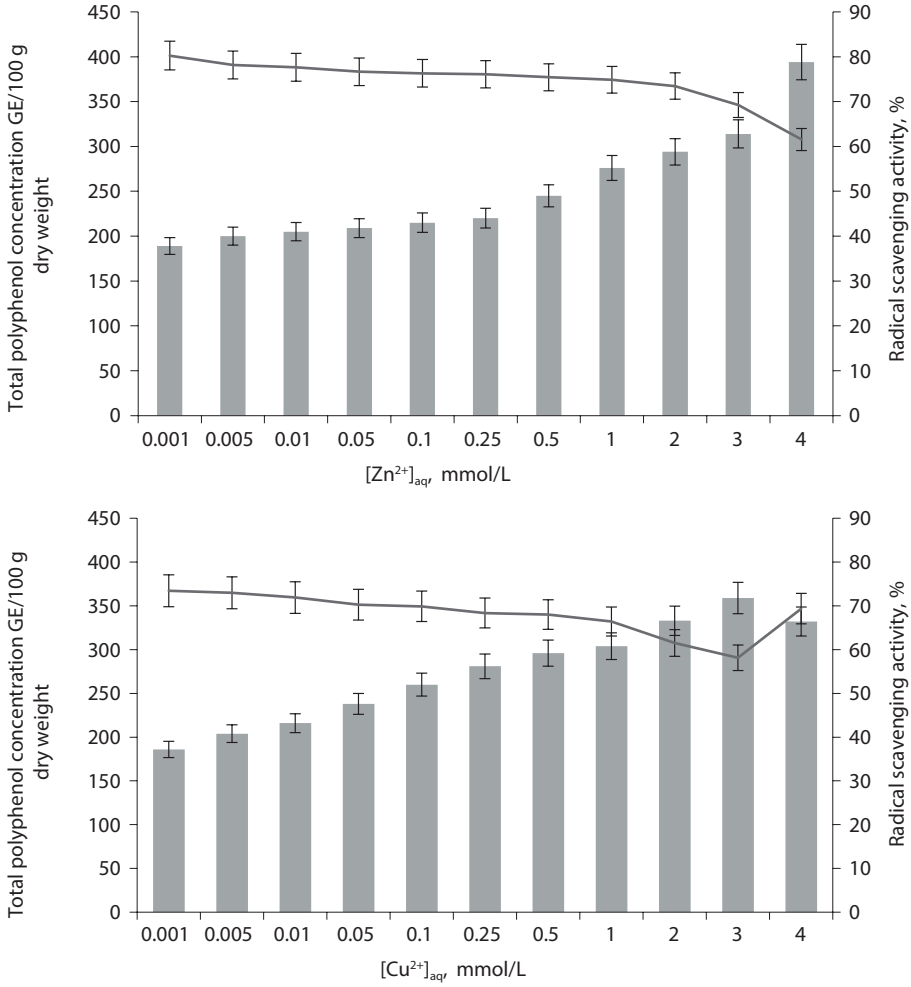

Figure 3.23. **Changes of total polyphenol concentration and radical scavenging activity in mosses *Pleurozium schreberi* and *Sphagnum magellanicum* after the treatment for 72 hrs with varying concentrations of $Zn(NO_3)_2 \times 3H_2O$, $Cu(NO_3)_2 \times 5H_2O$ solutions**

Most probably shikimic pathway contributing to synthesis of polyphenolic in mosses is not fully developed and thus production of phenolics with increasing pollution stress is not increasing as it is common in higher vegetation (Kylli, 2011).

To study impacts of metal ions on the moss metabolism, the analysis of changes of lipid composition in mosses subjected to increasing concentrations of metals during cultivation were done (Figure 3.24., 3.25.). Analysis of trends of changes on lipid pool on example of three major classes of lipids (hydrocarbons, including unsaturated substances, fatty acids and sterols) as reaction of increasing doses of metals (Pb^{2+} ions) revealed significant impact of metal exposure to lipid metabolism.

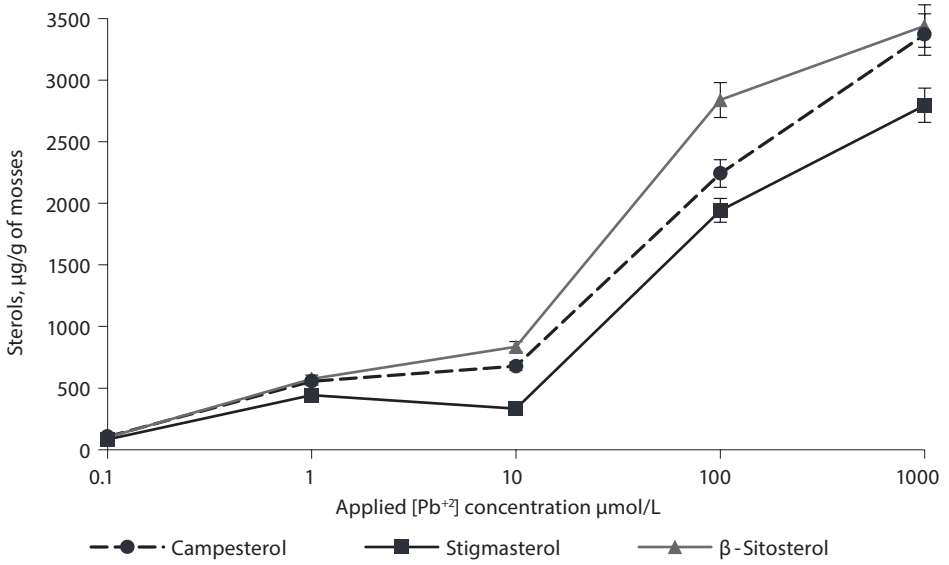

Figure 3.24. **Change of sterol concentration due to increasing doses of Pb^{2+}**

For some substances linear dependence of concentration change as a reaction on pollution stress was observed, for example, this kind of reaction is common for all selected sterols (campesterol, stigmasterol and β-sitosterol) as their concentrations are increasing with increase of metal concentration. However, also in this case differences amongst the substances might be observed, as the concentrations of campesterol and β-sitosterol are increasing smoothly, but concentration of stigmasterol do not much react on the Pb^{2+} concentration changes. However, then at concentration 10 µM significant increase happens – intensive production of this sterol is initiated with increasing metal concentration, thus indicating processes which are 'switched on' at some pollutant concentration. Contrary, concentrations of the selected fatty acids (nonanoic, decanoic and tetradecanoic acids) are decreasing with increase of the metal concentration (Figure 3.25., 3.22.).

Possible mechanisms of such metal impact can be related to explanations given by Guschina and co-authors (Guschina and Hardwood, 2000; Guschina *et al.,* 2002; Guschina and Hardwood, 2002). In studies of heavy metal impacts on accumulation of acetylenic triacylglycerols in moss *Dicranum scoparium*, Guschina and others suggested that metals can directly act as inhibitors on several enzymes involved in the synthesis chain of lipids. Reaction of hydrocarbon concentrations on the impact of heavy metals demonstrate different pattern for each of the selected substance: some were decreasing, others were increasing till some impact level, and decreasing afterwards (Figure 3.25., 3.23.).

As hydrocarbons are a part of moss waxes, at first their concentration is much lower, but their value as possible biomarkers is much lower.

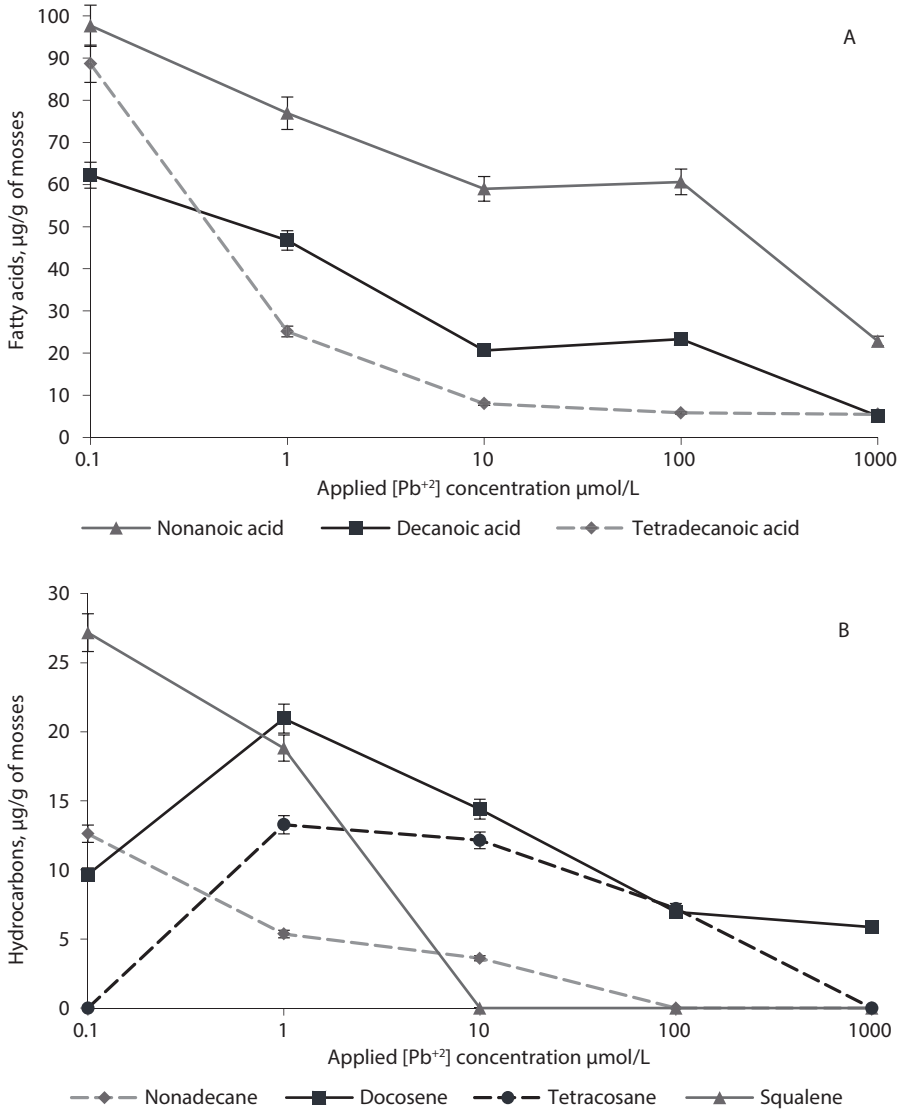

Figure 3.25. **Concentration change of fatty acids (A) and hydrocarbons (B) to increasing doses of Pb^{2+}**

Thus this study has convincingly demonstrated possibilities to use moss metabolite pool (in this case, lipids) for indication of pollutant impact below the threshold values, to indicate impact of pollutants at molecular level. Of course further research is needed, however already the first results demonstrate potential of such approach.

CONCLUSIONS

1. The study of elemental composition of selected 16 moss species biomass, supported by pyrolysis-gas chromatography/mass spectrometry, CPMAS ^{13}C NMR and FTIR spectrometry, demonstrated that their major components are various carbohydrates, while lignin cannot be found in the studied mosses. Elemental and structural composition analysis of mosses can be applied as a tool for their chemotaxonomy studies.
2. Selection and optimisation of moss secondary metabolite extraction methods can support elaboration of environmentally friendly approaches to obtain extracts with selected and specific activity (for example, antioxidant activity) resulting in high yields.
3. Analysis of moss secondary metabolite composition lead to the identified presence of moss genus characteristic substances as well as a group of biologically active substances found in the studied mosses for the first time.
4. Solid phase extraction fractionation is an efficient tool to support separation of secondary metabolites in groups of substances with high biological activity or possible significance for bioindication and studies of biogeochemical processes in the environment.
5. Cell tests of the studied moss extracts demonstrates different aspects of their biological activity (antioxidant, antimicrobial, antitumor) and, thus, the topicality of further studies of moss composition for bioprospecting purposes.
6. Impacts of environmental stress (droughts/wetness, pollutants) on mosses result in significant changes of their secondary metabolite pool as well as parameters, characterising their composition, and it can support identification of environmental stress biomarkers.

