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University of Turku

Supercritical CO_2 extraction of triterpenoids from *Inonotus obliquus*

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Nghia Huynh

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Abstract

Inonotus obliquus (Fr.) Pilát, is a basidiomycete, which can be found mainly above the 40th parallel north of Europe, Asia and America. The usage of the sterile conk, also known as Chaga, as folk medicine in Baltic countries goes back to the 17th century. Traditionally, Chaga extracts have been used for treating cancer. Chemical investigations have shown that Chaga contains high quantities of bioactive compounds such as triterpenoids, which have been connected to the health benefits and immuno-modulating effects of Chaga.

Supercritical fluid extraction (SFE) of Chaga was carried out with pure CO_2 to recover triterpenoids, while a modified Folch extraction method was used as comparison. Along with the extraction, one of the main purposes of this work was to determine the optimal extraction parameters for SFE by analysing the compositions of the extracts. The main attractions of SFE are less laborious and time-consuming operation but also the fact that hazardous solvents can be omitted from the extraction process. The high selectivity and inert nature of CO_2 also makes it possible to target and remove certain substances from the sample material without remarkably affecting the structure of the material. Three temperature-pressure combinations were tested in SFE varying between 40-50°C and 281-350 bars, in order to find suitable conditions for triterpenoid extraction. The extracts were saponified and the recovered triterpenoids analysed as trimethylsilyl (TMS) ethers. A gas chromatographic system coupled with flame ionization detector (GC-FID) was used for quantification, while mass spectrometry (GC-MS) was used for the identification.

Six sterol and triterpenoid constituents were identified and quantified from supercritical fluid (SF) extracts: ergosterol (17.9±1.3 mg / 100 g dry weight), lanosterol (65.5±7.2 mg / 100 g), β-sitosterol (3.6±0.5 mg / 100 g), stigmastanol (1.4±0.1 mg / 100 g), betulin (13.2±4.3 mg / 100 g) and inotodiol (97.7±11.5 mg / 100 g). Folch extracts produced higher yield in ergosterol (36.7±1.5 mg / 100 g), inotodiol (127.7±5.5 mg / 100 g), betulin (34.7±4.2 mg / 100 g), lanosterol (74.1±1.0 mg / 100 g), β-sitosterol (5.0±0.4 mg / 100 g) and stigmastanol (1.8±0.2 mg / 100 g). The difference was found to be statistically significant between Folch method and SFE ($p < 0.05$). Trametenolic acid was found only in Folch extracts, in relatively high amounts (44.8±1.0 mg / 100 g).

Despite the lower yield obtained by SFE, this method showed several advantages including significantly shorter extraction time and production of solvent waste. This work could be a starting point for further studies on green extraction methods of bioactive compounds from Finnish natural resources. The utilized methods can be further developed to achieve more efficient extraction conditions compared to Folch method.

Contents

Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
DAG	Diacylglycerol
DM	Diabetes mellitus
DOE	Design of experiment
ECD	Electron capture detector
EOS	Equation of state
ESI	Electrospray ionozation
FFA	Free fatty acid
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometer
HD	Hydrodistillation
HDMS	Hexamethyldisilazane
HIV	Human immunodeficiency virus
I.D.	Internal diameter
ISTD	Internal standard
LDL	Low-density lipoprotein
MAG	Monoacylglycerol
O.D.	Outer diamater
PAH	Polyaromatic hydrocarbon
PCB	Polychlorinated biphenyls
PL	Phospholipids
PDMS	Polydimethylsiloxane
RSM	Response surface methodology
SC	Side chain
SC-CO_2	Supercritical carbon dioxide
SCW	Subcritical water
SF	Supercritical fluid
SFE	Supercritical fluid extraction
TAG	Triacylglycerol
TLC	Thin layer chromatography
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl (ether)
Tri-Sil	Silylation reagent (HDMS/TMCS/Pyridine)

1 Introduction

The consumption of wild mushrooms has been more favored compared to cultivated fungi in many countries of Europe (Markkula & Rautavaara, 1996). Mushroom picking is a relatively popular hobby in Finland especially during autumn season and several kilos of fresh mushrooms are consumed per household annually. By average, mushroom dishes are prepared 2.4 times per month in Finnish households (Pekkarinen *et al*., 1980). In Finland and other European countries, mushrooms are considered as a delicacy particularly for their specific aroma and texture. Fresh and preserved fruiting bodies of tens of mushroom species can be prepared for different purposes from meals to functional foods. The typical edible mushrooms have low fat content, high moisture percentage (81.8–94.8 %) and high fiber supply. Mushrooms also contain varying concentrations of phenolic compounds and sterols, which are considered as important bioactive compounds responsible for antioxidant and cholesterol lowering properties (Mattila *et al*., 2001). The distinction between edible and medical mushroom is not easy to define since many of the common edible species have health promoting properties and several medical mushrooms are also eaten as food (Guillamón *et al*., 2010). In total, over 200 edible mushroom and polypore species have been identified in Finland. The most common edible mushrooms found in Finnish forests include *Agaricus bisporus* (bottom mushroom), *Cantharellus cibarius* (chanterelle) and *Boletus edulis* (cep), which belong to the morphological division of Basidiomycota (Salo *et al.,* 2005). Polypores are one of the best-known wood-decaying fungi, they are also basidiomycetes but have a distinctive physical appearance compared to typical edible mushrooms (Zheng *et al*., 2010). The visual characteristics of polypores are typically represented by a rigid texture, shelf-like appearance and the pores located on the underside of the spore-bearing surface (Largent & Stuntz, 1973). Some of the most common polypores that can be found in Finnish forest include *Ganoderma lucidum* (Lingzi), *Fomes fomentarius* (tinder fungus) and *Inonotus obliquus* (chaga). In Finland, polypores have been utilized for hundreds of years as tinder, pincushions, dyes, nutrients and functional food (Halmetoja, 2004). Many other polypores e.g. *Laetiporus sulphurous* (sulphur polypore), *Fomitopsis pinicola* (red belt conk), *Piptoporus betulinus*, and *Laricifomes officinalis* (quinine conk), have been utilized similarly in Europe. The mycochemical studies regarding traditional polypores have shown presence of multiple primary and secondary metabolites such as triterpenes, polysaccharides and phenolic compounds. The crude extracts obtained from traditional polypores possessed anti-inflammatory, cytotoxic, and antimicrobial activities (Grienke *et al*., 2014). Up to date, the medicinal mushrooms are growingly commercialized as dietary supplements or consumed in the form of capsules, tablets or extracts in many European, Asian and Baltic countries (Zaidman *et al*., 2005). In the case of *I. obliquus*,

the long heritage in traditional mushroom medicine is generally well acknowledged in normal households. However, the knowledge behind its health benefits, nutritional values and bioactive compounds are less well known compared to other natural products.

Studies have shown that *I. obliquus* contains variety of triterpenoids and sterols, which have been found to support the health of cardiovascular system. These are typically caused by high level of serum LDL cholesterol concentration. The active use of health benefiting components in food, such as plant sterols, has become growingly popular in normal households. The increasing demand of these food products combined with the growing concerns about the use of organic solvents and their disposal has created a need for greener methods. SFE has shown promising results as a more sustainable method of lipid extraction, especially for the food processing and pharmaceutical industries.

The focus of this thesis work is on triterpenoid extraction from *I. obliquus*, which is one of the most traditional medicinal polypores used for treating various diseases. A modified Folch method and supercritical carbon dioxide (SC-CO_2) extraction were utilized to extract triterpenoids from *I. obliquus* in this work. The triterpenoids were analysed by gas chromatography (GC), using flame ionization (FID) and mass spectrometric (MS) detection. The compounds were identified according to their retention times and measured mass spectra by comparison with spectral libraries and available reference standards. The effects of different parameters, such as pressure, temperature and extraction time, on the supercritical fluid extraction of *I. obliquus* were investigated. The data and knowledge acquired from the literary section was utilized to conduct the research. The modified Folch extraction was used to represent conventional extraction methods in this work. The comparison between the results of the supercritical fluid extraction and Folch extraction provide an insight of the potential for SFE to eventually replace the traditional methods.

2 Chaga

Inonotus obliquus (Fr) Pilát, also known as Chaga is a slow-growing, white-rot fungus belonging to the family *Hymenochaetaceae* (Yusoo *et al.*, 2000). Chaga has been extensively used as functional food and traditional folk medicine in Russia, China, Poland and most of the Baltic countries. There is no clear evidence on how the positive effects were discovered but reports have shown that Chaga has been used since the 16th century (Song *et al*., 2013). The earliest reported use of Chaga in Finland dates back to late 17th century when Chaga tea was used as a substitute for coffee (Halmetoja, 2004). In 2010, the Finnish food safety agency, Evira, classified Chaga as novel food denying sales for alimentation purposes. This was justified by the lack of significant trade for Chaga as foodstuff before 1997 within the European Union. For selling purposes in Finland, Chaga was then categorized as cosmetic. Eventually, this ban lasted only until September 2011, which also raised the public interest. The attention in the media and prevailing food trends lead to an increase of Chaga knowledge among households. In 2013, Chaga was recognized as medicinal herb by the Finnish health product retailer alliance due to observed long-term health enhancing effects (Halmetoja, 2013).

Chaga can be found in cold habitats in approx. 40° N–68° N latitude, growing as parasitism on living trunks of mature birch trees in North America, Baltic countries, NE Europe and western Siberia. Sporadically, it can also be found in China and Japan. *I. obliquus* grows usually on birch but also rarely on Ulmus, Alnus and Fraximus (Zhong *et al*., 2009; Song *et al*., 2013). The growth of Chaga (sterile conk) causes decay of the heartwood and the fruiting body can persist for 80 years. Once the host tree dies, the Chaga also dies due to lack of nutrients. The fungi produces one to two sclerotia on the main stem and branches of the tree. The sclerotium is perennial but the fruiting body occurs once in the infection cycle and appears as a crust-like layers of pores (Lee *at al*., 2007). Typically, Chaga appears as a dark-colored tumor figure approx. 25-40 cm in diameter. The outer surface is relatively hard with charcoal black and irregular appearance. Below the crust, the inside is light yellow with round pores (Halmetoja, 2014).

Chemical investigations have shown that *I. obliquus* produces a diverse range of bioactive metabolites including triterpenoids, sesquiterpene, benzoic acid derivatives, hispidin analogues, melanins, and polysaccharides. These molecules have shown to possess antioxidant, antitumor, and antiviral activities and the ability of improving immunity against infection of pathogenic microbes (Villares et al., 2012; Song *et al*., 2013). Chaga extracts have been used for treating various diseases such as tuberculosis, diabetes mellitus and different types of cancer. They are proven to possesses inhibitory effects on many cancer types e.g. melanoma, brain cancer, breast cancer, cervical cancer, bowel cancer, Ewing's sarcoma, leukaemia and liver cancer (Jaakko Halmetoja. 2013; Koyama *et al*., 2008).

Besides *I. obliquus,* the use and interest for medicinal mushrooms such as *G. lucidum*, *Lentinus edodes* (shiitake), *Ophiocordyceps sinensis* (caterpillar fungus)*, Grifola frondosa* (maitake) and *Agaricus blazei* (almond mushroom) have also grown significantly over the years. Similar to *I. obliquus*, these medicinal mushrooms have shown e.g. anti-tumour (Tang *et al*., 2006; Nanba *et al.,* 1987*;* Standish *et al*., 2010), anti-oxidation (Kodama *et al*., 2006; Takaku *et al*., 2001) and serum cholesterol level lowering effects (Winkler, 2009).

2.1 Pharmacological effects of Chaga

Most of the cancer treatments (chemotherapeutic agents) can destroy tumours and arrest cancer progress but at the same time damage healthy cells and tissues. Thus, new anticancer drugs from natural products are expected to play an important role in the development of more effective and safer agents to inhibit the onset of cancer (Cancer Research, 1998).

I. obliquus, being a slow-growing basidiomycete, has prolonged exposure to environmental stress factors such as UV irradiation, freeze, and the pathogenic microbes. Chemical investigations have shown that accumulation of bioactive metabolites in Chaga is connected to its defence strategy (Hoshino *et al*. 1998; Zucconi *et al*., 2002). Over the years, the knowledge on the pharmacological effects of Chaga has grown significantly due to extensive conducted studies. The wide range of active secondary metabolites found in Chaga have been demonstrated to possess cytotoxic, antibacterial, antimalarial, anti-inflammatory, anthelmintic and antioxidant properties (Glamočlija *et al*., 2015; Zhong *et al*., 2009; Ham *et al*., 2009). *I. obliquus* has also been reported to inhibit the protease of human immunodeficiency virus (HIV) (Ichmura *et al*., 1998). Melanins and lanostane-type triterpenoids extracted from *I. obliquus* arrested cancer cell in the early phase and induced cancer cell apoptosis or prevented cancer cell differentiation. Additionally, polysaccharides have also shown being indirectly involved in anticancer processes mainly via stimulating the immune system. The anti-oxidative ability due to presence of phenolic compounds may also prevent generation of cancer cells (Song *et al*., 2013).

Cui *et al*. studied the antioxidant capacities of ethanol extracts Chaga, which showed protective activity against cell oxidative stress. Furthermore, the anti-oxidative ability and free radical scavenging activity of *I. obliquus* extracts prevented generation of cancer cells *(*Cui *et al*., 2004). In another study, ethanol extracts of Chaga exhibited cytotoxicity against A549, HT29, Hela or L1210 tumour cell lines with moderate to strong effects (Zhao *et al*., 2015). Lee *et al*. reported that hot-water extracts of *I. obliquus* exerts inhibitory activity against the proliferation of human colon cancer cells. Hu *et al*. studied the (DPPH) radical-scavenging activity, anti-proliferative effects and ability to induce apoptosis in human colon cancer of different extracts from Chaga.

The crude water extracts and ethanol extract from Chaga showed positive cancer inhibiting effects (Hu et al., 2008). Anti-tumour experiments with n-hexane extracts of *I. obliquus* indicated a significant cytotoxic effect of triterpenoids, especially inotodiol, against Walker 256 carcinosarcoma and MCF-7 human mammary adenocarcinoma *in vitro*. *I. obliquus* is especially rich in triterpenoids such as inotodiol and trametenolic acid. Inotodiol and other lanostane-type triterpenoids including inonotsuoxide A and B extracted from Chaga have shown cytotoxic activity *in vitro* and antitumor activity *in vivo* prolonging the survival days of cancer bearing mice (Nakata *et al*., 2007). In several *in vivo* studies, inotodiol has shown to exhibit anti-proliferative and cytotoxic effects against the leukaemia cells in mice (Kahlos *et al*., 1987; Nomura *et al.,* 2008). Inotodiol and trametenolic acid extracted from Chaga have also been found to have inhibitory effect on α-amylase activity, free radical scavenging effects and cytostatic effect on cancer cells (Lu *et al*. 2009; Handa *et al.,* 2010). Inotodiol extracted from Chaga has demonstrated inhibitory effects on Walker-256 carcinosarcoma, breast cancer and positive properties against diabetes mellitus (DM) due to α-glucosidase inhibitory effects (Hu *et al*. 2006, Ying *et al*. 2014). Other triterpenoids found in *I. obliquus* include ergosterol, lanosterol, β-sitosterol, betulin and betulinic acid (Nakamura *et al*., 2009). Lanosterol is the basic substrate for the synthesis of triterpenoids in funguses including *I. obliquus*. Lanosterol itself has been found to contribute to the anticancer, antioxidant and immuno-modulating activity (Kahlos & Hiltunen 1987; Togashi *et al.* 1998). Ergosterol is an important vitamin D_2 precursor and it has a significant role in the fungal lifecycle (Barreira *et al*., 2014). Ergosterol is a plant and fungal sterol; it is an important component of the fungal cell membrane. In animal cells, cholesterol is present instead of ergosterol. β-sitosterol is a component found in many plants. Ergosterol has been demonstrated to possess health-promoting properties such as antioxidative, anti-inflammatory and anti-hyperlipidemic activities (Hu *et al*. 2006). β-sitosterol, betulin and betulinic acid can be commonly found in birch trees, which is also the main reason for their presence in *I. obliquus* (Abyshev, *et al.,* 2007). Ergosterol is involved in the activation of specific proteinase inhibitors together with enzymes participating in the defence response of *I. obliquus* (Lochman & Mikes, 2005). Ergosterol, ergosterol peroxide and trametenolic acid showed anti-inflammatory activities and cytotoxicity on human prostatic and breast carcinoma cells (Ma *et al*. 2012).

Polysaccharides including β-glucans and heteroglucans have been previously extracted from Chaga. The polysaccharides in *I. obliquus* were discovered to be indirectly involved in anticancer processes mainly via stimulation of the immune system (Mizuno *et al*. 1999; Rhee *et al*. 2008). Additionally, the polysaccharides fractionated from water extracts of Chaga demonstrated remarkable antioxidant activity (Hu *et al*. 2009). Polysaccharide extracts from Chaga showed

superoxide radical scavenging activities in a study conducted by Cui *et al*. In the same study, the separated polyphenolic extract from Chaga showed strong antioxidant activity protecting cells against hydrogen peroxide-induced damage (Cui *et al*., 2005). Other polyphenolic compounds identified from Chaga include e.g. 3,4-dihydroxybenzalacetone, inonoblins A, phelligridins D (Sung *et al*., 2008). Hot water, hexane and ethanol extracts from Chaga containing polysaccharides and polyphenols have also shown to be active in inhibiting tumour growth and metastasis (Burczyk *et al*. 1996; Rzymowska 1998; Chen *et al*. 2007). The phenolic compounds in Chaga include e.g. protocatechuic acid, vanillic acid, syringic acid, inonoblins A, B and C. The polyphenolic extracts of Chaga reportedly exhibits strong antioxidant activity e.g. protective effects against oxidative damage to DNA in human lymphocytes (Lee *et al*., 2007; Park *et al*., 2004).

2.2 Plant sterols and stanols

Sterols are an important group of bioactive lipid compounds present in all plant sources; similar to lipids they are insoluble in water but soluble in organic solvents. Over 40 plant-derived sterols (or phytosterols) have been identified. Of these, β-sitosterol, campesterol, and stigmasterol are the most abundant in nature. These are all 4-desmethyl sterols i.e. containing no methyl groups at carbon atom C-4 (**Figure 2**) (Law, 2000). Plant sterols can exist as free plant sterols or conjugated forms such as esterified plant sterols, steryl glycosides and acylated steryl glycosides (Normén *et al*., 1999) Plant sterols (**Figure 2**) resemble cholesterol (**Figure 1**) in their chemical structure and in biological function, while the latter is exclusively an animal sterol. Sterols are biosynthetic precursors to more oxidized steroids, which are composed of three terpene units, often also referred to as triterpenoids (Goad & Akihisa, 1997). The sterol ring is common to all sterols and the differences in sterol structure are mainly in the side chain (Piironen *et al*., 2000; Law, 2000). The triterpene family contains more than 100 different phytosterols and there are more than 4000 other types of triterpenes (Goad, 1991).

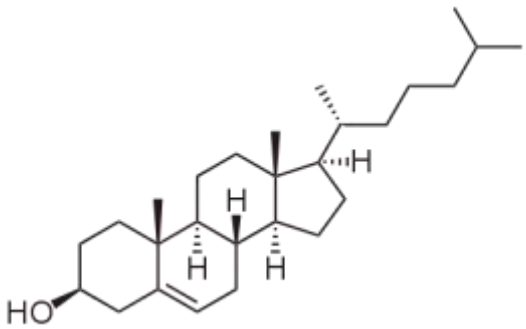

Figure 1. Cholesterol structure, one of the most commonly known triterpenoid.

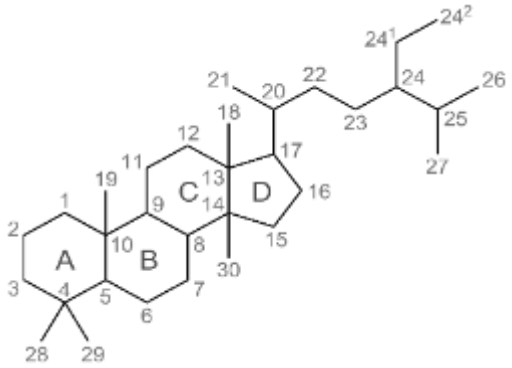

Figure 2. The general triterpene skeleton with carbon numbering.

Plant sterols and stanols (saturated or hydrogenated) have proven to decrease the incorporation of dietary and biliary cholesterol into micelles resulting in decrease of cholesterol absorption (de Jong *et al*., 2003). Dietary supplementation with phytosterols from the 4-desmethylsterol family effectively decrease low-density lipoproteins (LDL) cholesterol concentrations. Sitosterol, a 4-desmethylsterol, is the most common plant sterol, covering approx. 70% of all plant sterols in nature. However, 4,4-dimethylsterols such as α-amyrin and lupeol do not have the same decreasing effect on serum LDL cholesterol.