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Laura Kļaviņa

LAPU SŪNU, METABOLĪTU SASTĀVS UN VIDES STRESA IETEKME

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SATURS

SAĪSINĀJUMI

CP MAS ^{13}C NMR – ^{13}C kodolmagnētiskās rezonanses spektroskopija, izmantojot spektra uzņemšanu zem maģiskā leņķa (angļu val. *cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance spectroscopy*)

DMSO – dimetilsulfoksīds (angļu val. *dimethylsulphoxide*)

BSTFA – N,O-Bis(trimetilsilil)trifluoroacetamīds

DPPH – 2-difenil-1-pikrilhidrazils (angļu val. *2-diphenyl-1-picrylhydrazyl*)

GC/MS – gāzes hromatogrāfija-masspektrometrija (angļu val. *gas chromatography – mass spectrometry*)

HCA – hierarhiskā klāsteranalīze (angļu val. *hierarchical cluster analysis*)

FTIR – Furjē transformācijas infrasarkanā spektrometrija (angļu val. *Fourier transform infrared spectrometry*)

Py-GC/MS – analītiskā pirolīze – gāzes hromatogrāfija – masspektrometrija (angļu val. *analytical pyrolysis – gas chromatography – mass spectrometry*)

SPE – cietfāzes ekstrakcija (angļu val. *solid phase extraction*)

UPLC – ultra augstas izšķirtspējas šķidruma hromatogrāfija (angļu val. *ultra performance liquid chromatography*)

UPLC–TOF – ultra augstas izšķirtspējas šķidruma hromatogrāfija ar nolidojuma laika masspektrometriju (angļu val. *ultra performance liquid chromatography with time-of-flight mass spectrometry*)

IEVADS

Lapu sūnas pieder pie vienkāršākajiem sauszemes augiem, bet taksonomiski tās pieskaitāmas pie ar sugām bagātākās augu valsts grupas – sūnaugiem (Goffinet and Shaw, 2008). Neskatoties uz sūnaugu svarīgo lomu augu evolūcijā un augsto izplatību visa pasaulē, kā arī nozīmību vides un dabas procesos, pētījumu par sūnaugiem ir ievērojami mazāk nekā pētījumu par augstākajiem augiem. Sūnaugu sastāvā ir identificētas daudzas bioloģiski aktīvas vielas, kas savukārt veicina interesi par sūnaugu ķīmisko sastāvu un to funkcijām (Asakawa *et al.*, 2013). Jaunu vielu identifikācija sūnaugos parāda, ka šī augu grupa ir perspektīva izmantošanai turpmākos pētījumos biofarmācijas jomā. Tāpat sūnaugi var tikt uzskatīti par vērtīgiem augiem bioekonomikas attīstībai, ņemot vērā to izmantošanas augsto potenciālu. Neskatoties uz lielo sugu skaitu, tikai nelielam skaitam sūnaugu sugu ir pētīts to ķīmiskais saturs, vairums ķīmiskā sastāva pētījumi ir veikti aknu sūnām (Asakawa, 2007), it īpaši aknu sūnu eļļas ķermenīšiem. Sūnaugi ir vieni no vecākajiem sauszemes augiem, tādejādi tiem ir garš evolūcijas process un līdz ar to ķīmiskā sastāva izpēte varētu sniegt jaunu izpratni par augu ķīmisko un bioloģisko evolūciju.

Lielākā sūnaugu grupa ir lapu sūnas, kas ir īpaši svarīgs ekosistēmu elements īpaši Ziemeļu puslodē, kur tās ir galvenais purvu un kūdras veidojošais augs (*Sphagnum* sūnas). Tāpat būtisku lomu lapu sūnas ieņem mežu ekosistēmās.

Galvenā strukturālā savienojumu grupa sūnaugos ir ogļhidrāti (Maksimova *et al.*, 2014; Klavina, 2015), taču tie satur arī citus savienojumus ar iespējams augstu bioloģisko aktivitāti. Lapu sūnu ķīmiskais sastāvs ir maz pētīts īpaši saistībā ar tā izmaiņām vides apstākļu ietekmē. Vēl viens iemesls lapu sūnu izpētei ir to unikālais metabolisms, kas potenciāli atšķiras no augstākajiem augiem un ir atbildīgs par lapu sūnu unikālajām īpašībām un ķīmisko sastāvu. Lapu sūnām ir raksturīga augsta izturība pret dažādiem vides stresa faktoriem (sausums, mitrums, sals, karstums) un tāpēc sekundāro metabolītu pētījumi var palīdzēt noskaidrot mehānismus, kas par to ir atbildīgi.

Darba mērķis

Promocijas darba mērķis ir pētīt lapu sūnu sastāvu, to metabolītus un vides stresa ietekmes uz tiem.

Hipotēze

Sūnaugu kā zemāko augu ķīmiskais sastāvs atšķiras no augstākajiem augiem, bet to ķīmiskais sastāvs (pamatsastāvs un sekundārie metabolīti) ir būtisks, lai saprastu augu evolūciju un vides stresa atbildes reakcijas.

Darba uzdevumi

1. Lapu sūnu ķīmiskā sastāva analīze izmantojot multiparametru analīzes metodes, lai veicinātu ķīmiskās taksonomijas izmantošanu lapu sūnu identifikācijai;
2. Lapu sūnu sekundāro metabolītu ekstrakcijas un ekstraktvielu frakcionēšanas metožu izstrāde;
3. Lapu sūnu sekundāro metabolītu bioloģiskās aktivitātes raksturojums, lai veicinātu jaunu to izmantošanas jomu attīstību;
4. Vides mainības un vides piesārņojuma stresa ietekmes novērtējums uz lapu sūnu ķīmisko sastāvu.

Zinātniskā novitāte

1. Pierādīts, ka sūnu sastāvu veido ogļhidrāti, bet lignīns atrodas zīmju daudzumos;
2. Sūnu sastāva ķīmiskā analīze var tikt izmantota sūnu taksonomijai;
3. Ekstrakcijas apstākļu izpēte un optimizācija ļauj iegūt lielu skaitu sūnu metabolisma produktu izmantojot videi draudzīgas ekstrahentu sistēmas;
4. Sūnu sekundārie metabolīti uzrāda antimikrobiālu aktivitāti un tie uzskatāmi par augu ekstraktvielu grupu, kas perspektīva jaunu farmakoloģiski aktīvu savienojumu iegūšanai.

Sasniegumi

1. Metodoloģija kompleksai lapu sūnu ķīmiskā sastāva analīzei un raksturošanai;
2. Metodes lapu sūnu sekundāro metabolītu izdalīšanai un bioloģiskās aktivitātes testēšanai.

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1. LITERATŪRAS ANALĪZE

1.1. Sūnaugi vidē

Pasaulē ir atrodamas vismaz 25 000 sūnaugu sugas visdažādākajās ekosistēmās. Sūnaugi iedalās 3 grupās – lapu sūnas (*Musci*) ~18 000 sugas, aknu sūnas (*Hepaticae* ~6000 sugas) un ragvācelītes (*Anthocerotae* ~1000 sugas). Latvijā ir sastopamas vismaz 550 sūnaugu sugas, galvenokārt lapu sūnas, aknu sūnas un dažas ragvācelīšu sugas (Strazdina *u. c*., 2012). Balstoties uz evolūcijas teoriju, sūnaugi atrodas starp aļģēm un augstākajiem vaskulārajiem augiem (Goffinet and Shaw, 2008). Sūnaugu izpēte varētu veicināt izpratni par to kā notikusi augu attīstība no zemākajiem augiem līdz augstākajiem. Sūnaugi savā evolūcijā ir attīstījuši bioķīmiskos procesus, lai nodrošinātu savas augstās izdzīvošanas spējas, taču augstāko augu gadījumā notika to vadaudu un citu anatomiski fizioloģisku procesu attīstība. Piemēram, viens no ķīmiskajiem savienojumiem, kas ir atrodams augstākajos augos un kalpo par to šūnu sieniņu galveno elementu ir lignīns, kas līdz šim nav pārliecinoši identificēts zemākajos augos, tomēr tajos iespējams atrast citus savienojumus, kas pilda līdzīgas funkcijas (Popper and Fry, 2003; Popper, 2004).

Sūnaugi savas unikālās bioloģijas, bioķīmijas un adaptācijas spēju dēļ ir atrodami dažādos augšanas apstākļos visa pasaulē (Goffinet and Shaw, 2008; Glime, 2007). Neskatoties uz sūnaugo salīdzinoši nelielo izmēru, to augstās adaptācijas spējas un citas īpašības tos padara par svarīgu daudzu ekosistēmu sastāvdaļu. Sūnaugi, īpaši lapu sūnas, ir nozīmīgi tādās ekosistēmās kā purvi un tundra, kurās tās ieņem nozīmīgu lomu kūdras veidošanā (Turetsky, 2003). Tāpat sūnaugi nostiprina augsnes virskārtu un pasargā no erozijas, kā arī aiztur sedimentus un citas organiskās vielas no aizskalošanās. Sūnaugu slānis uztur arī mitrumu un stabilizē arī temperatūras izmaiņas, šādi apstākļi ir īpaši labvēlīgi dažādu sēņu, aļģu, mazu bezmugurkaulnieku un sēklu attīstībai un augšanai (Turetsky, 2003; Glime, 2007). Attīstītās adaptācijas un izdzīvošanas spējas ir iemesls tam, kādēļ sūnaugi ir tik plaši izplatīti. Sūnaugiem piemīt augstāka vides un piesārņojuma stresa tolerance kā lielākajai daļai augstāko augu (Glime, 2007). Tāpat sūnaugi spēlē būtisku lomu oglekļa un slāpekļa bioģeoķīmiskās aprites procesos.

Neskatoties uz plašo sūnaugu izplatību un sugu dažādību to unikālais ķīmiskais sastāvs ir salīdzinoši maz pētīts (Zinsmeister and Mues, 1990; Glime, 2007) un tiek lēsts, ka pašlaik ir izpētīts tikai 2% no lapu sūnu un 6% no aknu sūnu ķīmiskā sastāva.

1.2. Sūnaugu sastāvs

Lielākā daļa lapu sūnu ķīmiskā sastāva pētījumi koncentrējas tieši uz augu sekundārajiem metabolītiem, pateicoties šo savienojumu iespējamai bioloģiskajai aktivitātei (Asakawa *et al.*, 2013; Zinsmeister and Mues, 1991). Daudzi faktori apgrūtina

sūnaugu ķīmiskā sastāva studijas, taču pēdējos gados attīstoties analītiskajām metodēm arī pētījumu skaits un interese pieaug.

Būtiski sūnaugu pētījumos ir ne tikai analītisko metožu pielietošana, bet arī ekstrakcijas metožu attīstība, kas nodrošina jaunu savienojumu grupu izdalīšanu un identifikāciju. Izmantojot dažādas ekstrakcijas metodes un ekstrahentus, iespējams panākt atšķirīgu savienojumu izdalīšanu, tādēļ ekstrakcijas metožu optimizācija ir būtiska ķīmiskā sastāva analīzē. Pētījumos ir aprakstītas daudzas dažādas ekstrakcijas metodes, taču parasti tās ir optimizētas izmantojot augstākos augus, bet tikai daži pētījumi par modeļorganismiem izmanto sūnaugus (Klavina *et al.*, 2015; Irudayaray *et al.*, 2010). Pieejamā literatūra norāda, ka daži no ekstrakcijas principiem ir līdzīgi augstākajiem un zemākajiem augiem, tomēr pastāv būtiskas atšķirības starp abām augu grupām, tādēļ ir svarīgi optimizēt tieši ekstrakciju no sūnaugiem (Klavina *et al.*, 2015). Plaša profila savienojumu analīzei optimāli ir izmantot dažādus šķīdinātājus, tādejādi ekstrahējot vielas ar dažādām ķīmiskajām īpašībām.

Sūnaugu un augstāko augu ķīmisko sastāvu veidojošo savienojumu grupas būtiski neatšķiras. Galveno sūnaugu masu veido hemiceluloze un pektīns (30–60%), tāpat sastāvā atrodama celuloze (15–25%), olbaltumvielas (5–10%), polifenoli (5–10%) un neorganiskās vielas (3–10%) (Orlov *et al.*, 2005). Sūnaugu elementsastāvu tāpat kā augstākajos augos galvenokārt veido C, H, N un O, taču S un citi elementi ir atrodami ļoti zemās koncentrācijās.

Dažas vielu grupas sūnaugos ir interesantākas un perspektīvākas metabolisma izpētei nekā citas. Lai gan lielākā daļa bioloģiski aktīvo savienojumu sūnaugos ir sekundārie metabolīti, tiek pētīti arī primārie metabolīti un ogļhidrāti, kas ir īpaši interesanti augu funkciju izpratnei. Ogļhidrātu saturs ir nozīmīgs sūnaugu augstajai stresa tolerance, kaut arī to sastāvs atšķiras no augstākajiem augiem. Parasti ogļhidrāti augos tiek izmantoti kā enerģijas avots, sūnaugos tie tiek izmantoti galvenokārt kā šūnu veidojošs materiāls un lai palīdzētu augam adaptēties vides izmaiņām.

Visplašāk pētītā sekundāro metabolītu grupa ir lipofīlās vielas: terpenoīdi, steroīdi, bis-bibenzili, nepiesātinātās taukskābes un citas vielas (Asakawa *et al.,* 2013). Svarīga vielu grupa sūnaugos ir lipīdi, kas balstoties uz to funkcijām un molekulas polaritāti tiek iedalīti: neitrāli lipīdi, polārie lipīdi un glikolipīdi (Gunstone 1996). Neskatoties uz to, ka procentuālais lipīdu saturs sūnaugos ir zems (1–9%), tiem ir nozīmīga loma augu funkcionēšanā (Dembitsky, 1993; Asakawa *et al.,* 2013). Lipīdu sastāvs būtiski atšķiras starp dažādām sūnaugu klasēm, tāpat novērojamas atšķirības salīdzinot ar augstākajiem augiem. Novērotas arī atšķirības starp dažādām sūnaugu sugām, kas paver iespēju izmantot ķīmiskā sastāva pētījumus ķīmiskajā taksonomijā. Lipofīlo vielu pētījumi sūnaugos ir ne tikai svarīgi, lai labāk izprastu evolūciju, adaptācijas mehānismus un potenciālo bioloģisko aktivitāti, bet arī tāpēc, ka šo vielu analīze var palīdzēt izprast kūdras veidošanās procesus un kas to ietekmē. Sūnaugos ir atrasti vairāki steroli, kuriem varētu būt organismu regulējošas funkcijas: fitosterols, kampesterols, stigmasterols, sitosterols un citi (Chiu *et al.,* 1985).

Līdz šim vairāk kā 300 jauni terpenoīdi ir izolēti un identificēti no sūnaugiem. Lielākā daļa terpenoīdi ir atrasti aknu sūnas, taču arī lapu sūnas ir atrasti unikāli, citām augu klasēm neraksturīgi terpenoīdi (Asakawa *et al.,* 2013; Saritas *et al.,* 2001).

1.3. Sūnaugu un to sekundāro metabolītu izmantošanas iespējas

Augu valsts materiālu jaunu izmantošanas jomu pētījumi ir pamatā bioekonomikas attīstībai, kas ir gan ES, gan Latvijas tautsaimniecības attīstības un viedās specializācijas stratēģijas virziens (Sillanpaa and Ncibi, 2017; Latvijas bioekonomikas stratēģija, 2030).

Bioekonomikas attīstībai nozīmīgi ir jaunu augu izcelsmes produktu meklējumi un pieejamo augu valsts resursu izmantošanas potenciāla pētījumi. No šāda viedokļa sūnas uzskatāmas par nenovērtētu un nepietiekoši izpētītu augu valsts resursu. Sūnaugu plašās izplatības dēļ vēsturiski tie plaši tiek izmantoti tautas medicīnā un citās nozarēs daudzās kultūrās. Aukstākos reģionos lapu sūnas tiek izmantotas ziemeļbriežu barībai, lai gan kaloriskā vērtība ir zema tomēr šķiedru saturs ir augsts (Glime, 2007; Prins, 1981). Tāpat bada gadījumos lapu sūnas ir izmantotas kā piedeva maizes cepšanai (Bland, 1971). Vispazīstamākais lapu sūnu izmantošanas veids ir kā uzsūcošs materiāls apsējos, kas tika uzskatīts par labāku materiālu kā kokvilnas vate. Tāpat lapu sūnas gan vēsturiski gan mūsdienās tiek izmantotas kā pakošanas materiāls, kas nevien nodrošina labu mitruma uzsūktspēju, bet arī nodrošina antimikrobiālu efektu (Hotson, 1918; Mellegard *et al.,* 2009).

Mūsdienās sūnaugi, tajā skaitā lapu sūnas, iespējams kultivēt lielos apjomos izmantojot bioreaktorus vai atklātas teritorijas (Wichtmann *et al.,* 2016). Viens no potenciāliem lapu sūnu izmantošanas veidiem, kas pašlaik jau tiek izmantots ir to pielietojums kā substrāts, aizstājot kūdru. Daudzi pētījumi ir pierādījuši, ka lapu sūnas pilda visas nepieciešamās funkcijas, lai varētu tikt uzskatīts par labu substrāta materiālu (Glatzel and Rochefort, 2017; Kumar, 2017). Briofītiem pierādīta antimikrobiāla aktivitāte (Cansu *et al.,* 2012; Mellegard *et al.,* 2009). Pēdējās desmitgadēs plaši pētīts briofītu ekstraktvielu sastāvs un to izmantošanas iespējas jaunu zāļvielu meklējumos (Asakawa *et al.,* 2013; Saboljevic *et al.,* 2010; Sing *et al.,* 2006; Spjut *et al.,* 1986; Hotson, 1921). Pierādīts plašs terapeitiskās iedarbības spektrs vairākām lapu sūnu un īpaši aknu sūnu sugām (Cansu *et al.,* 2012; Asakawa 2007; Zinsmeister *et al.,* 1991).

1.4. Vides piesārņojuma ietekme uz sūnaugu sastāvu

Vides piesārņojums var būtiski ietekmēt sūnaugu vielmaiņas procesus un sastāvu un līdz ar to, tos var izmantot par vides piesārņojuma bioindikatoriem. Tradicionāli vides piesārņojuma iedarbības izpētei izmanto augstākos augus, ņemot vērā to izplatību un nozīmību cilvēka dzīvē, tomēr zemākos augos reakcija uz piesārņojošo vielu iedarbību var būt ievērojami citādāka (Kaiser, 2001). Tomēr sūnaugu izmantošanai vides piesārņojuma novērtēšanai ir ievērojams potenciāls, jo tie var akumulēt piesārņojošās vielas, jutīgi reaģēt uz to iedarbību un tie ir plaši izplatīti dažādās ekosistēmās. Sūnaugu spējas akumulēt metālus ir plaši pētītas (Steinnes, 1995; Berg and Steinnes, 1997; Rühling and Steinnes, 1998) un mūsdienās tiek izmantotas vides piesārņojuma monitoringa programmās. Metālu uzņemšana notiek caur visu sūnauga virsmu (Sun *et al.,* 2007), gan no metāliem jonu formā, gan metāliem, kas saistīti ar putekļu un aerosolu daļiņām, gan arī atkarībā no sugas un arī no substrāta, kurā augs attīstās, tomēr mazāk ir pētīta metālisko elementu iedarbība uz sūnaugu sastāvu, bet īpaši metabolismu (Aceto *et al.,* 2003). Sūnaugu spējas

uzkrāt metāliskos elementus tiek izmantotas arī tā saucamajā "sūnu maisiņu" metodē (Robalds un Kļaviņš, 2011; Ares *et al.,* 2012).

Tipisks reakciju kopums vidi piesārņojošo vielu iedarbības rezultātā ir brīvu radikāļu veidošanās augā, kas ir pakļauts piesārņojošo vielu iedarbībai (Hall, 2002; Bhaduri and Fulekar, 2012). Jāatzīmē gan, ka sūnaugos notiekošo procesu izpētei (ja neņem vērā metālu akumulāciju auga masā), vērība pievērsta visai maz. Pētīts brīvo radikāļu veidošanās process sūnās *Hypnum plumeaforme* (Sun *et al.,* 2009; Sun *et al.,* 2011) un parādīts, ka metālu jonu iedarbības rezultātā pieaug enzīmu aktivitāte, kas nodrošina reaģētspējīgu savienojumu (piemēram, ūdeņraža peroksīds) sagraušanu. Pētīts arī glutationa veidošanās raksturs sūnās *Fontinalis antipyretica* (Bruns *et al.,* 2001) un tā producēšanas izmaiņas piesārņojuma iedarbībā. Metālisko elementu (Pb) un arsēna iedarbība uz sūnas *Taxithelium nepalense* vielmaiņas produktiem parāda lipīdu peroksidācijas procesu pastiprināšanos, kā arī hidroperoksīdradikāļu un superoksīdanjona veidošanos (Guschina and Harwood, 2002; Choudhury and Panda, 2004). No otras puses, pierādīta superoksīddismutāzes aktivitātes pieaugums (Choudhury and Panda 2004) metālu jonu iedarbības rezultātā.

Tomēr atzīmējams, ka vidi piesārņojošo vielu iedarbības izpētei veltīto pētījumu skaits ir niecīgs. No otras puses, jāuzsver, ka ir svarīgi izprast ne tikai piesārņojošo vielu iedarbību uz sūnaugos noritošo procesu raksturu, bet arī identificēt vielas (biomarķieri), kas var uzrādīt iedarbības efektus un raksturot mehānismus, kas nosaka piesārņojuma iedarbības efektus. Šis jautājums ir īpaši svarīgs ņemot vērā vides faktoru (piemēram, sausums, pārmērīgs mitrums, UV starojums ietekmes), kas var būtiski apdraudēt sūnaugu cenozes.

2. MATERIĀLI UN METODES

2.1. Paraugu ievākšanas vietas un ievākšanas plāns

Lapu sūnu (2.1.tabula) ievākšanas plāns tika izstrādāts ņemot vērā: 1) nepieciešamību izmantot tikai Latvijā plaši pieejamas sūnu sugas (Strazdiņa u. c., 2011), 2) nepieļaut iespējamību izjaukt dabīgo lapu sūnu augšanas vidi, 3) esošos pētījumus par lapu sūnu ķīmisko sastāvu un bioloģisko aktivitāti (Asakawa *et al.,* 2013), 4) iespējami zemu cilvēka ietekmi uz ievākšanas vietu (vismaz 300 m no ceļiem un mājām), kā arī citu piesārņotāju ietekmi uz lapu sūnu ķīmisko sastāvu, 5) iespēja ievākt liela daudzuma paraugus (> 300 g slapju sūnu), 6) attālumu līdz ievākšanas vietai un ievākšanas izmaksas. Balstoties uz noteiktajiem faktoriem, tika izvēlēti jauktu koku meži un purvi Latvijas teritorijā. Visas ievāktās sūnas ir epigeīdi, kas nozīmē, ka barības vielas tiek uzņemtas gan no atmosfēras, gan no substrāta (Glime, 2007). Šajā pētījumā lapu sūnas ir iedalītas pēc to augšanas vietas – meža un purva.

2.1. tabula. **Pētīto lapu sūnu saraksts un tām raksturīgie augšanas apstākļi**
(pielāgots no Strazdiņa u. c., 2011)

Suga	Kods	Augšanas apstākļi
Aulacomnium palustre	AP	Purva mala un mitras vietas uz kokiem, kas sākuši trūdēt; veido blīvu sūnu puduri
Climacium dendroides	CD	Zālāji, mitras vietas mežā, zāļu purvi un pelēkās kāpas
Dicranum polysetum	DP	Jauktu koku un skuju koku meži, pārpurvotas vietas
Hylocomnium splendens	HS	Lapu un skuju koku meži, zālāji, virsāji
Polytrichum commune	PC	Skujkoku mežs, mitras vietas, dažreiz aug kopā ar *Sphagnum girgensohnii*
Polytrichum juniperinum	PJ	Skujkoku mežs, augstais un pārējas tipa purvs, trūdošu koku tuvumā vai uz tiem
Pleurozium schreberi	PS	Lapu koku un skujkoku mežs ar trūcīgu augsni
Ptilium crista-castrensis	PCC	Jauktu koku mežs, saulainās un vidēji mitrās vietās
Rhytidiadelphus triquetrus	RT	Jauktu koku mežs
Sphagnum girgensohnii	SG	Mitri skujkoku meži ar skābu augsni
Sphagnum angustifolium	SA	Purva vidus, purva lāmas un ļoti mitras vietas
Sphagnum magellanicum	SM	Purvs un purva lāmas
Sphagnum fallax	SF	Purvs un purva lāmas
Sphagnum tenellum	ST	Sūnu purva vidusdaļa starp ciņiem un lāmu malās, aug kopā ar *Sphagnum cuspidatum*
Sphagnum capillifolium	SS	Purva mala, purvs
Sphagnum rubellum	SR	Purvi un purvājs

Lapu sūnu paraugi tika ievākti 2014–2016. gada augustā. Paraugi tika ievākti vietā, kur izvēlētās lapu sūnas veidoja homogēnu klājumu un to sugas daļēji tika identificētas uz vietas. Ar šķērēm tika ievākta augu augšējā daļa, aptuveni 3 cm. Laboratorijā paraugi tika šķiroti, attīrīti, precīzi identificēti, žāvēti un ievietoti uzglabāšanai –20 °C saldētavā.

2.2. Lapu sūnu elementsastāva raksturošana

Elementu analīze (C, H, N) tika veikta izmantojot Elemental Analyzer modeli EA-1108 (Carlo Erba Instruments). Pētīto sūnu sagatavošana metālu analīzei tika veikta izmantojot zināmas metodes (Ekholm *et al.,* 2007; Šakalys *et al.*, 2009; Schroder and Pesch, 2010). Atomabsorbcijas spektrometrija tika izmantota, lai noteiktu lapu sūnu makroelementus (Ca, Fe, K, Mg, Na) un mikroelementus (Cu, Mn, Zn, Pb).

Analītiskā pirolīze, veicot pirolīzes produktu gāzes hromatogrāfisko analīzi ar masspektrometrisko detekciju (Py-GC/MS) tika veikta izmantojot Frontier Lab Micro Double-shot Pyrolyser (Py-2020iD) pirolīzes iekārtu. Veidotos pirolīzes produktus identificēja, izmantojot gāzes hromatogrāfu ar masspektrometrisko detekciju (GC/MS). Analīzi veica Oskars Bikovens Latvijas Koksnes ķīmijas institūtā.

2.3. Lapu sūnu spektroskopiskais raksturojums

Furjē transformācijas infrasarkanie (FTIR) spektri tika uzņemti, izmantojot FTIR spektrometru Spectrum BX (Perkin-Elmer Instruments) KBr tabletēs. Cietvielas ^{13}C kodolmagnētiskās rezonanses (NMR) spektri tika uzņemti, izmantojot krospolarizācijas zem maģiskā leņķa (CP/MAS) metodi. Spektri tika uzņemti, izmantojot Bruker Avance NMR spektrometru, izmantojot 4 mm MAS paraugu turētāju. ^{13}C NMR spektrus uzņēma Oskars Purmalis.

2.4. Lapu sūnu ekstrakcija

Konvencionālā ekstrakcija. Sausu lapu sūnu paraugu (0,3 g) aplēja ar 50 mL ekstrahenta un kratīja 24 h 140 rpm. Izmantotie ekstrahenti bija etanols (96% – 20%), acetons, dioksāns 10% un 5% sālskābe, 10% un 5% skudrskābe, DMSO (100% – 20%).

Ekstrakcijai ar ultraskaņu tika iesvērti 0,3 g sausa lapu sūnu parauga, kam pievienots 50 mL ekstrahenta. Ultraskaņas apstrāde veikta ultraskaņas vanniņā (100 W) divos režīmos – 20 min un 40 min. Izmantotie ekstrahenti bija etanols (96% – 20%), acetons, dioksāns, 10% un 5% sālsskābe, 10% un 5% skudrskābe, DMSO (100% – 20%). Pēc ultraskaņas apstrādes paraugi tika kratīti 24 h 140 rpm.