The LDL lowering mechanism associated to sterols and stanols is linked to the increased expression of the LDL receptor. These proteins are essential for many processes in the cell, one of which is regulation and decrease of dietary cholesterol absorption. Consequently, the cholesterol synthesis is also increased counteracting the cholesterol lowering effect of plant sterols to some extent. The absorption of cholesterol and plant phytosterols is a selective process, many internal factor and different inhibitors may affect the absorption rate. The absorption of plant sterols and stanols (0.02-3.5%) is significantly lower when compared to cholesterol (35-70%), and it depends on many factors, such as age, gender or diet. (Turley *et al*., 2003; de Jong *et al*., 2003). The presence of phytosterols and concentrations in mushrooms are species-dependent and influenced by environmental factors. Stanols are suggested to be more effective in cholesterol lowering and are found in much smaller quantities in plant foods. In a study related to children with familial hypercholesterolemia showed that 6 g/*die* sitosterol reduced LDL cholesterol by 20% while 1.5 g/*die* sitostanol reduced LDL cholesterol by 33% (Becker *et al*., 1993) The recommended daily intake required for reducing serum LDL cholesterol concentrations is 2.5 g/*die* (Brüll & Mensink, 2009). Ergosterol and β-sitosterol have been proven to lower serum LDL concentrations effectively. Both have been isolated from the sterile conk of *I. obliquus* (Chaga) and ergosterol is present in majority of common mushroom species (Kim *et al*., 2011).

Statin drugs are typically used for treating patients with ischemic heart diseases. The reduction in the concentration of LDL cholesterol is greater with statins compared to plant sterols and stanols. However, the possibility to add sterols and stanols to foodstuff such as margarines, cream cheese, salad dressing, and yoghurt, have increased their popularity to some extent. Fats are needed to solubilize sterols, which makes the combination with margarines ideal (Law, 2000).

2.2.1 Comparison of sterol quantities in different natural sources

Plant sterols are found in all food of plant origin. Legumes, mushrooms, nuts, seeds, vegetable oils and berries are nutritional sources of sterols. Normén *et al.* analyzed the total sterol content in commonly consumed vegetables and fruits in Sweden. The plant sterol content in vegetables was found to be 3.8-50 mg/100 g edible portion, while the median was 14 mg/100 g edible portion. The highest concentrations of sterols were found in broccoli, Brussels sprouts, cauliflower and olives. The plant sterol content of fruits was 3-44 mg/100 g edible portion and the median being 16 g/100 g of edible portion (Normén *et al*., 1999). The lipid and sterol quantity in mushrooms is typically higher compared to vegetables and fruits. Hammann *et al*. extracted sterols from common edible mushrooms *A. bisporus, L. edodes, Pleurotus eryngii*, and *Pleurotus ostreatus*. The free ergosterol quantity was the highest in button mushroom, *A. bisporus* and *L. edodes* (415-544 mg/100 g dry weight) and the lowest in *P. eryngii*, and *P. ostreatus* (<350 mg/100 g dry weight). The vitamin D and sterol content of wild and cultivated *A. bisporus, P. ostreatus and L. edodes* mushrooms were studied by Mattila *et al*. The most abundant sterol found in these mushrooms was ergosterol, which was found in higher quantities in cultivated mushrooms (602.1–678.6 mg/100 g dry weight) than in wild mushrooms (296–489 mg/100 g dry weight). Other sterols that they found in significantly lower quantities were ergosta-7,5-dienol (28.5-94 mg/100 g dry weight), fungisterol (14.6-16.7 mg/100 g dry weight) and ergosta-7,22-dienol (13.5-62.7 mg/100 g dry weight) (Mattila *et al*., 2002).

Phillips *et al*. analyzed the sterol composition of some edible mushrooms including *A. bisporus, L. edoles, Cantrarellus spp.* and *G. frondosa*. The mean quantity of ergosterol was found to be 503 mg/100 g by dry weight (Phillips *et al*., 2000).

Zheng *et al*. found that the ergosterol content in Chaga was 40.0 mg/100 g by dry weight. They also found that mycelium of wild Chaga contained lanosterol (45.5 % of the total sterols), inotodiol (25.4 %) and another ten sterols (30.2 %). The lanosterol concentration in cultured mycelia was lower compared to field grown samples (Koyama *et al*., 2008; Zheng *et al*., 2010). Yusoo *et al*. extracted sterols from Chaga mycelium by using ethanol extraction and column chromatography separation. Their extraction yielded 50 mg/100 g of ergosterol, 21 mg/100 g of lanosterol and 33 mg/100 g of ergosterol peroxide (dry weight) (Yusoo *et al*., 2001). Du *et al*.,

on the other hand, reported concentrations of 13.0 mg/100 g of inotodiol and 7.0 mg/100 g of trametenolic acid (Du *et al*., 2011). There seems to be a clear difference in ergosterol concentration between Chaga and typical edible mushrooms. The sterol composition in *I. obliquus* is affected by the long harsh growth conditions, which have been shown to inhibit the biosynthesis of ergosterol (Zheng *et al*., 2007).

3 Supercritical fluid extraction

3.1 Supercritical fluid

The supercritical fluid state is predominant when a substance subjected to a temperature and pressure above its critical point lacks a distinctive gas and liquid phases. The density of such fluid depends on pressure and the temperature. An example of a phase diagram in which the supercritical fluid (SF) state is highlighted is represented in **Figure 3**, where P_C and T_C points represents the critical temperature and pressure, respectively.

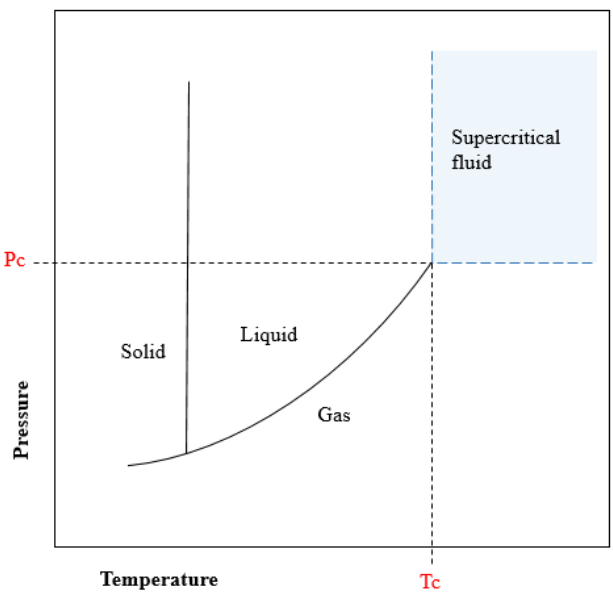

Figure 3. The modelled phase diagram for a pure compound.

The SFE methodology was studied and introduced by two researchers Hannay and Hogart already in 1879. They discovered that the solubility of the studied inorganic salts increased when the ethanol was heated above its critical temperature, then later they also discovered the significance of pressure. They found that sufficient pressure can dissolve the solutes and pressure decrease causes the dissolved materials to precipitate. (Hannay & Hogart, 1897; McHugh & Krukonis, 1993). Reportedly, the first observation of a supercritical state was by Baron Cagniard de la Tour over 50 years before Hannay and Hogart's studies. At the time, the lack of knowledge about supercritical fluids was most likely the reason of low interest on developing and exploiting this technique further. A major development step took place in 1964 when Kurt Zosel patented a commercial decaffeination process of coffee beans by using carbon dioxide (CO_2) which commenced a wider interest in this technology and the potential was being realized (Dean *et al.*, 1993).

3.2 SFE instrumentation

Supercritical fluid extraction systems have developed considerably over the years to become more efficient, cost-effective with higher selectivity and more versatile extraction techniques. Nowadays, the sample size ranges from milligrams (analytical) to industrial bulk samples. SFE can be used for extracting selected components from the sample, making possible to remove and reduce undesired sample components for example cholesterol from meat. Supercritical fluids can be used as online extraction medium allowing direct coupling of the extraction system to analytical apparatus and the acquisition of real-time results.

The SFE system used in the present work is shown in **Figure 4**. The syringe pump (A) supplies the CO_2 from the tank, the globe valves (C and D) are used to control the flow inlet/outlet. The secondary pump (B) supplies co-solvents, which were not used in this present work. The control panel (E) is used for adjusting extraction parameters such as extraction pressure, temperature, and time. The extraction takes place in the extraction chamber (G), where the sample cartridge (I) is loaded. The solvent flow from the CO_2 tank goes through the heated restrictor (H) prior to the outlet. The velocity of the solvent flow can be controlled by adjusting the length of the capillary between the outlet and the sample collection vial. A longer capillary increases the pressure at the outlet restricting the solvent flow. The heated restrictor prevents the capillary from being clogged by dry ice at the outlet.

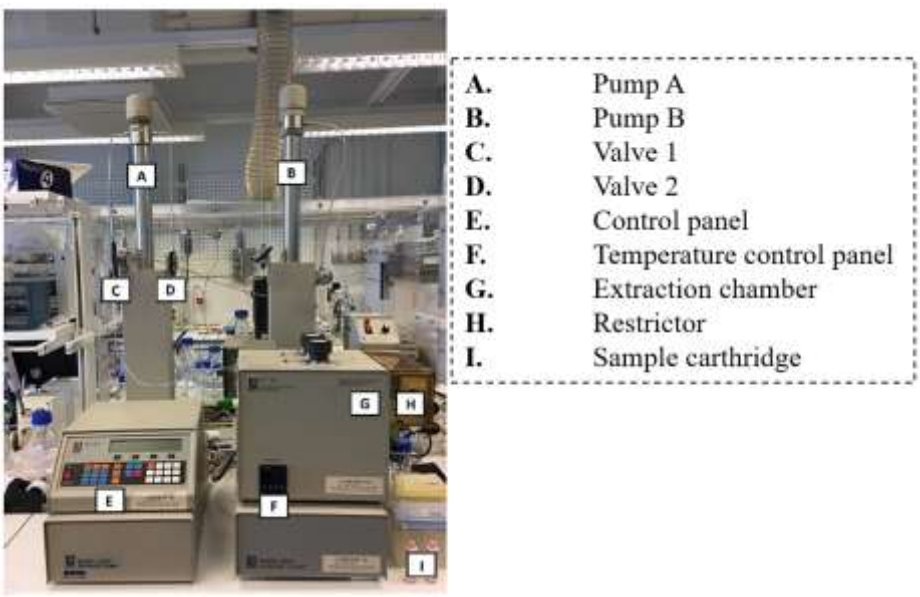

Figure 4. The Isco Extractor SFX 220 with two model 260D syringe pumps.