Mikroviļņu ekstrakcija tika veikta, izmantojot Milestone Ethos One mikroviļņu ekstrakcijas iekārtu 120 °C un 150 °C temperatūrā, ar 1500 W jaudu. Lapu sūnu paraugi tika iesvērti ekstrakcijas traukos un pievienots 50 mL etanols (96% – 20%).

Superkritiskā CO_2 ekstrakcija. Sausas lapu sūnas (15 g) tika iesvērtas metāla kolonnā, kolona ievietota uzsildītā (60 °C) krāsnī, CO_2 plūsma 10 mL/min un spiediens 20 MPa, ekstrakcijas laiks 30 min un 60 min.

Soksleta ekstrakcija tika veikta Soksleta ekstraktorā, tika izmantoti 20 g lapu sūnu parauga un 96% etanols. Ekstrakcija tika veikta 80 °C, 8 h un 24 h.

2.5. Lapu sūnu ekstraktu summāro rādītāju raksturojums

Kopējo polifenolu sastāvs tika noteikts izmantojot Folina-Čikolto reaģentu (Singleton *et al.,* 1999). Rezultāti aprēķināti izmantojot standartlīkni un izteikti kā galluskābes ekvivalents (GE) / 100 g sausu sūnu (Singleton *et al.,* 1999; Silverstein *et al.,* 2005). Flavonoīdu daudzums noteikts spektrofotometriski, kā standartu izmantojot kvercetīna dihidrātu (Mitrovič *et al.,* 2011). Kopējais ogļhidrātu daudzums noteikts ar Čaplina metodi un kalibrācijas līknei izmantojot glikozes šķīdumu (Silverstein *et al.,* 2005). Antiradikālā aktivitāte tika noteikta izmantojot 2-difenil-1-pikrahidrazil reaģentu (DPPH) (Mitrovič *et al.,* 2011).

2.6. Lapu sūnu sekundāro metabolītu frakcionēšana

Sūnu lipīdu ekstraktu frakcionēšana, izmantojot cietfāzes ekstrakcijas metodi, tika veikta, izmantojot silikagēla, aminopropilsilikagēla un C18 SPE kolonnas, izmantojot kā eluentus heksānu, hloroformu, dietilēteri un etiķskābi. Eluātus ietvaicēja slāpekļa plūsmā. Pirmās eluāta frakcijas pēc izšķīdināšanas hloroformā analizēja ar GC/MS, bet atlikušās pēc izšķīdināšanas acetonitrilā un derivatizācijas ar BSTFA.

2.7. Lapu sūnu sekundāro metabolītu hromatogrāfiskā analīze

Ultra augstas izšķirtspējas šķidruma hromatogrāfiskā analīze (UPLC) tika veikta izmantojot Waters Acquity hromatogrāfu: kolonna Acquity UHPLC BEH C8. Hromatogrāfijas datu apstrāde tika veikta, izmantojot Empower 3 sistēmu.

Lai nodrošinātu lipīdu gāzes hromatogrāfisko analīzi ar masspektrometrisko detekciju (GC/MS), paraugi pirms analīzes (piemēram 20 mg) tika derivatizēti apstrādājot ar BSTFA. Analīze tika veikta, izmantojot Clarus 680 hromatogrāfu ar Clarus SQ 8C masspektrometru un Elite 5Ms kapilāro kolonnu. Signālu aiztures laiks un masas spektri tika salīdzināti ar datu bāzi NIST vai literatūras datiem, lai nodrošinātu vielu identifikāciju.

Sūnu metanola ekstrakti tika analizēti, izmantojot ultraaugstas izšķirtspējas šķidruma hromatogrāfu Agilent 1290 Infinity (X Bridge C8 kolonna ar nolidojuma laika (TOF) detekciju. Augstas izšķirtspējas masas spektri tika uzņemti, izmantojot Agilent 6230 TOF LC/MS ar elektronu impakta jonizāciju. Dati tika apstrādāti izmantojot MassHunter B05.00 programmu. Analīzes veica LU Ķīmijas fakultātes docente Ilva Nakurte.

2.8. Lapu sūnu ekstraktu bioloģiskā aktivitāte

Lapu sūnu ekstraktu pretvēža aktivitātes testēšanai tika izmantotas sekojošas vēža šūnu līnijas; žurkas glioma (C6), cilvēka epidermas karcinoma (A431), cilvēka plaušu karcinoma (A549), peļu melanoma (B16-F10), cilvēka krūts adenokarcinoma (MCF-7), cilvēka resnās zarnas karcinoma (CaCo-2). Analīzes veica Irina Šestakova, Organiskās sintēzes institūts.

Lapu sūnu etanola ekstrakta antimikrobiālā aktivitāte tika noteikta ar agara bedrīšu difūzijas metodi (Perez *et al.,* 1990). Mikroorganismi iegūti no Latvijas Mikroorganismu līniju kolekcijas. Antimikrobiālā aktivitāte tika testēta uz: *Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus mirabilis, Escherichia coli.*

2.9. Metālu akumulācijas ietekme uz lapu sūnu ķīmisko sastāvu

Eksperimentā tika izmantotas lapu sūnas *Pleurozium schreberi* un *Sphagnum magellanicum*. Lapu sūnu paraugi tika ievietoti 5 × 8 × 2 cm seklās plastmasas kastītēs un apstrādāti ar Zn un Cu nitrātu šķīdumiem dažādās koncentrācijās. Augi tika apstrādāti ar 10 mL Mora maisījuma (Carginale *et al.,* 2004), pH 6,5 (KNO_3 100 mg, $CaCl_2 \times 4H_2O$ 10 mg, $MgSO_4$ 10 mg, KH_2PO_4 136 mg, $FeSO_4$ 0,4 mg uz 1000 mL destilēta ūdens). Mora maisījums izmantots kontroles parauga apstrādē, kā arī lai iegūtu nepieciešamās metālu jonu šķīdumu koncentrācijas – 0,001; 0,005; 0,01; 0,05; 0,10; 0,25; 0,50, 1, 2, 3, 4 mM ($Zn(NO_3)_2 \times 3H_2O$ un $Cu(NO_3)_2 \times 5H_2O$). Pēc apstrādes ar metāla jonu šķīdumiem un 72 h inkubācijas, paraugi tika nomazgāti ar dejonizētu ūdeni, žāvēti un veiktas tālākās analīzes.

3. REZULTĀTI UN DISKUSIJA

3.1. Pētīto lapu sūnu ķīmiskais sastāvs

3.1.1. Pētīto lapu sūnu pamatsastāvs

Augu elementsastāvs raksturo to uzbūvi, sniedz informāciju par galvenajām ķīmiskajām struktūrām, kas tos veido, kā arī raksturo to augšanas vidi un vides piesārņojumu. Pētītās sūnas raksturo relatīvi maz izteikta to elementsastāva mainība (3.1. tabula). Pētīto sūnu elementsastāva mainības robežas ir sekojošas: C – 40–43%; H – 5,5–6%; N – 0,4–2%; S – ~0%. O saturs mainās no 48% līdz 53%. Salīdzinot ar literatūrā aprakstīto sūnu sastāva mainības intervāliem: C – 45–63%; H – 3,6–7,7%; N – 0,4–5,8%; S – 0,5–1,5%; pelni – 0,1–1,2% (Zaccone *et al.,* 2007) konstatējama izteikta līdzība. Gan elementsastāva rādītāji, gan sūnas veidojošo elementu atomattiecības C/H, C/O rāda, ka ar skābekli bagātas molekulas (piemēram, ogļhidrāti) ir sūnas veidojošie nozīmīgākie struktūrelementi. Tomēr starp pētītajām sūnām to sastāvā vērojamas atšķirības, jo, piemēram, slāpekļa saturs *Sphagnum* sūnās ir ievērojami zemāks nekā citās sūnās.

3.1. tabula. **Pētīto lapu sūnu elementsastāvs (%)**

Suga	C	H	N	O
Aulacomnium palustre	43,51±0,01	5,72±0,02	0,515±0,015	50,25±0,02
Climacium dendroides	41,32±0,02	5,39±0,02	1,683±0,019	51,61±0,01
Dicranum polysetum	42,15±0,02	5,85±0,03	1,325±0,019	50,67±0,04
Hylocomnium splendens	42,12±0,02	5,43±0,01	1,215±0,007	50,23±0,10
Pleurozium schreberi	43,24±0,01	5,83±0,11	1,230±0,040	49,78±0,10
Polytrichum commune	43,74±0,02	5,95±0,02	1,123±0,015	49,18±0,01
Polytrichum juniperum	41,99±0,02	5,89±0,02	1,988±0,019	50,13±0,01
Ptilium crista-castrensis	42,24±0,02	5,68±0,03	1,219±0,019	50,86±0,04
Plagiochila asplenioides	42,10±0,02	5,80±0,02	1,488±0,003	50,61±0,01
Rhytidiadelphus triquetrus	42,47±0,02	5,52±0,01	1,121±0,007	50,89±0,10
Sphagnum angustifolium	41,75±0,01	5,53±0,11	0,430±0,040	52,28±0,10
Sphagnum capillifolium	40,98±0,01	5,58±0,01	0,416±0,012	53,01±0,01
Sphagnum girgensohnii	42,05±0,02	5,75±0,02	1,050±0,030	51,16±0,04
Sphagnum magellanicum	42,21±0,01	5,54±0,01	0,516±0,013	51,72±0,01
Sphagnum fallax	42,08±0,01	5,32±0,01	0,402±0,012	52,20±0,01
Sphagnum rubellum	42,15±0,02	5,42±0,02	0,850±0,030	51,58±0,04

3.2. tabula. **Metālu saturs lapu sūnās (mg/kg)** (kodi atbilstoši 2.1. tabulā norādītajiem)

Suga	Na	Mg	K	Ca	Fe	Mn	Ni	Cu	Zn	Cd	Pb	Cr	V
AP	178±12	1254±22	4641±30	2662±20	170±5	262±12	8,2±0,1	6,5±0,2	31±2	0,20±0,05	3,2±0,1	0,12±0,03	0,41±0,04
CD	98±6	1839±22	10140±40	9548±40	121±5	77±6	0.5±0.1	5,8±0,2	41±2	0,35±0,05	2,4±0,1	0,28±0,03	0,51±0,04
DP	622±15	2039±22	3060±30	7353±40	206±5	241±12	1,9±0,1	6,3±0,2	204	0,27±0,05	2,9±0,1	0,26±0,03	0,37±0,04
HS	104±6	1620±22	8438±40	3327±20	36±1	51±6	1,5±0,1	5,3±0,2	34±2	0,25±0,05	1,6±0,1	0,22±0,03	0,24±0,04
PC	87±6	944±10	6210±40	2561±20	779±8	126±12	1,7±0,1	8,1±0,2	42±2	0,17±0,05	3,0±0,1	0,18±0,03	0,34±0,04
PJ	405±15	1344±22	3140±30	3390±40	80±1	38±3	0,6±0,1	18,4±0,2	179±8	0,21±0,05	1,7±0,1	0,32±0,03	0,55±0,04
SS	171±12	1238±22	7715±40	2461±20	208±5	175±12	1,6±0,1	11,2±0,2	152±9	0,26±0,05	1,3±0,1	0,38±0,03	0,34±0,04
RT	170±12	1693±22	7311±40	3963±40	64±1	241±12	0,9±0,1	5,5±0,2	87±2	0,23±0,05	1,9±0,1	0,42±0,03	0,54±0,04
PCC	226±12	1287±22	10123±40	4126±40	103±5	256±12	1,8±0,1	14,1±0,2	48±2	0,26±0,05	1,1±0,1	0,28±0,03	0,21±0,04
PS	240±12	1022±22	4779±30	3182±20	157±5	282±12	0,3±0,1	4,2±0,2	34±2	0,33±0,05	1,4±0,1	0,29±0,03	0,30±0,04
SM	643±15	1040±22	2739±20	3695±40	507±8	28±3	1,3±,1	5,0±0,2	42±2	0,10±0,05	1,5±0,1	0,12±0,03	0,23±0,04
SF	615±15	1697±22	3637±30	6341±40	162±8	50±6	1,0±0,1	12,4±0,2	88±5	0,11±0,05	1,6±0,1	0,24±0,03	0,34±0,04
SA	544±15	1185±22	3785±30	4560±40	99±1	85±6	1,2±0,1	11,8±0,2	93±5	0,15±0,05	3,2±0,1	0,18±0,03	0,56±0,04
SG	253±12	1128±22	7535±40	2912±20	83±1	171±12	1,4±0,1	3,5±0,2	190±10	0,22±0,05	2,4±0,1	0,27±0,03	0,43±0,04
SR	572±15	1093±22	5073±30	2801±20	123±5	29±2	0,6±0,1	3,3±0,2	71±5	0,15±0,05	2,3±0,1	0,34±0,03	0,73±0,04