3.3 SFE and conventional extraction methods

Conventional solvent extraction methods are still mainly in use for separation of organic compounds in many analytical and industrial processes. However, due to stricter environmental

regulations and constantly improving technological innovations, SFE has been gaining wider acceptance during previous years. SFE has been already applied to a multitude of edible and non-edible natural materials e.g. seeds, fruits, leaves, flowers, roots and fungi (Reverchon *et al*., 1996). SFE can constitute an industrial alternative to solvent extraction, by providing faster, more efficient and less laborious extractions. The extraction can be carried out without other solvents than CO_2, reducing safety hazards and avoiding harmful solvent waste production (King & List, 1996). Carbon dioxide is the most commonly used in supercritical fluid extractions for several practical reasons. It is easy and relatively cheap to produce with very high purity. The low critical pressure (74 bar) and temperature (31 °C) make CO_2 easily pressurized into liquid or fluid state. Storing and transportation of CO_2 is also safe in normal conditions because it is non-flammable and possesses a low toxicity. However, excessive amount of CO_2 in the air causes a risk of suffocation, which can be taken into account with premises with proper ventilation.

The high selectivity of SFE means that it is possible to control density, viscosity and hence polarity of fluid on a molecular level (Lang & Wai, 2000). Fluid properties can be adjusted for to match the solubility of desired lipophilic compounds (Hawthorne, 1990). By choosing suitable temperature and pressure parameters it is possible to target and remove certain substances from the sample material without causing a significant thermal damage or remarkably affecting its structure (Clarke, 1991). Supercritical fluids have low viscosity and no surface tension, making effusion through solids possible, similarly to gas with faster mass transfer (meaning faster extraction) (Lang & Wai, 2000) .For example, SFE can be utilized in sample matrices permeated by conventional solvents.

The most significant drawback of supercritical CO_2 is its relatively weak interaction with both analytes and matrices. Pure CO_2 provide poor efficiencies in the extraction of more polar compounds such as polychlorinated biphenyls (PCB) (Hawthorne, 1990). This problem has been overcome either by addition of cosolvents (e.g. methanol, pentane, toluene) to increase the polarity of the extraction medium, or by reducing the polarity of the analytes by complexation, esterification and reverse micelle formation (Luque & García-Ayuso; 1997). Another drawback of SFE is the discrepancies in efficiency (Langenfeld *et al.*, 1993). Remarkably, the supercritical fluid extraction efficiency is generally lower compared to conventional solvent extraction methods, like Soxhlet, hence still inconvenient for medicinal and food industry.

3.3.1 Soxhlet and Folch extraction

The simplest and the most practical extraction processes are as old as the recorded history. Many names have been used to describe the variations of this process e.g. maceration, infusion, decoction, lixiviation and displacement (Jensen, 2007). For example, lixiviation, which is also commonly known as leaching, is extensively applied in metal processing industry for removing the metal constituents as soluble salts from impure mixtures (Geankoplis, 2004). Another leaching technique, Soxhlet, has been in use for a continued time. The Soxhlet extractor was first introduced in 1879 by the German agricultural chemist, Franz Ritter von Soxhlet. The extractor was originally applied in determination of fat in milk (Luque & García-Ayuso, 1997; Jensen, 2007). The typical Sohxlet apparatus consists of Sohxlet extractor, condenser, distillation flask and a system for heating the distillation flask. The sample is placed inside an extraction thimble and the distillation flask with solvent is heated, which gradually fills the thimble holder with condensed fresh solvent. As the liquid reaches the overflow level, a siphon unloads the solvent back into the distillation flask with the extracted compounds. The Soxhlet extraction has been a standard technique for over a century and it has remained the reference, to which the performance of other extraction methods are compared. Typically, samples are extracted close to the boiling point of the solvent for a long period. A very similar but significantly simpler technique was described by Morfit, which involved packing of the sample in a cylindrical percolator. The percolator was then filled with a heated solvent (typically alcohol or ether) which was allowed percolate slowly through the sample and to drain out an opening in the bottom, where it was collected into a sample vessel. This process can be repeated several times using fresh quantities of solvent and the combined extracts then evaporated to recover the extracted matter (Morfit, 1857; Luque & García-Ayuso, 1997). The Soxhlet apparatus is relatively cheap, reusable and simple to use. Additionally, it has the possibility to extract higher quantities at once compared to most of the latest methods. The possibility of thermal decomposition of the target compounds cannot be ignored when vulnerable analytes are being extracted (Luque & García-Ayuso, 1997). Conventional extraction methods generally require multiple repetition and washing steps, which produces large amounts of solvent waste.

Folch extraction is a method of liquid-liquid extraction, which is extensively used for lipid extraction from tissues. It has proven to be a very complete lipid extraction method. Similar to Soxhlet, Folch extractions often require multiple repetitions, which is time consuming and producing large quantities of solvent waste. In addition, the Folch method requires chlorinated solvent, which is a critical drawback from the environmental point of view. The mandatory

filtration and rinsing steps after extraction increase overall process time and the already relevant solvent. Additionally, a multi-step process increases the risk of sample loss and contamination.

3.3.2 Other conventional extraction techniques

Many conventional extraction techniques can be performed as an independent system or can be assisted with different performance accelerating set-ups. For example, hydrodistillation (HD) which is one of the main methods to obtain volatile essential oils from the plant materials along with other similar methods including; maceration, empyreumatic distillation (dry distillation), and expression. HD can also be carried out as microwave accelerated hydrodistillation, in which the sample is placed inside the microwave reactor with a frequency of 2.45 GHz, which is well known to have an enhancing effect on the results in many processes in the chemical and food industry (Ferhat *et al.,* 2006).

For the extraction of total lipids, a more popular method is the solid-phase extraction (SPE) method. The use of SPE has increased in the past few years because it is applicable to both polar and non-polar components. In SPE, the mobile phase containing analytes is passed through the solid phase where the desired analytes are retained for the duration of the sampling process. The analytes are then recovered from the solid phase by elution. A less popular lipid extraction method is the Gülbaran extractor. It is used for the extraction of fat from foods, for example for extraction of oil from seeds. The extraction system consists mainly of a rolling mill, which has two pairs of cylinders, a percolation type extractor, solvent and water heating vessels, screw conveyor and miscella recovery system (Türkay *et al.*, 1985). The general disadvantages of conventional extraction techniques include, long extraction times, losses of volatile compounds, toxic solvent residues, degradation of unsaturated compounds and the unwanted off-flavours due to heat (Khajeh *et al*., 2004).

3.3.3 SFE of lipids and triterpenoids from natural matrices

The increasing interest in SFE started in the early 1980s when this technology was applied to oilseeds and lipids. Lipids consist of diverse selection of biomolecules varying from polar to nonpolar. Lipids include several classes such as aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), wax esters, steryl esters, methyl esters, acylated glycerol ethers, triacylglycerols, free fatty acids, aliphatic alcohols, sterols, diacyglycerols, monoacyglycerols, glycoglycerolipids, pigments and phospholipids (Parrish, 1998) The technological advances and conducted researches during the past four decades have resulted in many patents regarding the extraction and fractionation of oil by means of supercritical fluids (Hawthorne, 1990). From the

food industry point of view, the ability of obtaining products that are free from solvent residues is particularly attractive (Clifford & Walker, 1996). The adjustable density of supercritical CO_2 makes it possible to extract lipids even without cosolvents. The supercritical fluids have been shown to possess mass transfer and penetrating properties typical for gas combined with solvating properties comparable to liquids (de Melo *et al*., 2014).
The consumption and production of vegetable fats and oils has increased dramatically, while the price of classic solvent, such as hexane, have also increased. Hexane is produced by refining crude oil, whose price has increased significantly. Demands on cost efficiency and eco-friendliness creates the need for further developments in SFE applications.

Triterpenoids have been previously extracted by SFE e.g. from birch and *Eucalyptus* bark (Yuhong *et al*., 2003; Domingues *et al*., 2012), mulberry leaves (Boszormenyi *et al*., 2009), saw palmetto berries (Catchpole *et al*., 2002), marjoram herb (Vagi *et al*., 2005), dandelion seeds (Simandi *et al*., 2002) and different mushroom species (Gil-Ramírez *et al*, 2013; Kitzberger *et al*., 2009). There are no reports available regarding extraction of triterpenoids from Chaga by SFE. Extraction of Chaga by subcritical water (SCW), on the other hand, has been studied but the properties and results of SCW and SF extractions are not comparable due to significantly higher polarity of SCW compared to supercritical fluids commonly used for extraction process. Seo and Lee studied the antioxidant activity of Chaga extracts obtained by SCW extraction. Extraction were done at various temperature and time combinations, the results indicated that SCW extracts possessed significant antioxidant activities but did not specify which compounds were accountable for the results (Seo & Lee, 2010).

3.4 SFE parameters

The extraction parameters are typically selected depending on the targeted analytes. The polarity of the analytes must be evaluated case by case, since more polar compounds may not be extracted by pure CO_2 and require a cosolvent. SFE is generally defined as a high-pressure technology because the pressure value is more preferably tuned compared to temperature. Different pressure variations directly affect the density value, which can be used to perceive how effectively the fluids reach a liquid-like solvent power. (de Melo *et al*., 2014) The most utilized temperature and pressure combinations for extraction of vegetable matrices are presented in **Figure 5**. The lines of constant CO_2 density (straight line) and Hildebrand solubility parameter (segmented line) are also shown.

Majority of the works have concentrated on temperature region within 40-60 °C and the multiplicity is greater closed to the lower value. This indicates that researchers have been eager to explore pressure impact on results at lower temperatures in order to protect thermally labile compounds.

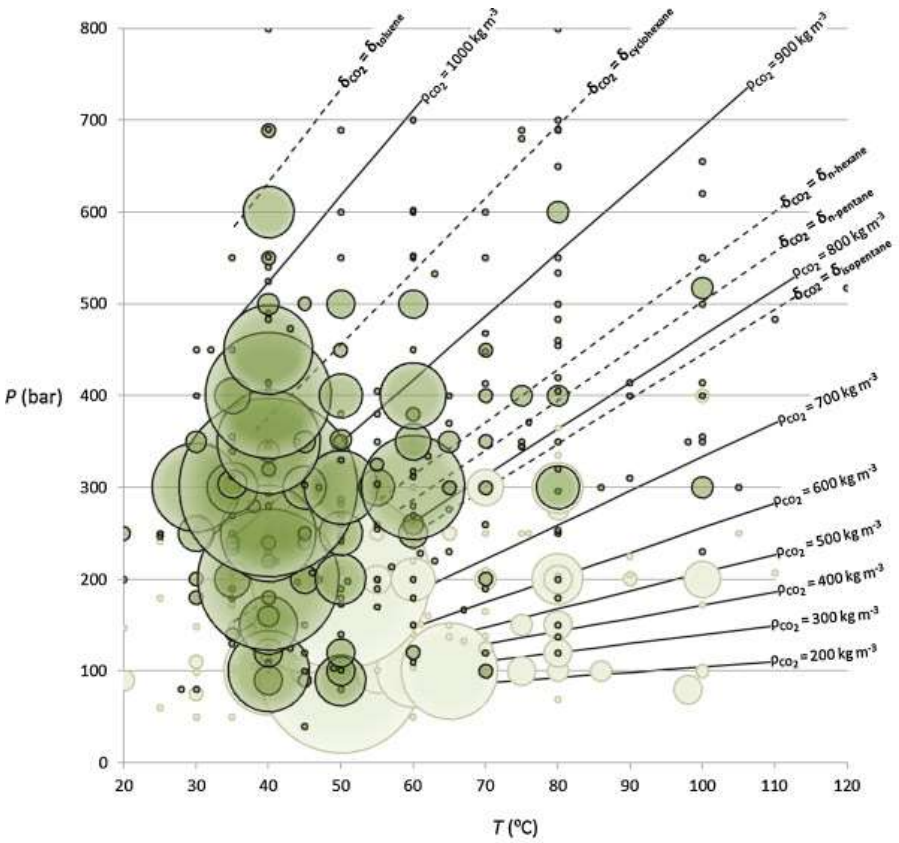

Figure 5. Most studied temperature and pressure combinations in SFE (de Melo et al., 2014)

The most utilized temperature and pressure combinations for extraction of vegetable matrices are presented in **Figure 5**. The Hildebrand solubility value (δ) represent the total van der Waals force, demonstrated as a simple solubility value. This parameter enables a rough comparison between different solvents with a constant solute (de Melo et al., 2014; Burke, 1984). It is noticeable that most studied extraction conditions correspond to a CO_2 density above 800 kgm^{-3}, at which the solvent power is comparable to typical apolar organic solvents. The general rule is that the higher is the pressure, the larger is the solvating power and the smaller is the extraction selectivity. The solubility is strongly linked to fluid density, which again is defined by the prevalent temperature and pressure. As can be observed in **Figure 6**, an elevation of pressure at given temperature increases the fluid density. Consequently, when the extraction pressure is higher, the smaller is the volume of the fluid needed for the extraction. However, high pressure is not always recommended for complex matrixes, since the resulting higher solvating power decreases the fluid selectivity, making extract purification and analysis more complicated. The presence of co-

extracting solutes may also change the solubility level of the solute of interest. The solubility behaviour becomes hard to estimate and degree of freedom increases when the amount of present components increases. Additionally, many other variables such as analyte vulnerability, extraction time and yield losses need to be taken into account when choosing the most effective extraction parameters (Hixon & Bockelmann, 1942).

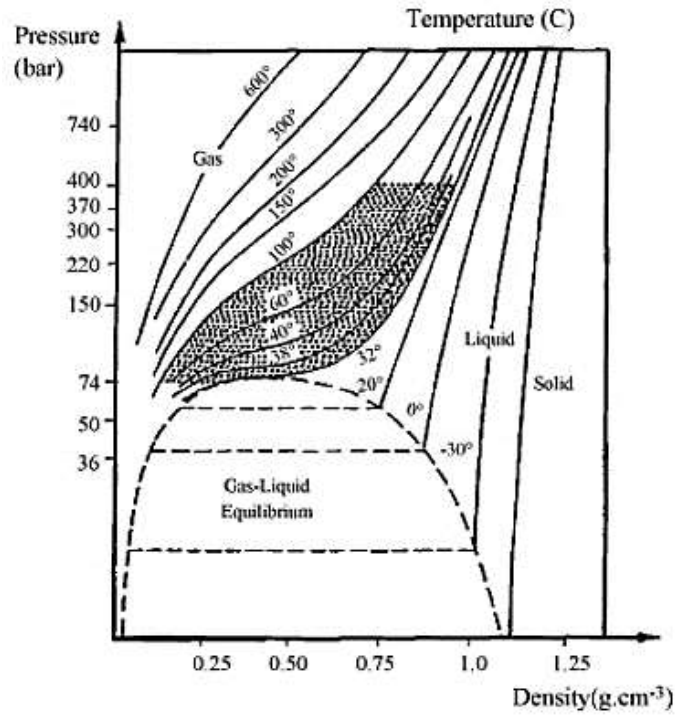

Figure 6. Pressure vs. density diagram for CO_2. The shaded area in highlights the supercritical phase of carbon dioxide after the critical pressure and temperature values have been exceeded (Andersen et al., 1990).

3.4.1 Extraction kinetics

The analyte mass transfer in SFE from the sample matrix can be broken down into four steps in order to understand the kinetic factors and stages of extraction on the molecular level. The diffusion of the analytes through the internal volume of the sample is the first stage and this requires analyte solubility with the solvent. Then the surface desorption of the analyte within the sample, sometimes the adsorption of analytes onto another sample surface takes place. In order to obtain quantitative recovery of analytes, the analytes must be efficiently partitioned from the sample matrix into the supercritical fluid. The diffusion of the analytes happens through a surface boundary layer, which is defined by the particle size, packing density and sample properties such as water content. The single solute molecules are then finally transported into the bulk SF phase, which is defined by the extraction time and flow rate (Anklam *et al*., 1998; Sovova & Stateva, 2011).

The desired analyte must be sufficiently soluble in the fluid in order to carry out a successful extraction. The solvating effect of the fluid mainly depends on the fluid density. At constant pressure, the solvating effect is lowered by the elevation of temperature due to decrease in density (Clifford & Walker, 1996). However, the vapour pressure is increased at higher temperatures, which again increases solubility. The proper selection of temperature requires optimization of these two opposing effects, and this generally requires extensive testing (de Melo *et al*., 2014).
At the optimization phase, there are several crucial parameters to consider that define the total solubility. Firstly, the pressure at which the analyte starts partitioning into the fluid (threshold pressure), typically not the same as the optimal extraction pressure. The next relevant point of interest is the pressure at which the solute reaches the maximum solubility, which can be determined mainly by collecting extensive extraction data or by employing mathematical models. Both ways have their benefits and disadvantages. Collecting solubility data experimentally is laborious and time consuming. Fortunately, the amount of valuable data on solubility has grown significantly in recent years and is mostly readily available. Determining solubility by using mathematical models, e.g. design of experiments (DOE) or response surface methodology (RSM) is getting more and more common nowadays. These mathematical analysis tools are implemented to determine how a response is affected by a set of quantitative variables in specified regions (de Melo *et al*., 2014). Solubility and diffusion values along with the properties of the sample matrix generally define the recovery rate in SFE. Knowing the solubility is one of the most fundamental piece of information, which provides a good starting point for the method development stage. The solubility of the desired analytes and the coextraction of the undesired compounds is particularly important when extracting flavours, fragrances and other easily degradative compounds including some lipids (Reverchon *et al*., 1996). The knowledge on the physical properties of the solute for example melting point is also important especially when extracting vulnerable compounds. This facilitates the understanding on degradation of desired composites at higher temperatures.

3.4.2 Extraction yield in SFE

The extraction yield is commonly linked to recovery rate, which represents the amount of the analyte extracted compared to the original amount in the sample. Suitable extraction parameters result in high recovery rate with time and cost-effective extraction results. A sufficient extraction time in most cases does not mean an absolute recovery of the desired analytes. As visualized in **Figure 7**, the extraction by supercritical fluids start very rapidly and is then followed by a kinetically limited region. The permeability of a plant tissue can be extremely low, which is why

the diffusion of a solute through the plant surface is also very slow. The initial period of rapid extraction of easily accessible solutes can be easily distinguished from the final period with much slower extraction (Sovova & Stateva, 2011). However, the original analyte amount inside the sample can be calculated with relatively high precision (**Equation 1**).

$$m_0 = m_1 + \frac{m_2^2}{(m_2 + m_3)}$$

Equation 1. Extrapolation equation for calculating the original solute mass in the sample (Bartle et al., 1990).

In **Equation 1**, the m_0 represents the original amount (mass) of the analyte. While m_1 and m_2 are the mass of two extractions with different extraction times.

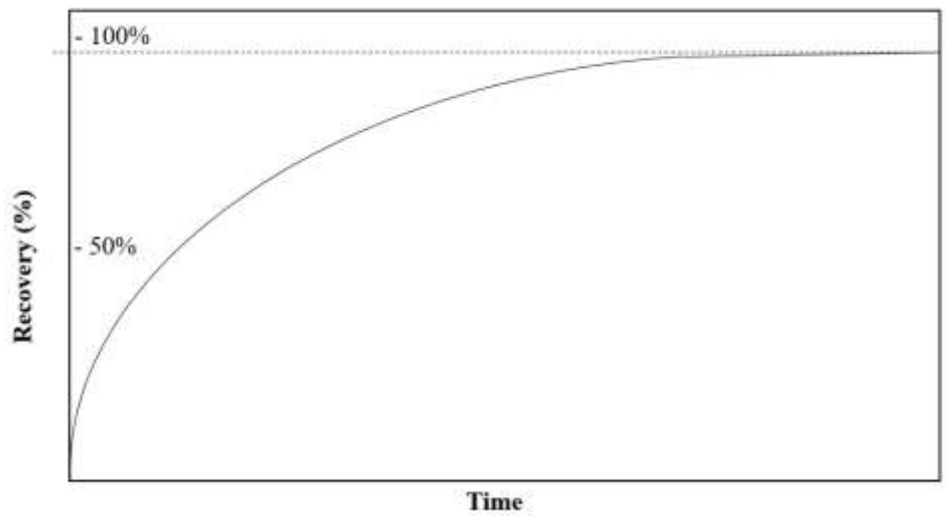

Figure 7. A schematic curve of the extraction yield (%) versus time for SFE.

In order to understand the recovery rate inhibiting mechanisms during the extraction, King and France contrived a simple model to represent SFE of an analyte from a single sample particle. The extraction begins with the desorption of the analyte from the sample. The analytes are then solubilized into the solvent flow, and carried out of the sample matrix. Being interaction between fluid and analyte an equilibrium, both excessive sample adsorption and desorption are possible. The first halts the extraction, the second leads to clogging of extraction apparatus. It is impossible to remove the adverse sub-processes completely. However, by choosing the optimal operating conditions it is practicable to overrule the adverse effects by the positive ones (King & France, 1992).

3.4.3 Solvent density and solvating power

The most commonly utilized equation of states for SC-CO_2 density calculations can be separated into three major types: Redlich-Kwong (RK-type), Benedict-Webb-Rubin (BWR-type), and Span-Wagner (SW-type) (Wang *et al*., 2015) Numerous equations of states are mentioned in the literature with varying relative errors in different temperature-pressure combinations. For example, Haghbakhsha *et al*. compared their experimental density data to three different density models and the results showed clear differences (**Figure 8**).