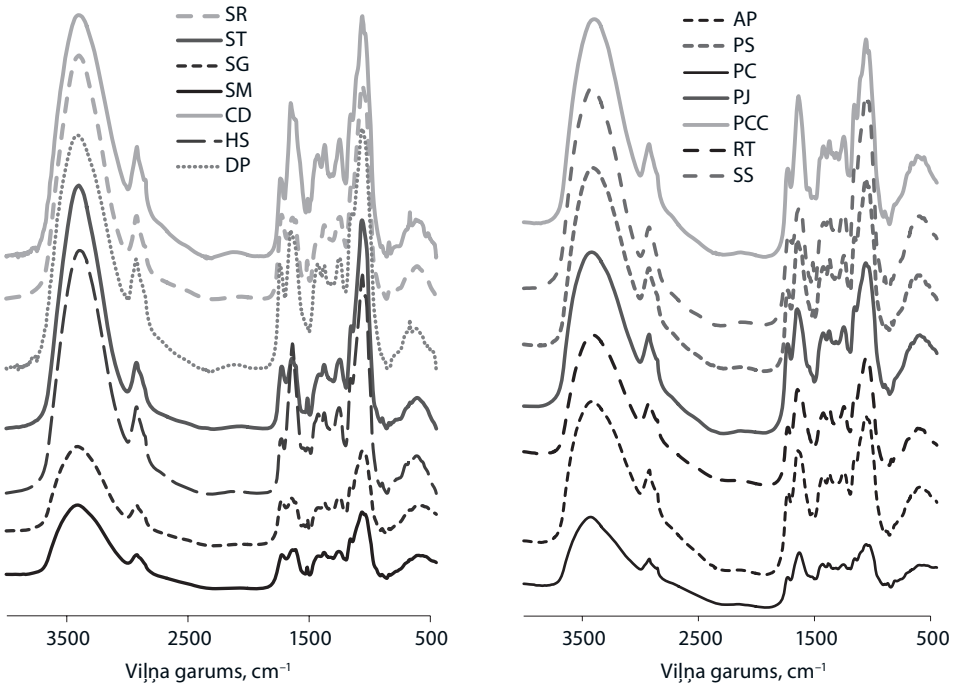
Nozīmīga elementu grupa, kas raksturo sūnu sastāvu ir metāliskie elementi (3.2. tabula). Tā kā sūnu paraugu ievākšanas vietas tika izvēlētas tālu no tiešiem piesārņojuma avotiem, ir pamats uzskatīt, ka makroelementu sastāvu ietekmē dominējoši dabiskie procesi, bet vairāku mikroelementu saturu ietekmē arī vides piesārņojums (Berg and Steinnes, 1997).

3.1.2. Lapu sūnu sastāva izpēte

Lapu sūnu sastāva izpēte tika veikta izmantojot Furjē transformācijas infrasarkano spektrometriju (FTIR), analītisko pirolīzi – gāzes hromatogrāfiju/masspektrometriju (Py-GC/MS) un ^{13}C kodolmagnētisko rezonansi (CP MAS ^{13}C NMR).

Sūnu FTIR spektri (3.1. attēls) var tikt iedalīti reģionos, kas parāda tās veidojošo savienojumu galveno funkcionālo grupu sorbciju. Sorbcijas maksimumi pie 3600–2800 cm^{-1} ir plaši un sorbciju šajā reģionā nosaka –OH grupu klātbūtne. Sorbcija pie 2950 un 2850 cm^{-1} raksturo CH_3– un CH_2– grupas attiecīgi. Sorbcijas maksimumi ap 1700 cm^{-1} (1725–1700 cm^{-1}) ir raksturīgi karbonilgrupām, bet pie 1690–1500 cm^{-1} amīdu saitēm. Reģionā 1625–1610 cm^{-1} sorbcija raksturo aromātisko C=C un karbonilgrupu, kā arī hinonu C=O klātbūtni. Pie viļņu skaitļa 1470–1370 cm^{-1} novērojama sorbcija, kas raksturīga C–H, O–H un C–O saitēm.

Arī veiktā sūnu FTIR spektroskopiskā analīze uzrāda vispārēju līdzību to FTIR spektros, tomēr atzīmējamas būtiskas atšķirības vairāku signālu intensitātē, kas savukārt parāda atšķirības sūnas veidojošajos savienojumos (3.1. attēls).



3.1. attēls. **Lapu sūnu FTIR spektri** (kodi atbilstoši 2.1. tabulā norādītajiem)

Analītiskā pirolīze, izmantojot pirolīzes produktu gāzes hromatogrāfiju ar masspektrometrisko detekciju ir metode, kas ļauj izpētīt sūnas veidojošos ķīmiskos savienojumus (Schellekens *et al.,* 2009). 3.3. tabulā sniegts pētīto sūnu pirolīzes produktu galveno vielu grupu daudzums. Sūnu pirolīzes produktu starpā dominē zemas molekulmasas alifātiski savienojumi, fenols, 4-etenilfenols un 1,6-anhidro-β-D-glikopiranoze un citas vielas, kuras uzskatāmas par ogļhidrātu termiskās sadalīšanās produktiem.

^{13}C NMR CP/MAS spektri ļauj iegūt tiešu informāciju par galvenajiem augu materiālu veidojošajiem savienojumiem (Spaccini *et al.,* 2006). Signāli šajos spektros raksturo oglekļa savienojumus, kas saistīti dažādās struktūrās. Arī šajā gadījumā pētīto sūnu spektri kopumā ir līdzīgi un ļauj novērtēt oglekļa atomu daudzumu, kas ir saistīti dažādās funkcionālās alifātiskās struktūrās, aromātiskos savienojumos vai, piemēram, ogļhidrātos.

3.3. tabula. **Lapu sūnu pirolīzes produktu galveno vielu grupu relatīvais (%) daudzums**
(kodi atbilstoši 2.1. tabulā norādītajiem)

Suga	MA	C	Ar	L	Lp	N
AP	14,30 ± 0,28	10,39 ±0,23	0,76 ± 0,01	0	1,17 ± 0,03	0
PS	20,68 ± 0,31	5,96 ± 0,18	2,52 ± 0,05	0	1,35 ± 0,03	0
PC	17,03 ± 0,25	6,05 ± 0,11	0,72 ±0,02	0	1,97 ± 0,04	0,08 ± 0,002
PJ	16,16 ± 0,24	5,75 ± 0,13	0,60 ± 0,02	0	1,23 ± 0,03	0
PCC	19,90 ± 0,39	4,74 ± 0,11	0,79 ± 0,02	0	1,45 ± 0,03	0
RT	20,68 ± 0,31	5,96 ± 0,14	2,52 ± 0,06	0	1,35 ± 0,03	0
SA	17,72 ± 0,26	6,38 ± 0,15	3,78 ±0,07	0	1,10 ± 0,02	0
SS	15,84 ± 0,42	7,72 ± 0,21	3,06 ± 0,06	0	1,35 ± 0,03	0
SG	16,87 ± 0,21	5,58 ± 0,17	4,26 ± 0,09	0	1,47 ± 0,03	0,02 ± 0,001
SM	14,26 ± 0,21	9,12 ± 0,31	3,48 ± 0,07	0	1,24 ± 0,03	0

MA – nenoteiktas izcelsmes alifātiskie savienojumi C < 6; C – furāni, kuru izcelsmes avoti ir ogļhidrāti, pirāna un ciklopentāna atvasinājumi; Ar – aromātiskie savienojumi (neieskaitot fenolu metoksiēterus); L – fenolu metoksiēteri; Lp – savienojumi, kuru avoti ir lipīdi C > 6; N – N-saturoši savienojumi

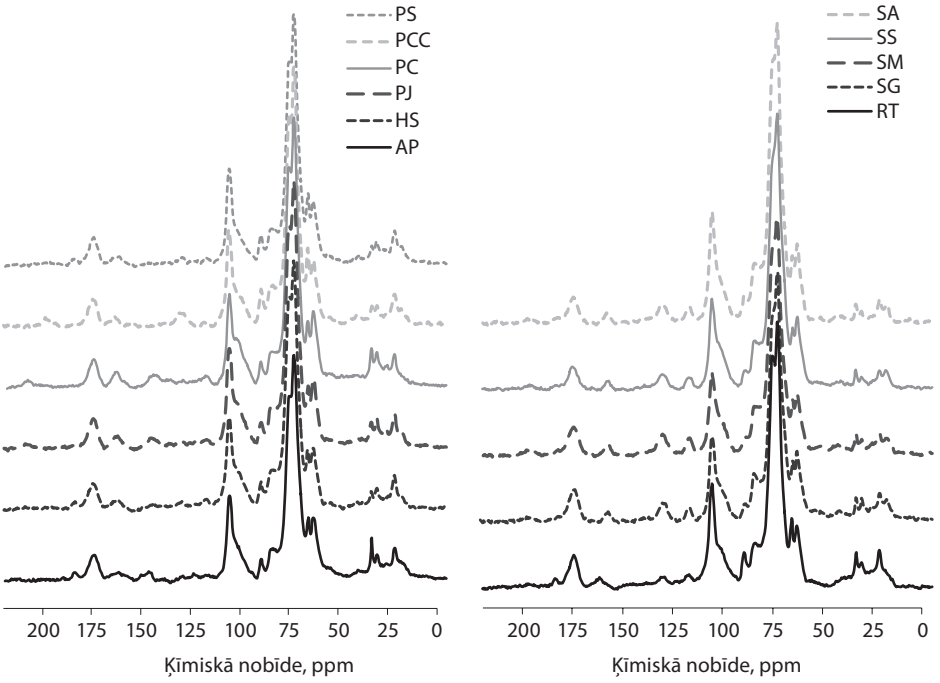
Ķīmiskās nobīdes reģions no 50 līdz 92 md atbilst ogļhidrātu sastāvā ietilpstošiem oglekļa atomiem. Visos spektros novērojami izteikti signāli pie 63, 65, 73, 84 un 89 md, kuri raksturo oglekļa atomus sekojošās struktūrās: O–CH_3 (63 md), HO–CH_2– (65 md), –CH(OH) – (73 md). Ķīmiskās nobīdes reģions no 112 līdz 136 md raksturo aromātisko struktūru sastāvā ietilpstošu oglekļa atomu daudzumu. Reģions no 136 līdz 159 md raksturo fenolu sastāvā un N-aizvietotu aromātisko savienojumu sastāvā ietilpstošu aromātisko savienojumu daudzumu. Reģions no 159 līdz 190 md atbilst oglekļa savienojumiem taukskābēs, to esteros un amīdos. ^{13}C NMR CP/MAS spektru analīze līdz ar to ļauj iegūt izšķirošu priekšstatu par aromātisko savienojumu klātbūtni sūnu sastāvā, bet īpaši par lignīna klātbūtni, kas ir ilgstošu zinātnisko diskusiju jautājums (Hajek *et al.,* 2011; Ballance *et al.,* 2012). ^{13}C NMR CP/MAS spektru analīze sniedz viennozīmīgu atbildi – lignīns nav

atrodams sūnu sastāvā. Šī atziņa ir būtiska, analizējot ķīmiskās evolūcijas procesu un pāreju no zemākajiem augiem (aļģes, sūnas) uz augstākajiem augiem, kā arī jaunu bioloģiski aktīvu savienojumu meklējumus augos.

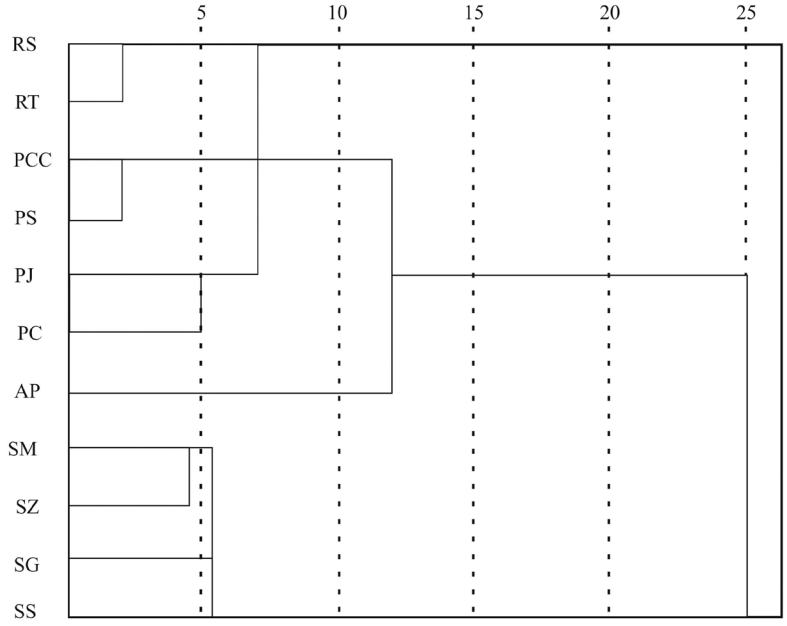
3.4. tabula. **Ķīmiskās nobīdes intervāli ^{13}C NMR CP/MAS spektros (%)**

Suga	212–190	190–159	159–136	136–112	112–92	92–50	50–0
Aulacomnium palustre	nd*	5,49	0,71	0,72	14,57	64,96	13,56
Pleurozium schreberi	0,03	5,29	nd*	1,66	15,46	66,45	11,12
Polytrichum commune	0,43	4,75	1,95	2,62	14,80	62,33	13,11
Polytrichum juniperum	0,13	5,02	1,22	2,08	16,07	64,88	10,59
Ptilium crista-castrensis	0,66	4,44	0,07	1,82	15,06	67,83	10,12
Rhytidiadelphus triquetrus	0,14	5,54	0,49	3,42	16,19	63,88	10,35
Sphagnum angustifolium	0,28	3,67	1,05	4,18	15,40	69,51	5,91
Sphagnum capillifolium	0,23	3,24	0,44	3,10	14,58	71,14	7,27
Sphagnum girgensohnii	0,22	5,05	0,53	3,40	13,29	66,28	11,23
Sphagnum magellanicum	0,53	4,72	0,93	5,05	14,03	65,44	9,31

*nd – nav atrasts



3.2. attēls. **Sūnu ^{13}C NMR CP/MAS spektri** (kodi atbilstoši 2.1. tabulā norādītajiem)



3.3. attēls. **^{13}C NMR CP/MAS spektros noteikto sūnas veidojošo ķīmisko struktūru procentuālā daudzuma klāsteranalīze** (kodi atbilstoši 2.1. tabulā norādītajiem)

^{13}C NMR CP/MAS spektros noteikto sūnas veidojošo ķīmisko struktūru procentuālā daudzuma klāsteranalīze ļauj novērtēt pētīto sūnu līdzību saskaņā ar tās veidojošo savienojumu līdzību. Veiktā analīze parāda līdzību *Sphagnum* sugas sūnu starpā, bet no otras puses, atšķirības no citām pētītajām sūnu sugām.