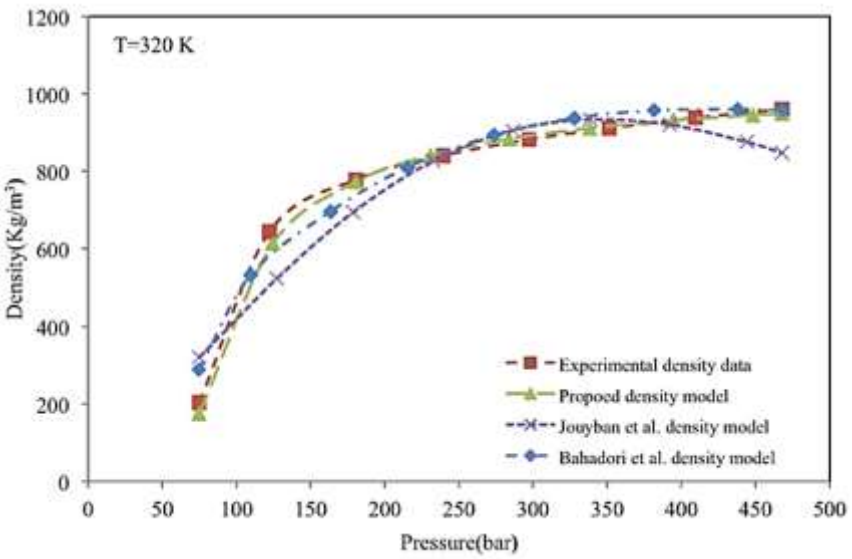

Figure 8. Different density models for SC-CO_2 at fixed temperature of 46.9°C degrees (Haghbakhsha et al., 2013).

In most cases, the complexity of biological and environmental samples leads to extracting ranges of analytes instead of a single compound. For example, ergosterol was partially extracted with a final concentration 0.7 g/mL, while complete extraction was estimated as 0.9 g/mL and within this range also other lipid compounds e.g. free fatty acids, waxes are coextracted (Young & Games, 1993).

3.4.4 Dynamic and static extraction modes

Offline extractions can be done as dynamic or static extractions. The dynamic extraction mode allows continuous supply of pure extraction fluid into the sample without extensive plumbing between extraction cell and outlet, therefore minimizing loss of extracted analyte (Lang & Wai, 2000). In the static extraction, the solvent flow is momentarily stopped on purpose, after the extraction cell is filled with the solvent allowing the solvent to modify the sample matrix. Static mode consumes less fluid and requires less pumping capacity, it can also increase the extraction yield due to better access to the analytes. The actual benefit of static extraction depends on the

sample and analyte properties. In some cases there is no significant increase in yield due to readily good access of analytes. For example, Klink *et al.* extracted fatty acids and sterols from *Nymphaea alba* (European white water lily) by using SFE, a 5-10 minute static extraction did not show any enhancing effect in the yield (Klink *et al.*, 1994). The efficiency of the static extraction can also be increased by adding a known quantity of modifier directly into the sample matrix (Lang & Wai, 2000).

3.4.5 Alternative fluids and cosolvents

Freons, ammonia, water and some organic solvents such as n-pentane have been used as solvents in SFE. Freon is more commonly utilized in environmental SFE. For example freons and sulfur hexafluoride (SF_6), which is a non-polar molecule, has been shown to selectively extract aliphatic hydrocarbons up to around C-24 from a mixture containing both aliphatic and aromatic hydrocarbons (Pourmortazavi & Hajimirsadeghi, 2007). Supercritical H_2O is corrosive and requires relatively high temperature and pressure (**Table 1**), which is why it has somewhat limited possibilities for practical applications in plant oil extractions. Supercritical H_2O has been mainly used for the destruction of hazardous organics (Luque & García-Ayuso; 1997). Near-critical water (also known as subcritical water or hot pressurized water) has been more commonly utilized due to its environmentally friendly properties similar to SC-CO_2. Carbon dioxide and subcritical water are considered the most promising solvents for the extraction of natural substances and various chemical processes. When pressurized at high temperature, water (near critical) becomes an excellent solvent for not only polar compounds but also non-polar substances. The critical temperature and pressure of water is significantly higher compared to critical points of CO_2 (see **Table 1**). The operating temperature of subcritical water is typically selected closer to the lower limit of the range. For example, Seo and Lee used 50-300 °C and 100 bar when performing SCW extractions from Chaga (Seo & Lee, 2010; Sovova & Stateva, 2011). The practical critical conditions are one of the reason CO_2 is the most commonly used SF solvent (Reverchon *et al.*, 2006).

Table 1. Critical properties of several solvents used in SFE.

Fluid	Critical temperature (°C)	Critical pressure (bar)
Carbon dioxide	31.1	73.8
Ethane	32.4	48.8
Methanol	-34.4	80.9
Ammonia	132.4	119.5
Nitrous oxide	36.7	72.4
Xenon	16.7	58.4
Water	374	221.1
Chlorodifluoromethane	96.3	49.7

The polarity adjustment is quite limited when using only pure CO_2 without cosolvents. The cosolvents are chosen in order to enhance the affinity of the solvent mixture towards polar compounds. The polarity of the supercritical fluid is increased by cosolvents such as ethanol, methanol or acetonitrile. Basing on 166 SFE publications, listed by de Melo *et al*., the most commonly used cosolvents were ethanol (53 %), methanol (21 %), solvent mixtures (7 %), water (5 %), dichloromethane (3 %), n-hexane (2 %) and vegetable oils (2 %). The remaining 7 % were labelled as "other" without containing additional details. (de Melo *et al*., 2014) The choice of the cosolvent is based on the nature of the solute of interest. As rule of thumb, the cosolvent should be able to dissolve the target analytes in its liquid state. There are three possible ways for cosolvent addition into the SFE system. In the more conventional method, separate pump and line are required for addition of a fixed amount of cosolvent to the feed. As alternative, the cosolvent can be added (spiked) to the matrix at the beginning of the experiment, and then extracted with pure fluid. The final option is the use of a pre-mixed fluid. (Lang & Wai, 2000; de Melo *et al*., 2014) With the online cosolvent addition, the mixture is generally more homogenous leading to more repeatable extraction results. It is important to keep in mind that cosolvent addition changes the critical parameters of the mixture, which also makes it harder to estimate the results in terms of solubility and efficiency. Addition of another liquid solvent into the mixture means it is recovered together with the extracts, and an additional purification process step is sometimes needed.

3.4.6 The effects of sample matrix on the extraction

Sample properties that must be taken into account when conducting SFE are moisture content, particle size and packing density. The packing density represents the amount of sample fitted into the volume of the extraction vessel. Larger particles decrease the packing density and smaller sample particle increase the packing density. Generally, decreasing particle size of the sample result in higher surface area for interactions, making the extraction more efficient. However, smaller particle size may lead to higher re-absorption rate of the analytes, decreasing extraction efficiency. High packing density, due for example to excessive sample grinding, also decreases the pressure inside the extraction chamber and hampers the extraction. An increased flow rate can restore the extraction efficiency, but not indefinitely. (Pourmortazavi & Hajimirsadeghi, 2007) The packing density has a key role in regulating the solvent and analyte flow and must be kept constant in order to achieve reproducible extraction results.

Water as a cosolvent can aid the extraction by increasing the polarity of the fluid and enable higher recoveries of polar compounds. However, in the case of samples with high moisture content, polar analytes will prefer to partition into the aqueous phase, decreasing its SFE yield. Relatively polar analytes, on the other hand, can be extracted anyway, due to the additional equilibrium between water and supercritical CO_2. Kerrola and Kallio studied the effects of water addition into the CO_2 extraction system on the relative amounts of the volatile components of *Angelica archangelica* (angelica). They found that the recovered yield was higher when an aliquot of water was added to the system. (Kerrola & Kallio, 1994) A suitable level of water content can also aid the extraction process by swelling the sample matrix, leading to pore opening and thereby allowing the fluid to access the analytes. (Lehotay, 1997) Sample moisture, typical for example of most vegetable matrixes, affects the SFE also of lipids. Analytes that are not soluble in water, once out of the matrix precipitate onto its surfaces in the presence of water, and even though the analyte may be highly soluble in the extraction fluid, the excess water in the sample could act as a barrier in transfer of the analyte to the fluid. Additionally, water may cause the plugging of the restrictor during the fluid depressurization because of its low solubility in CO_2 (0.3%).

In the case of plant tissues, the drying process can damage plant cell walls, facilitating the extraction process. However, the extract quality and composition can be compromised due to uncontrolled drying conditions. Milder drying conditions, for example freeze-drying, are recommended in order to avoid thermal degradation of vulnerable compounds. (Pourmortazavi & Hajimirsadeghi, 2007)

4 Aims and objectives of the thesis work

Various studies have been published proving SFE applicable for lipid extractions from diverse range of sample matrices such as mushrooms, wood, leaves and seeds. One of the main objectives of this thesis work is to extract, identify and quantify triterpenoids from Chaga.

The use of SFE at different conditions was compared with the Folch method, the latter being a complete total lipid extraction method. A simple ethanol extraction was carried out as well, with larger quantity of Chaga. The results of the comparison provides a brief insight on the benefits and possible shortcomings of these three methods.

The optimization of the SFE process was carried out by testing different temperature-pressure combinations and the impact of the restrictor. Other investigated parameters included solvent flowrate, which is regulated by the length of the capillary tubing at the outlet. The first step was to create an applicable SFE method for triterpenoid extraction from Chaga. TLC was utilized in order to confirm the presence of triterpenoids. Prior to the quantification, a saponification of the extract was required. Several saponification temperature, time and washing solvent combinations were tested to find a suitable procedure. Different oven temperature programs in the gas chromatographic analysis were tested in order to achieve the sufficient separation between the compounds.

5 Materials and Methods

5.1 Materials

Finnish company Eevia Oy supplied the *I. obliquus* sample analysed in this work. The mushrooms had been freeze-dried with remaining moisture content at 8 % and particle sizes varying from 10 to 20 mm.

Picture 1. Chaga sample as supplied before handling.

Dried sample of *I. obliquus* was crushed by using a mortar and pestle and a distribution of particle sizes around 1mm in diameter selected by an application of a centrifugal mill. The samples were then weighed and placed in 10 mL plastic extraction cartridges along with an inert filling material. Milled samples were stored in sealed clear water-white glass bottles away from light at room temperature and analysed no more than three days later from sample preparation.