3.2. Lapu sūnu sekundāro metabolītu raksturojums

3.2.1. Lapu sūnu ekstrakcija

Sūnu metabolītu sastāva izpētei nepieciešams veikt to ekstrakciju. Līdz šim plaši pētīts aknu sūnu vielmaiņas produktu sastāvs, turklāt galveno vērību pievēršot to eļļas ķermenīšu sastāvā ietilpstošām vielām, tomēr, lai izprastu briofītu, bet īpaši lapu sūnu metabolismu un vides faktoru ietekmi uz to, nepieciešams veikt ekstrakcijas apstākļu izpēti, kas nodrošina plaša spektra vielu ieguvi.

Sūnaugu ekstrakcijas apstākļu izpētei tika izmantotas divas sūnu sugas (*Rhytidiadelphus triquetrus* un *Sphagnum rubellum*), bet kā ekstrakcijas efektivitātes rādītāji tika izmantoti: a) ekstraktvielu daudzums (sausais atlikums un D_{280}), b) kopējais polifenolu daudzums, c) ekstraktvielu spēja saistīt brīvos radikāļus; d) UPLC hromatogrammā identificēto savienojumu daudzums. Ņemot vērā ekstraktvielu potenciālās izmantošanas jomas kā kritēriji ekstrahentu izvēlei tika izvēlēti: a) videi draudzīgi ekstrahenti ar iespējami

zemu to toksiskumu; b) gaistamība un atšķirīga polaritāte; c) zemas cenas. Ekstrakcijas procesa priekšmēģinājumos tika izvēlēti ekstrahenti ar iespējami plašu īpašību gradientu, piemēram, ūdens, metanols, etanols, acetons, dioksāns, dimetilsulfoksīds. Ekstrakcijas process tika pētīts, izmantojot vairākas ekstrakcijas metodes: a) konvencionālā ekstrakcija (maisot vai kratot paraugu ar ekstrahentu); b) Soksleta ekstrakcija; c) ekstrakcija veicot apstrādi ar ultraskaņu; d) ekstrakcija veicot apstrādi ar mikroviļņiem; e) ekstrakcija ar CO_2 superkritiskā stāvoklī. Mainot ekstrakcijas procesa parametrus, piemēram, ekstrakcijas laiks, temperatūra, sūnu sausās masas un ekstrahenta daudzuma attiecības, tika izvērtēta katra konkrētā procesa efektivitāte (3.5. tabula).

3.5. tabula. **Ekstrakcijas efektivitāte izmantojot dažādas ekstrakcijas metodes un *R. triquetrus* sūnas (ekstrahents: 60 % etanols).** Ekstrakcijas eksperimenti veikti 3 atkārtojumos

Ekstrakcijas metode	Ekstrakcijas apstākļi		Parauga/ekstrahenta attiecība		Kopējais polifenolu daudzums, GE mg/100 g	Antiradikālā aktivitāte, GE mg/100 g	Ekstrakcijas sausās masas iznākums, mg/100 g
	Laiks, h	T, °C	Ekstrahents, mL	Iesvars, g			
Soksleta	8,0	80	300	11,2	165,5±8,3	116,4±5,8	205,2±10,3
	24,0	80	300	11,2	239,6±11,9	142,6±7,1	231,5±11,6
Mikroviļņu	0,5	120	30	0,3	111,2±5,6	167,8±8,4	195,6±9,8
	0,7	150	30	0,3	486,9±24,4	172,9±8,6	150,2±7,5
Ultraskaņa	0,5	50	40	0,5	243,7±12,2	54,1±2,7	195,5±9,7
	0,7	70	40	0,5	254,6±12,7	63,3±3,2	150,6±7,5
Superkritiskā CO_2	0,5	102	150	15,0	230,4±11,5	162,4±8,1	125,1±6,3
	1,0	102	150	15,0	274,9±13,5	143,5±7,2	124,8±6,2
Konvencionālā	12,0	24	30	0,4	150,7±7,6	25,8±1,3	99,5±4,9
	24,0	24	30	0,4	194,3±9,7	36,9±1,8	97,2±4,9

No salīdzinātajām metodēm (3.5. tabula) par efektīvāko uzskatāma apstrāde ar mikroviļņu starojumu paaugstinātā temperatūrā (150 °C) un pie paaugstināta spiediena, kā arī ekstrakcija Soksleta ekstraktorā (kas gan ir laikietilpīga un kuras laikā nestabilākas vielas, kuras tajā pašā laikā nosaka ekstraktvielu spēju saistīt brīvos radikāļus, sabrūk). Arī šķīdinātāja patēriņš, izmantojot Soksleta ekstraktoru un konvencionālo ekstrakciju, ir ievērojami lielāks.

Lai iegūtu maksimāli augstu ekstraktvielu daudzumu tika veikta šķīdinātāja ietekmes izpēte, sekojot "zaļās ķīmijas" principiem un ņemot vērā solventa izmaksas. Augstāko polifenolu iznākumu nodrošina etanola un dimetilsulfoksīda izmantošana, turklāt ūdens piedevas paaugstina ekstraktvielu iznākumu (3.6. tabula). Jāatzīmē, ka optimālie ekstrakcijas apstākļi katrai pētītajai sūnu sugai ir atšķirīgi un, piemēram, augstāko *R. triquetrus* ekstraktvielu iznākumu nodrošina ekstrakcijas veikšana pie 120 °C, izmantojot 60% etanolu. Līdz ar to ir pamats uzskatīt, ka kaut arī kopumā ekstrakcijas apstākļi ir līdzīgi, tomēr tos ir svarīgi optimizēt katra izmantotā auga gadījumā.

3.6. tabula. **Ekstrakcijas optimizācija izmantojot dažādus ekstrahentus.**
Ekstrakcijas eksperimenti veikti 3 atkārtojumos

Ekstrahents		Kopējais polifenolu daudzums, GE/100 g		Antiradikālā aktivitāte, GE/100 g		Sausnes iznākums, mg/100 g	
		R. triquetrus	*S. rubellum*	*R. triquetrus*	*S. rubellum*	*R. triquetrus*	*S. rubellum*
Ūdens		230,0±11,5	237,2±11,9	10,5±0,5	11,9±0,6	36,8±1,8	51,8±2,6
Metanols	100%	183,1±9,2	132,9±6,6	11,6±0,6	45,9±2,3	75±3,8	91,6±4,6
	80%	208,6±10,4	132,9±6,6	32,4±1,6	48,7±2,4	91,7±4,6	91,4±4,6
	60%	189,7±9,5	135,3±6,8	24,1±1,2	42,6±2,1	91,8±4,6	75,1±3,8
	40%	182,8±9,1	120,4±6,0	7,6±0,4	31,2±1,6	108,3±5,4	50,2±2,5
	20%	171,9±8,6	116,2±5,8	6,2±0,3	27,6±1,4	75,0±3,8	50,8±2,5
Etanols	96%	254,0±12,7	210,4±10,5	11,5±0,6	22,5±1,1	652,5±32,6	345,0±17,3
	80%	304,0±15,2	215,7±10,8	50,4±2,5	26,4±1,3	667,5±33,4	287,5±14,4
	60%	296,0±14,8	252,6±12,6	24,0±1,2	33,5±1,7	195,0±9,8	97,5±4,9
	40%	334,0±16,7	207,5±10,4	7,9±0,4	11,5±0,6	187,5±9,4	179,5±9,0
	20%	320,0±16,0	193,2±9,7	6,3±0,3	1,6±0,1	96,6±4,8	78,0±3,9
Acetons	100%	174,5±8,7	127,9±6,4	9,1±0,5	15±0,8	36,8±1,8	67,5±3,4
	80%	195,7±9,8	178,1±8,9	47,2±2,4	52,6±2,6	91,5±4,6	58,3±2,9
	60%	238,1±11,9	177,6±8,9	46,2±2,3	52,1±2,6	116,3±5,8	50,7±2,5
	40%	214,2±10,7	160,9±8,0	31,2±1,6	49,5±2,5	125,6±6,3	41,6±2,1
	20%	191,1±9,6	128,6±6,4	12,5±0,6	42,6±2,1	50,4±2,5	28,4±1,4
Dioksāns		151,2±7,6	138,3±6,9	13,5±0,7	13,4±0,7	15,0±0,8	147,0±7,4
DMSO	100%	363,9±18,2	75,7±3,8	47,9±1,6	31,5±1,6	41,7±2,1	97,5±4,9
	40%	295,1±14,8	75,7±3,8	34,7±2,2	43,6±2,2	25,2±1,3	108,0±5,4
	20%	270,0±13,5	75,7±3,8	19,1±1,0	16,6±0,8	16,9±0,8	68,3±3,4

Ekstrakcijas apstākļu izpēte atsedz pētīto sūnu metabolītu būtiskas atšķirības no augstāko augu metabolītiem: ja augstākajos augos to spēju saistīt brīvos radikāļus būtiski ietekmē polifenolu klātbūtne (Tabart *et al.,* 2006; Ehala *et al.,* 2005), kuru biosintēze savukārt ir šikīmskābes ceļa nozīmīgs rezultāts, tad pētītajās sūnās starp polifenolu koncentrāciju un ekstraktvielu spējām saistīt brīvos radikāļus izteiktu kopsakarību nepastāv, kas arī atbilst iepriekš konstatētajam lignīna iztrūkumam sūnaugos. Šī atziņa ir būtiska metabolisma izpratnei sūnās.

3.2.2. Lapu sūnu sekundāro metabolītu raksturojums

Sūnu sastāvu pamatā veido polisaharīdi (Asakawa, 2007; Klavina, 2015), bet to sekundāro metabolītu sastāvā dominē mazpolāras vielas (lipīdi) un polāri savienojumi (ogļhidrāti, aminoskābes, polifenoli un citas vielas). Ņemot vērā plašo lipīdu grupas savienojumu definīciju, kas balstās uz vielu fizikālo īpašību līdzību (Gunstone, 1996), pie

tiem pieskaita ne tikai triglicerīdus, bet arī vaskus, taukskābes, garas virknes alkanolus, kā arī sterolus, terpenoīdus un citas vielas. Lai veiktu sūnu sekundāro metabolītu sastāva izpēti, ņemot vērā pieejas, kas ieteiktas augstāko augu metabolītu spektra (Samuelson and Bohlin, 2009) raksturošanai un veiktā pētījumu rezultātus, pētīto sūnu ektrakcijai izvēlēti ekstrahenti un ekstrakcijas apstākļi, kas nodrošina gan augstu kopējo ekstraktvielu daudzumu iegūšanu, gan īpaši ekstraktvielu spēju saistīt brīvos radikāļus (3.7. tabula).

3.7. tabula. **Pētīto lapus sūnu etanola ekstraktu kopējā rādītāji, etanola* un hloroforma** ekstraktu sausnes iznākumi, izteikti uz 100 g sausu sūnu** (kodi atbilstoši 2.1. tabulā norādītajiem)

Kods	Kopējie polifenoli*, GE	Kopējie ogļhidrāti*, GLE	Antiradikālā aktivitāte*, GE	Sausnes iznākums*, mg	Sausnes iznākums**, mg
AP	267,5±13,1	10,6±0,4	39,0±1,9	114,2±5,7	259±12,8
CD	272,1±14,3	7,6±0,3	42,3±2,8	98,6±4,7	412±22,7
DP	399,2±19,5	8,4±0,3	16,4±0,8	133,5±6,7	482±24,2
HS	298,4±14,6	6,9±0,3	59,3±3,1	102,6±5,1	443±23,2
PS	275,6±13,4	11,4±0,5	15,6±0,8	145,2±7,3	561±27,9
PC	800,7±39,1	12,1±0,5	12,6±0,6	154,8±7,7	513±26,1
PJ	416,2±20,2	5,6±0,2	24,1±1,2	88,5±4,4	510±26,1
PCC	237,9±11,7	9,7±0,3	19,6±1,0	145,5±7,3	464±23,9
RT	379,1±18,5	15,3±0,7	11,3±0,5	144,8±7,2	451±23,5
SF	783,2±38,1	8,9±0,4	17,6±0,9	142,5±7,1	213±10,1
SM	370,6±18,1	6,6±0,3	18,2±0,9	140,6±7,0	378±18,4
SR	345,7±16,7	10,3±0,4	16,3±0,8	65,3±3,3	181±9,8
ST	280,1±13,7	7,9±0,3	14,9±0,7	149,3±7,4	240±11,6

Pētīto sūnu ekstrakti satur relatīvi augstu ekstraktvielu daudzumu. Sūnas satur polifenolus (no 200 līdz 800 mg gallusskābes ekvivalentu/100g sūnu sausās masas), bet ekstraktvielu spēja saistīt brīvos radikāļus ir zemāka nekā augstāko augu līdzīgos ekstraktos (Sun *et al.,* 2009), turklāt arī šis pētījums apliecina, ka nepastāv korelācijas starp polifenolu koncentrāciju un ekstraktu spēju saistīt brīvos radikāļus.