5.2 Extraction

5.2.1 Soxhlet extraction

Several soxhlet extractions were performed with approx. 2 g Chaga samples and an extraction time varying between 2-12 hours. The samples were extracted with a relatively polar dichloromethane-methanol (3:1) mixture. Both dichloromethane, which has similar properties compared to chloroform, and methanol are commonly used in lipid extraction. After the extraction, the samples were purified from solvents in rotary evaporator at 40°C degrees with the speed of 140 rounds per minute (RPM) for approximately 10 minutes.

5.2.2 Folch extraction

The Folch extraction procedure was modified from the original method presented by Jordi Folch (Folch *et al*., 1956). The applied Folch extraction steps are shown in **Figure 9** and the following washing steps are shown in **Figure 10**.

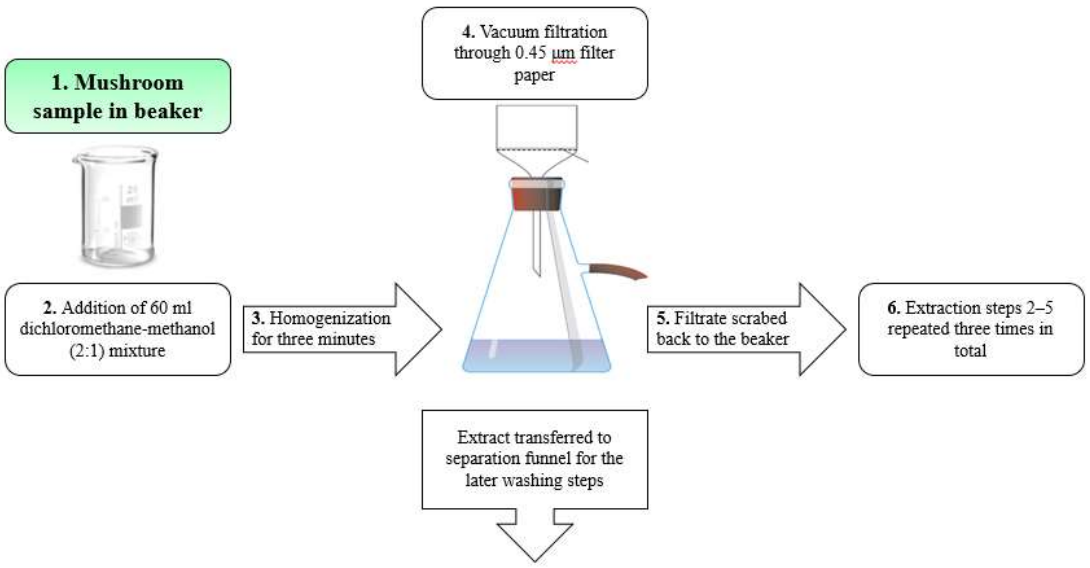

Figure 9. Extractions steps for applied Folch extraction.

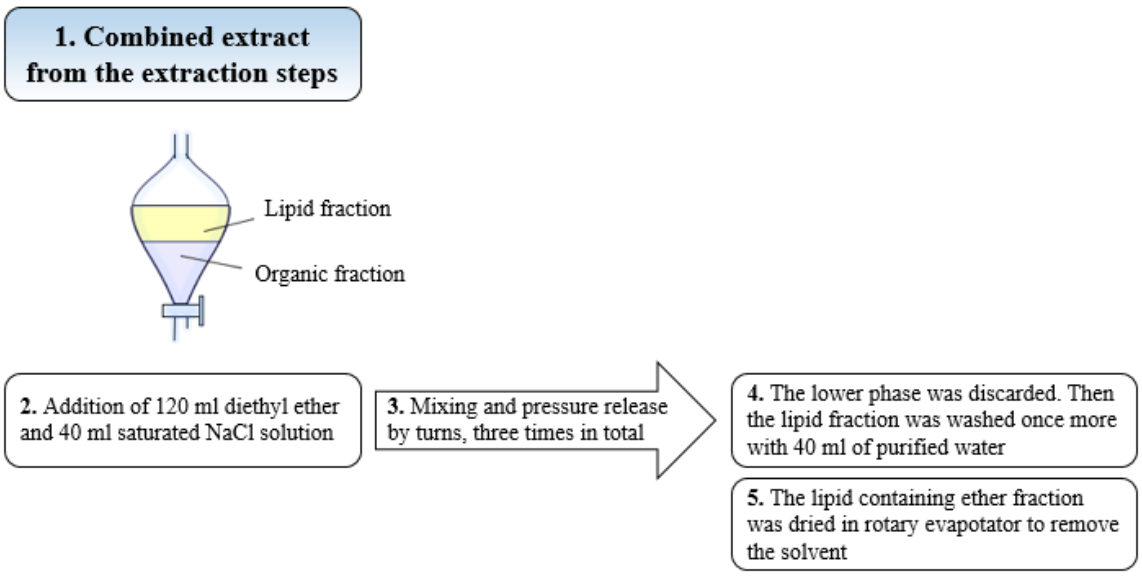

Figure 10. Washing steps for the extract.

A simple pre-treatment with water was tested by soaking 2 g of Chaga sample in 25 mL of purified water. The aim was to test how the freeze dried mushroom sample matrix would react and if the yield would increase. However, this failed because a slurry was formed which clogged the filtering paper during the vacuum filtration.

5.2.3 Ethanol extraction

The ethanol extraction was done by soaking 100 g of milled Chaga sample in 500 mL of ethanol at ambient temperature. The extraction was repeated three times, the extracts were finally combined and concentrated by rotary evaporator.

5.2.4 SFE

The Isco supercritical fluid extractor 200 (model Isco SFX 200) is shown schematically in **Figure 11** below. A photograph of the actual apparatus is shown in **Figure 4.** The integrated system consists of two Isco model 260D pressurizing syringe pumps, the Isco SFX 200 control panel, a temperature controlled reactor chamber with two ports (i.e. an heating extraction chamber), a pressure reducing restrictor capillary (i.e. the restrictor for short) and a stand for collection tubes. A 70 cm long capillary restrictor tube with an inner diameter of 70 µm was applied unless otherwise stated. The extractions were carried out as off-line extraction meaning the samples were collected for later analysis.

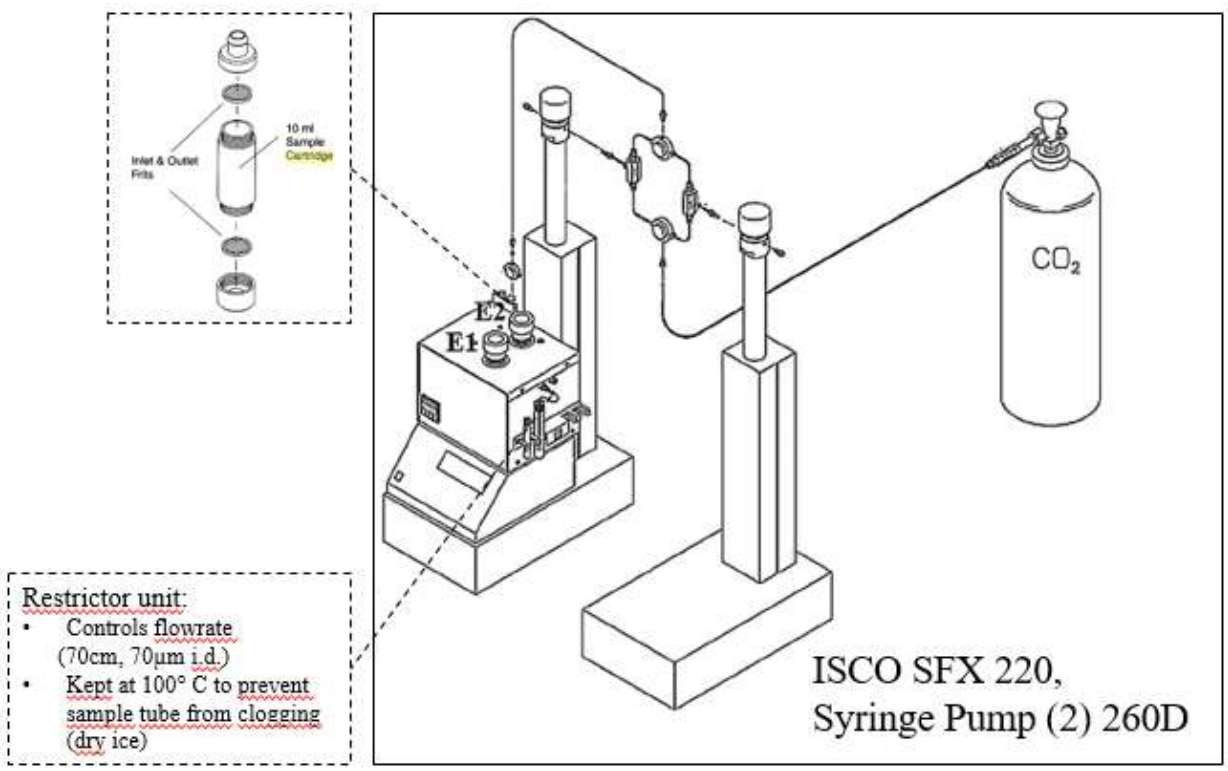

Figure 11. The scematic view of SFX apparatus.

Table 2. SFE apparatus' specifications.

Extractor SFX 220:	
Max. temperature	150 ºC
Max. operating Pressure	700 bar
Cylinder/Pump Capacity	266.05 mL
Flow Rate Range (<517 bar)	1 µl/min – 40 mL/min
Flow Rate Range (>276 bar)	1 µl/min – 90 mL/min
Refill Time	2.5 minutes

The chosen extraction conditions are summarized in **Table 3**. The extraction duration can be set to be limited by time or fluid volume and both conditions were tested. At the system testing phase, the time limit resulted relatively high standard deviation in the yield (23-40 %) between the samples. The initial absence of a heating restrictor led to clogging of the outlet capillary, which resulted in unstable solvent flow. After the restrictor installation and further testing, the extraction duration was set to be limited by the fluid volume. This showed higher consistency in yield. The chosen fluid volume was equal to five times the internal volume of the sample cartridge (50 mL). The average extraction duration was 13±0.47 minutes, which is nearly comparable to the time-limited extractions.

Table 3. Tested extraction conditions.

Extraction temperature and pressure	**Fluid density (kgm^{-3})**	**Restrictor**	**Limiting factor**	
40 °C, 281 bar	898.45	No	Time	15 minutes
40 °C, 281 bar	898.45	Yes	Time	15 minutes
40 °C, 281 bar	898.45	Yes	Fluid vol.	50 mL
50 °C, 350 bar	899.40	Yes	Time	10+10 minutes
50 °C, 350 bar	899.40	Yes	Fluid vol.	50 mL
40 °C, 350 bar	934.90	Yes	Fluid vol.	50 mL

The system cleaning between the extractions was done by performing a 10 minutes extraction with a blank cartridge. The adequacy of the cleaning was verified by doing two blank extractions and the second extract was analysed to confirm the absence of any triterpenoid residues. The 10 mL plastic extraction cartridges were washed with soap, dried and then sonicated in methanol. Prior to using the cartridges were always rinsed with ethanol and dried.