3.2.2.1. Lapu sūnu lipīdu sastāvs

Sūnu lipīdu sastāva izpētes pamatā ir V. Dembitska (Dembitsky 1993) pētījumi, bet mūsdienās šīs izpētes aktualitāti nosaka interese par jaunu bioloģiski aktīvu savienojumu meklējumiem, kā arī klimata pētījumiem, jo lipīdu sastāvu analīze nogulumos sniedz informāciju par klimata pārmaiņām pagātnē (Baas *et al.,* 2000). Lipīdu izdalīšanai izmantoti dažādi ekstrahenti (tipiski, hloroforms), tomēr to izmantošanas efektivitāte nav plaši pētīta. Veiktā pētījuma ietvaros veikts salīdzinājums (3.8. tabula) parāda dažādu ekstrahentu izmantošanas efektivitāti. Izmantotās sūnas (*Polytrichum commune, Dicranum polysetum*) satur < 12 mg/g lipīdu, tomēr, veicot intensīvu un atkārtotu ekstrakciju, iespējams iegūt līdz 21–60 mg/g lipīdu.

3.8. tabula. **Lapu sūnu lipīdu ekstraktu iznākums (mg/g)**

Suga	C_6H_{12}	CH_2Cl_2	$CHCl_3$	$C_2H_5OOCCH_3$	$(C_2H_5)_2O$
Polytrichum commune	7,2	6,8	10,8	8,8	8,0
Dicranum polysetum	4,0	11,7	12,4	9,2	12,0

Sūnu lipīdu sastāva analīze uzrāda liela skaita dažādu savienojumu klātbūtni (kopumā identificētas un kvantificētas 88 dažādas vielas), kurām ir nozīmīgas funkcijas sūnu metabolisma procesos un daudzas no kurām ietekmē sūnu ekstraktu bioloģisko aktivitāti. Atzīmējamas ievērojamas atšķirības pētīto sūnu lipīdu sastāvā un vairāki savienojumi konstatēti tikai vienas vai dažu, līdzīgu sugu sastāvā, turklāt atšķirības attiecas uz vielām, kuras raksturo potenciāli augsta bioloģiskā aktivitāte, piemēram, steroli un terpenoīdi.

Ptilium crista-castrensis sastāvā, relatīvi augstā koncentrācijā atrasts ergost-7-ēn-3-ols un pimārskābe, bet *Polytrichum commune* atrasti citi steroli, turklāt lielākos daudzumos nekā citās sūnās. Bagātīgākais lipīdu sastāvs konstatēts *Pleurozium shreberi* ekstraktos, bet vismazāk vielu – *Sphagnum magellanicum* sastāvā. Pētītajās sūnās pirmo reizi atrastas tādas vielas kā α-amirīns, uvaols, cikloartenols, ursolskābe, neofitadiēns un citas. Pētīto sūnu lipīdu ekstrakti satur daudzus alkānus, taukskābes, ieskaitot nepiesātinātās taukskābes un alkanolus. Ņemot vērā lomu metabolisma procesos, sterolu un terpenoīdu klātbūtne uzskatāma par īpaši nozīmīgu. Sūnu lipīdu ekstraktos ogļūdeņražu koncentrācijas ir relatīvi zemas (100±290 μg/g sausā masa). Alkānu vidū dominē C_{21+} nepāra oglekļa atomu skaita homologi. Steroli veido nozīmīgu lipīdu grupu pētīto sūnu ekstraktos un to koncentrācijas mainās no 450 līdz 1600 μg/g sausā masa. Terpenoīdu koncentrācija lipīdu ekstraktos mainās ievērojami vairāk nekā sterolu koncentrācijas. Terpenoīdu starpā dominē ursolskābe, amirīns un lupeols. Arī taukskābju koncentrācijas būtiski atšķiras atkarībā no pētītās sūnu sugas un to koncentrācija mainās no 200 līdz 1050 μg/g sausā masa. Vesela rinda taukskābju (piemēram, C_{24} vai C_{26}) ir raksturīgas tikai briofītiem (Karunen and Salin, 1980; Ficken *et al.,* 1998). Kopējā garas oglekļa virknes alkanolu koncentrācija mainās no 80 līdz 340 μg/g sausā masā. Lipīdu ekstrakti satur arī α-tokoferolu kā arī C_{25} un C_{27} metilketonus.

Sūnu lipīdu ekstraktu analīze, izmantojot GC-MS parāda, ka ir vielas, kuras ir atrodamas tikai vienas pētītās sugas sastāvā un līdz ar to var tikt izmantotas sugu identifikācijai, piemēram, sfagnskābe, 9-heksadecēnskābe. Vairāki triterpēni ir raksturīgi tikai vienai pētītajai sugai un līdz ar to var tikt izmantoti hemotaksonomijai, bet to analīze var būt būtiska bioloģiski aktīvu vielu klātbūtnes izpētei sūnu sastāvā.

3.2.2.2. Lapu sūnu ekstraktu frakcionēšana

Tradicionāli lipīdus iedala neitrālos lipīdos (triglicerīdos), fosfolipīdus, glikolipīdos un citos (Dembitsky 1993). Tomēr, lai iegūtu ieskatu sūnu bioloģiski aktīvu vielu sastāvā un to vielmaiņas raksturā, prioritāri aktuāli ir pētīt brīvi pieejamas vielas sūnu sastāvā, bet, ņemot vērā to lielo skaitu, ir svarīgi sākotnējo ekstraktvielu daudzumu sadalīt frakcijās, kas ļauj novērtēt vielu grupu daudzumu un paaugstināt analītiskās noteikšanas jutību.

Sūnu lipīdu frakcionēšanai perspektīva metode ir cietfāzes ekstrakcijas metodes izmantošana, kas veicama izmantojot polārus sorbentus (silikagēls un aminopropilsilikagēls). Ņemot vērā izpētei aktuālu vielu grupas, kā eluenti izvēlēti solventi ar mainīgu polaritāti.

Izmantojot heksānu kā eluentu iespējams izdalīt mazpolāros savienojumus (ogļhidrāti C_{10} līdz C_{30}). Citu vielu starpā var atzīmēt fitolu, dažus aldehīdus, fitadiēnu, α-tokoferolu, kampesterolu, stigmasterolu, γ-sitosterolu un cikloartenolu. Vienlaikus atzīmējams, ka pastāv būtiskas atšķirības starp rezultātiem, kas iegūti izmantojot silikagēlu un aminopropilsilikagēlu. Cietfāzes ekstrakcijas frakcionēšana izmantojama, lai analizētu sūnu sastāvā atrodamos ogļūdeņražus. Paaugstinot eluentu polaritāti, iespējams kā atsevišķu frakciju izdalīt karbonskābes un to esterus, fitolu un citas vielas.

Izmantojot noslēdzoši hloroformu kā eluentu iespējams izdalīt tādas vielas kā neofitodiēns, fukosterols un citas. Izmantojot aminopropilsilikagēlu un kā eluentu dietilēteri un etiķskābi (98:2), iespējams izdalīt visai polāras vielas kā atsevišķu frakciju. Līdz ar to cietfāzes ekstrakcija, mainot sorbentu materiālus un eluentu sistēmas, iespējams izmantot lipīdu ekstraktu frakcionēšanai, vienlaikus uzlabojot iespējas veikt ekstraktu sastāva analīzi.

3.2.2.3. Sūnu polāro ekstraktvielu analīze

Lai nodrošinātu sūnu ekstrakta bioloģiskās aktivitātes izpēti, veikta to ekstrakcija ar etanolu mikroviļņu ekstrakcijas iekārtā, bet sekojoši izmantojot šķidruma hromatogrāfiju ar nolidojuma laika (TOF) detekciju. Ekstrakcija izmantojot etanolu, nodrošina iespējas izdalīt tādas vielas kā polifenoli, ogļhidrāti, aminoskābes un citas. Sūnu metanola ekstraktos pirmo reizi atrastas vairākas vielas, kas iespējami ietekmē sūnu ekstraktu bioloģisko aktivitāti. Vairākos paraugos atrasts fitohormons abscisskābe, kā arī apigenīns. No otras puses, ja sfagnskābe līdz šim ir tikusi atrasta tikai *Sphagnum* sūnās, tad šajā pētījumā tā atrasta arī citās sūnās. Sūnas *Sphagnum rubellum* sastāvā atrasts alkaloids harmols un tā atvasinājumi.

3.9. tabula. **Galvenās vielu grupas pētīto lapu sūnu ekstraktos, mg / 100 g sausā masa**
(lapu sūnu hloroforma ekstraktu analīze izmantojot GC/MS un LC-TOF-MS)

Vielu grupas	AP	CD	DP	HS	PCC	PJ	PS	SF	SM	SR	ST
Taukskābes	329	720	471	1707	796	771	878	836	447	614	1017
Monogliceroli	46	3	24	167	123	56	284	161	86	107	64
Terpenoīdi	198	159	168	396	176	269	357	116	82	125	134
Alkoholi	80	94	166	215	168	74	178	130	74	226	121
Steroli	735	745	1619	1673	1716	715	2131	1696	633	686	633
Diterpēni	23	16	33	110	52	13	107	40	1	81	59
Alkāni	34	66	106	279	174	57	93	102	19	116	80
Vaski/vasku esteri	01	5	171	16	3	6	162	40	3	347	55
Triterpēni	38	156	203	373	263	69	487	10	654	539	274
Polifenoli	652	711	705	509	252	559	441	522	580	632	504
Aminoskābes	1440	1866	2810	118	988	2412	3266	2121	2536	590	2621

Sūnu lipīdu ekstraktu sastāvā (3.9. tabula) dominē taukskābes (329–1707 mg / 100 g sausā masa) un steroli (632–2130 mg / 100 g sausā masa). Sūnu etanola ekstraktu sastāvā dominē aminoskābes (590–3266 mg / 100 g sausā masa).

Atzīmējams, ka sūnu ekstraktu sastāvā ietilpst arī relatīvi daudz terpēnu, polifenolu un citu vielu.

3.2.3. Lapu sūnu ekstraktu bioloģiskā aktivitāte

Sūnaugu ekstraktvielās atrodas daudzas bioloģi aktīvas vielas (Spjut *et al.,* 1992; Asakawa, 2007; Nikolajeva *et al.,* 2012; Cheng *et al.,* 2012), kuras turklāt nav atrodamas citās augu grupās, kas var kalpot par pamatu jaunu, biofarmācijā izmantojamu vielu meklējumiem. Lai novērtētu sūnu ekstraktu potenciālu jaunu aktīvu vielu meklējumiem, tika veikta sūnu ekstraktu: a) antimikrobiālās aktivitātes izpēte izmantojot 5 mikroorganismu līnijas; b) spējas kavēt vēža šūnu attīstību, izmantojot 6 vēža šūnu līnijas.

Visi pētītie ekstrakti demonstrē spējas kavēt izmantoto baktēriju attīstību (inhibīcijas zonas diametrs no 9 līdz 15 mm), respektīvi, uzrāda antimikrobiālu aktivitāti. Visi sūnu ekstrakti uzrāda antibakteriālu aktivitāti pret *Bacillus cereus*. Augstākās spējas kavēt *Bacillus cereus* attīstību demonstrē *Climacium dendroide* un *Polytrichum commune*, ekstraktvielas, bet zemāko aktivitāti uzrāda *Hylocomnium splendens* un *Sphagnum magellanicum*. Kopumā augstāko antimikrobiālo aktivitāti uzrāda *Polytrichum commune* ekstrakti pret baktērijām *Staphylococcus aureus*. Vairāku sūnu sugu (*Climacium dendroides, Ptilium crista-castrensis, Rhytadiadelphus triquetrus*, *Sphagnum magellanicumi)* ekstraktvielas uzrāda spējas kavēt *E.coli* attīstību. *Hylocomnium splendens* und *Pleurozium shreberi* ekstraktvielas demonstrē spējas kavēt *Pseudomonas aeruginosa* attīstību. Veiktais sūnu ekstraktvielu antimikrobiālās aktivitātes skrīnings parāda atšķirības starp pētītajām sūnām, bet atsevišķos gadījumos, piemēram, *Polytrichum commune* ekstraktvielu tālākas un padziļinātas izpētes augsto potenciālu jaunu, bioloģiski aktīvu vielu meklējumiem.

Sūnu ekstraktvielu bioloģiskās aktivitātes izpēte tika turpināta, pētot vēža šūnu proliferāciju. Neviens no pētītajiem ekstraktiem neuzrādīja citotoksisku efektu. Labākais iedarbības efekts tika konstatēts izmantojot žurkas gliomas, cilvēka epidermas karcinomas un resnās zarnas karcinomas šūnas, turklāt augstāko aktivitāti uzrādīja sūnu *Sphagnum magellanicum, Dicranum polysetum, Pleurozium shreberi* ekstraktvielas. Sūnu ekstraktvielu iedarbības efektivitāte uz vēža šūnu attīstību bija zemākā uz plaušu karcinomas un peļu melanomas šūnām.

Veiktā sūnu ekstraktvielu bioloģiskās aktivitātes priekšizpēte parāda, ka tām piemīt antimikrobiāla aktivitāte un spējas kavēt vēža šūnu attīstību un līdz ar to ir perspektīvi uzsākt bioloģiski un farmakoloģiski aktīvu vielu meklējumus sūnu sastāvā. Veiktā daudzfaktoru analīze ļauj iegūt priekšstatu par to, kādas vielu grupas var ietekmēt sūnu ekstraktvielu iedarbību uz pētītajām šūnām un līdz ar to izmantošanas potenciālu.

3.3. Vides un piesārņojuma stresa ietekme uz lapu sūnu ķīmisko sastāvu

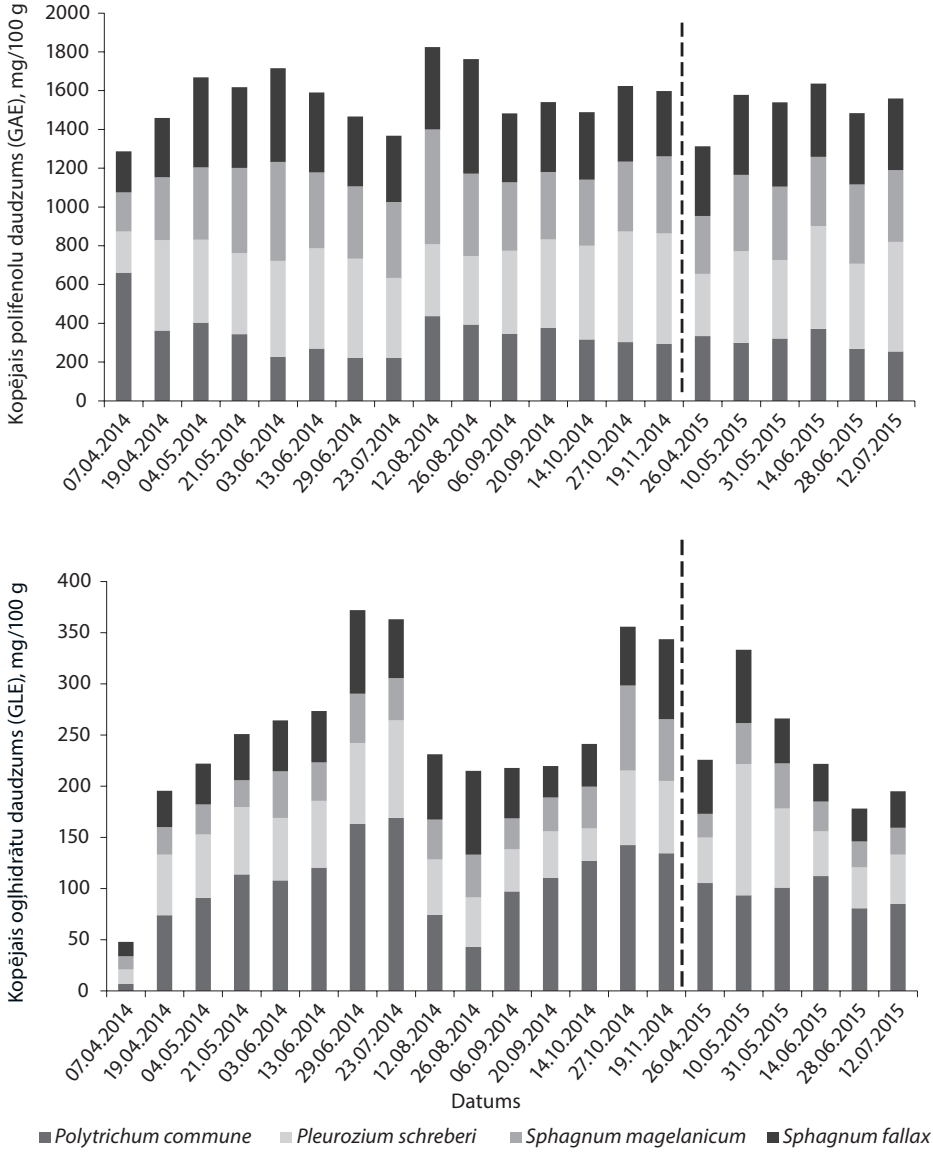
3.3.1. Lapu sūnu sastāva sezonālā mainība

Lai izprastu sūnās notiekošos procesus, mehānismus, kas nosaka sūnu augsto noturību pret vides apstākļu mainību, tajā skaitā sausuma/pārmērīga mitruma, UV starojuma radīto stresu, kā arī, lai piemeklētu apstākļus, kad sūnu ekstraktvielu sastāvā ir augstākās bioloģisko aktīvo vielu koncentrācijas, veikta 4 pētīto sūnu (*Sphagnum fallax, Sphagnum magellanicum, Polytrichum juniperinum, Pleurozium schreberi*) sastāva sezonālās mainības izpēte, paraugus ievācot 2 veģetācijas sezonas un analizējot gan sūnu sastāva, gan lipīdu ekstraktvielu, gan laikapstākļu mainības raksturu un to ietekmi uz sūnu sastāvu.

Pētījums parāda, ka polifenolu un ogļhidrātu saturs veģetācijas sezonas laikā ievērojami mainās (3.4. attēls) un ir visai atkarīgs no nokrišņu daudzuma un Saules spīdēšanas intensitātes. Iespējams konstatēt šīs mainības vispārējās likumsakarības: 2014. gada veģetācijas sezonas laikā ogļhidrātu daudzums pieaug, bet polifenolu daudzums samazinās. Ja sausuma, intensīvu nokrišņu periodi nav izteikti un ilgstoši, arī ogļhidrātu un polifenolu mainības intensitāte nav augsta.

Kopsakarību izpēte starp klimatiskajiem faktoriem (temperatūra, nokrišņu daudzums) pētījuma laikā un sūnu ekstraktvielas raksturojošiem summārajiem parametriem vairākos gadījumos parāda ciešas korelācijas starp pētītajiem rādītājiem. Kopējais ekstraktvielu daudzums korelē ar kopējo nokrišņu daudzumu. Sūnu ekstraktvielu daudzums korelē ar to spēju saistīt brīvos radikāļus un ir augstāks veģetācijas perioda uzsākumā, bet tās gaitā pakāpeniski samazinās.

Klimatisko procesu ietekme uz sūnaugu sastāvu pētīta molekulārā līmenī, analizējot lipīdu sastāva mainību pētīto sūnu ekstraktvielu sastāvā. Atzīmējams, ka gan individuālu vielu koncentrācijas, gan to skaits pētītajos ekstraktos ir ievērojami mainījies sezonas laikā. Visu izvēlēto lipīdu pārstāvju (kampesterols, stigmasterols, β-sitosterols, oktadekānskābe, pimārskābe, dehidroabietīnskābe un citas vielas) koncentrācijas sezonas laikā ievērojami mainās un šī mainība ir atkarīga gan no auga attīstības rakstura sezonas laikā, gan to ietekmē klimatiski procesi. Daudzu pētīto sterolu augstākās koncentrācijas konstatētas veģetācijas sezonas sākumā un sūnu intensīvākās augšanas laikā, bet rudenim uzsākoties samazinās. Atzīmējams arī, ka atšķirīgām sūnu sugām to lipīdu koncentrācijas maksimālās vērtības novērojamas atšķirīgos laika posmos, ko, iespējams, ietekmē to augšanas vide. Sūnu sastāva un metabolītu sastāva sezonālās mainības rakstura izpēte ir nozīmīga, lai ne tikai izprastu sūnās notiekošos procesus, klimata mainības/pārmaiņu iespējamās ietekmes, bet arī, lai izvēlētos sezonu sūnu paraugu ievākšanai jaunu bioloģiski aktīvu vielu meklējumiem sūnu sastāvā.



3.4. attēls. **Kopējo polifenolu un ogļhidrātu daudzuma mainība 2014., 2015. gada veģetācijas sezonu laikā**

3.3.2. Vides piesārņojuma ar metāliem ietekme uz lapu sūnu elementsastāvu un sekundāro metabolītu sastāvu

Augu izmantošana vides piesārņojuma monitoringam ir plaši pētīta vides kvalitātes izpētes un monitoringa pieeja, tomēr metodes tālākai attīstībai ir svarīgi saprast piesārņojošo vielu ietekmi molekulārā līmenī, kā arī identificēt ietekmes uz augu sastāvu (piemēram, metabolisma procesiem), lai identificētu to iedarbības biomarķierus. Lai pētītu metālu (izmantojot Cu un Pb) ietekmi uz sūnu sastāvu un to metabolismu veikta *Pleurozium schreberi* un *Sphagnum magellanicum* kultivēšana vidē, kas piesārņota ar minētajiem metāliem, apstākļos, kas ieteikti iepriekš veiktos pētījumos (Sun *et al.,* 2011; Basile *et al.,* 2012). Veiktie pētījumi, protams, parāda sūnu spējas akumulēt metālus, kas tajā pašā laikā ir atkarīga no izmantotās sugas, jo *Sphagnum magellanicum* spējas akumulēt izmantotos metālus ir augstāka nekā *Pleurozium schreberi.*

Vienlaikus jāatzīmē, ka fizikālā sorbcija un hemosorbcija nav vienīgi faktori, kas ietekmē metālu aizturēšanu auga biomasā un metāla jonu iedarbību. Ir pierādīts, ka vides piesārņojums ar metāliem ietekmē tādus procesus, kā brīvo radikāļu veidošanos un izmaiņas augu metabolismā. Pētot metālu jonu (Zn^{2+} un Cu^{2+}) ietekmes uz sūnu *Pleurozium schreberi* un *Sphagnum magellanicum* metabolismu pierādītas ietekmes uz brīvo radikāļu saistīšanas kapacitāti, kas turklāt ir atšķirīga atkarībā no pētītā metālu jona. Atzīmējamas polifenolu koncentrācijas izmaiņas piesārņojuma iedarbībā: ja augstākajos augos polifenoli sniedz ieguldījumu piesārņojuma stresa mazināšanā, tad kā pierādīts šajā pētījumā sūnās lignīna nav un arī polifenolu daudzumi ir ievērojami mazāki un līdz ar to pieaugot piesārņojuma ar metāliem intensitātei sūnās, polifenolu koncentrācija būtiski nepieaug.

Lai izvērtētu vides piesārņojuma ar metālu joniem ietekmes uz sūnu metabolismu analizētas to lipīdu sastāva izmaiņas kultivējot sūnas pieaugošu metālu jonu daudzumu klātbūtnē. Nosakot gan lipīdu grupu (taukskābes, steroli un citas), gan individuālu metabolītu daudzumu sūnu ekstraktos, pētīts vides piesārņojuma ar metālu joniem (Pb^{2+}) ietekmes raksturs uz sūnu metabolismu. Vairāku vielu koncentrācijas pieaug piesārņojošo vielu pieaugošu daudzumu iedarbības rezultātā, piemēram, šāds iedarbības raksturs tipisks steroliem (kampesterols, β-sitosterols). Tajā pašā laikā stigmasterola koncentrācija, pieaugot metāla jonu koncentrācijai, pieaug tikai sasniedzot noteiktu metāla koncentrāciju vidē. No otras puses, taukskābju koncentrācijas, pieaugot metālu jonu koncentrācijai vidē, samazinās.

Līdz ar to sūnu metabolītu sastāva izpēte izmantojama, lai pētītu piesārņojošo vielu iedarbību pie to subletālām koncentrācijām un sūnu metabolītu sastāvā iespējams identificēt vielas – biomarķierus, kas izmantojami vides piesārņojuma stresa izpētei.

SECINĀJUMI

1. Lapu sūnas galvenokārt sastāv no dažādiem ogļhidrātiem, taču lignīns nav atrodams pētīto lapu sūnu sastāvā. Lapu sūnu elementsastāvs un strukturālo elementu ķīmiskais sastāvs sniedz informāciju, ko iespējams izmantot ķīmiskās taksonomijas studijās.
2. Lapu sūnu sekundāro metabolītu ekstrakcijas optimizācija, var veicināt un pielietot videi draudzīgu pieeju kā arī iegūt ekstraktus ar specifisku aktivitāti (piemēram, antiradikālā aktivitāte) un augstu sausnes iznākumu.
3. Lapu sūnu sekundāro metabolītu ķīmiskā analīze uzrāda dažādām sūnu sugām raksturīgu savienojumu klātbūtni, kā arī bioloģiski aktīvas vielas, kas konkrētajās sūnās ir identificētas pirmo reizi.
4. Cietfāzes ekstrakcija ir efektīva frakcionēšanas metode lapu sūnu ekstraktiem un uzlabo sekundāro metabolītu sadalīšanu dažādās vielu grupās ar atšķirīgu bioloģisko aktivitāti vai ar iespējamo pielietojamību bioindikācijas pētījumos un, lai izvērtētu bioģeoķīmiskos procesus vidē.
5. Pētot lapu sūnu ekstraktus, tika noteikta to bioloģiskā aktivitāte (antiradikālā, antimikrobiālā un pretvēža aktivitāte), kas paver iespējas lapu sūnu ekstraktu iespējamai izmantošanai zāļvielu iegūšanai.
6. Vides stress (sezonālu laikapstākļu maiņa, piesārņojums) ietekmē lapu sūnu ķīmisko sastāvu, mainot lapu sūnu kvalitatīvo un kvantitatīvo sastāvu. Iegūtie rezultāti norāda uz iespējām identificēt vides stresa ķīmiskos biomarķierus.

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