5.2.5 Restrictor

The flow velocity of the extraction is mainly controlled by the outlet capillary tubing, which is part of the restrictor system at the end of the extraction cycle. Several test extractions were done in order to find the optimal capillary length. The fused silica capillaries (100µm I.D., 363µm

O.D.) were cut and tested from between 0.3-2.5 m, which resulted flowrates of 0.9-15 mL/min. The desired flowrate was approximately 3 mL/min, which was achieved with a 1.0 m capillary. The temperature of the restrictor was kept at 55 °C degrees to keep the capillary tubing from clogging by dry ice.

5.3 Analysis

5.3.1 Thin layer chromatography

Following the extractions, thin layer chromatography (TLC) was used to confirm the coextracted lipid classes. Silica coated (adsorbent) and aluminum foil supported TLC plates were used, with hexane-diethyl ether-formic acid (80:20:2 by volume) as the mobile phase. The hexane-diethyl ether-formic acid mixture has been commonly used for analyzing neutral lipid samples such as fatty acids, tri-, di-, and monoglycerides (Peyrou *et al*., 1996).

5.4 Sampling procedures

Random and systematic errors caused by e.g. sample preparation, chromatography and detection cannot be fully avoided. Internal or external standard addition to lower the random and systematic errors in the quantitative analysis improves the results in term of reproducibility but also reliability.

A concentrated cholesterol standard solution of 1 g/mL of the internal standard (ISTD) was prepared and an aliquot of this solution was added at the same concentration to every sample prior to the extraction. ISTD was prepared to a hexane solution, which proved to be repeatable after pipetting tests. In addition, dichloromethane was tested resulting lower accuracy and repeatability due to higher vapour pressure and density compared to hexane. Prior to the milling in Folch method, 300 µL of the ISTD solution was added into the Chaga sample. In the SFE samples, the same amount of ISTD was added directly into the extraction cartridge and dried under nitrogen flow to remove the hexane. Multiple point internal standard method was used in this work. The ratio of the analyte area to the area of the ISTD was determined and plotted with the ratio of analyte concentration to the ISTD concentration.

5.4.1 Saponification

Saponification simplifies the gas chromatographic analysis of sterols by removing triacylglycerol and other free fatty acids as salts. Several saponification methods were tested by varying the saponification temperature and washing solvents. For example, a cold saponification procedure was tested by treating the extract with 20 mL of 1.2 M potassium hydroxide in ethanol, and saponified in room temperature for 24 hours.

The saponification procedure applied for all extracted samples is as follows: extracted sample was treated with 20 mL of 1.2 M potassium hydroxide in ethanol, and then heated while mixing at 70 °C degrees for 60 minutes. To reduce undesired sterol oxidation, the saponification vessel was purged with nitrogen prior to heating, and then sealed with parafilm and aluminum foil to create anaerobic conditions.

5.4.2 GC sample derivatization

The sterol samples and standards were initially pipetted into 2.5 mL vials and held under nitrogen flow to evaporate the solvent. After drying, the Tri-Sil reagent was added for the derivatization step. The GC samples were quantitatively transferred into 300 µL inserts, which were placed inside 2.5 mL sample vials (**Figure 13**). Due to the circumstances at the time, the Tri-Sil amount had to be lowered in order to analyse all samples.

Figure 12. Sample vial with 300 µl insert inside.

The derivatization is necessary in gas chromatographic analysis due to low volatility of some analytes (Knapp, 1979). In addition, some analytes may respond poorly on a certain detector types and possibly needs to be altered with a different functional group in order to improve the detection e.g. chlorine improves response on an electron capture detector (ECD). Derivative formation of sterols may also give enhancement in peak resolution and improve stability of thermally labile compounds. Silylation is the most prevalent derivatization method as it readily volatizes the sample and is therefore very suitable for non-volatile samples for GC analysis (Orata, 2012). Trimethylsilyl (TMS) ethers along with acetates are commonly the most employed derivatives used in sterol and lipid analysis due to the suitability for quantitative purposes (Goad & Akihisa, 1997; Sellers, 2010). GC Samples were derivatized by treating them with 100 µL of Tri-Sil

reagent by the following procedure. Lipid sample were first dried from solvents prior to silylation reagent addition, vials were then mixed vigorously and heated to 70°C degrees for 1 hour. The samples we let to cool down to room temperature before the analysis.

Figure 13. Example of Tri-Sil treatment for ergosterol.

5.5 GC-MS

The identification of sterols was done mainly by using gas chromatography–mass spectrometry (GC-EI-MS) analysis. The mass spectrometer was operated in electron impact ionization mode. The GC-MS equipment and analysis parameters used in the present work are summarized in **Table 5**. Sterol samples were silylated by using Tri-Sil reagent and analysed as trimethylsilyl (TMS) esters. Analysis was conducted by using a Hewlett Packard HP 6890 and Hewlett Packard 5973 single-quadrupole GC-MS with a DB5 cross bond 5% diphenyl, 95% dimethyl polysiloxane GC column (30 m × 0.25mm × 0.25µm df) with 3:1 split injection. The column inlet pressure was set at 117 kPa for a carrier gas flow of 1mL/min and linear velocity of 30 cm/s.

Table 4. GC-MS equipment and used parameters.

Equipment	**Model**	**Manufacturer**
Gas chromatograph-mass spectrometer	HP 6890 GC-MS	HP Inc., Palo Alto, USA
Injection port	Split 3:1	-
Liner	0.75 mm ID splitless/split liner	Krotek, Espoo, Finland
Software	Agilent Chemstation, version HP 6890	Agilent Technologies, Alto, CA, USA
Column	DB5 (low polarity) 30m×0.25mm×0.25µm	Agilent J&W
	Parameters	
Inlet	250 ºC, pressure 117 kPa, total flow 30 mL/min, purge flow 18.0 mL/min, purge time 3 min	
Column	Pressure 44.7 kPa, flow 0.9 mL/min, average velocity 34 cm/sec	

Mass spectrometer	Ionization energy: 70 eV, scanning frequency: 2.6/second, 30—600 Da, interface temperature (AUX-value): 250 ºC, ion source: 230 ºC, quadrupole: 250 ºC

*Table 5. Oven temperature program as visualized on **Figure 12.***

Increase (°C/min)	T (°C)	Holding time (min)
-	255	1
0.75	265	25
10	325	5

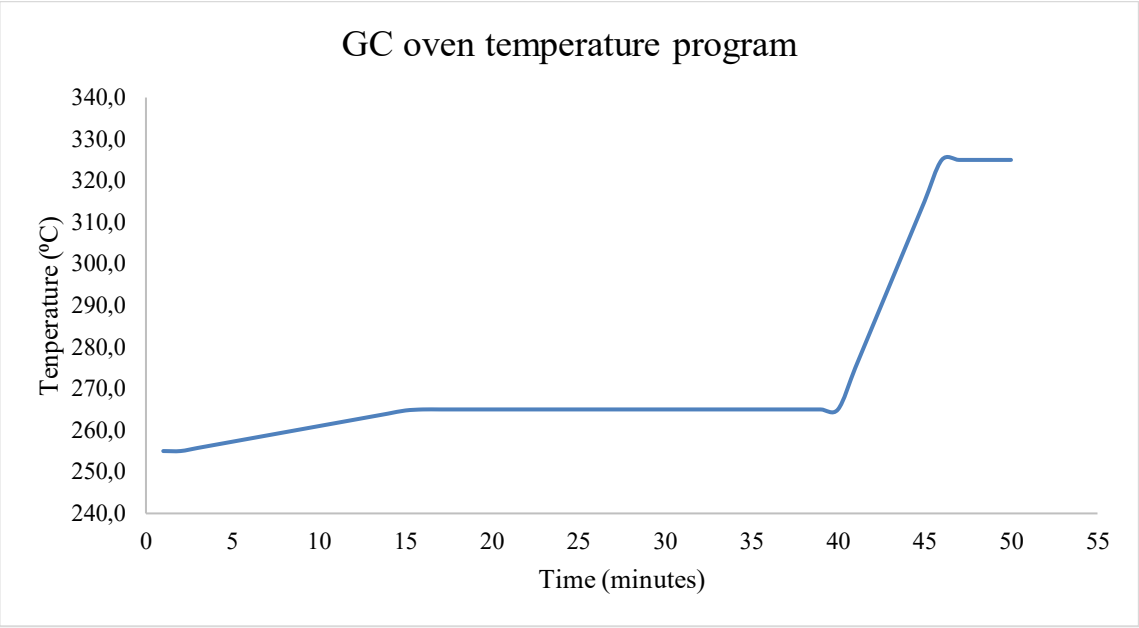

Figure 14. Gas chromatograph oven temperature program used.

5.6 GC-FID

Gas chromatography with flame-ionization detection (GC-FID) was used in the quantitative analysis of the sterol samples. An equivalent oven temperature program was used compared to GC-MS.

Table 6. GC-FID method.

Equipment	Model	Manufacturer
Gas chromatograph-flame-ionization detection	GC-2010 plus, Shimadzu	HP Inc., Palo Alto, USA
Injection port	300 ºC, Split 5:1	
Liner	0.75 mm I.D. splitless/split liner	Krotek, Espoo, Finland
Software	Agilent Chemstation, version HP 6890	Agilent Technologies, Alto, CA, USA

Column	DB1-MS 30m×0.25mm×0.25µm	Agilent J&W

	Parameters
Inlet	240 ºC, pressure 117 kPa, total flow 30 mL/min, purge flow 18.0 mL/min, purge time 3 min
Column	Pressure 44.7 kPa, flow 0.9 mL/min, average velocity 34 cm/sec
Detector	325 ºC

5.7 Statistical evaluation of results

Differences between triterpenoid contents in different extraction conditions were analyzed by a one-way analysis of variance (ANOVA) together with Tukey's t-test or by a T-test ($p<0.05$). Statistical analyses were performed using SPSS 16.0 (SPSS Inc. H, Chicago, IL) and Origin (OriginLab Corp., Northampton, MA). Differences reaching a confidence level of 95% were considered as statistically significant.

6 Results

6.1 Extraction yields

Table 7. Sohxlet extractions with Chaga sample.

	Extraction time (h)	Sample size (g)	Weight of lipid extract (g)
Sample 1S	6	2.0018	0.06
Sample 2S	12	2.0032	Failed
Sample 3S	2	2.0039	1.14
Sample 4S	2	2.0097	0.04

Soxhlet extraction conditions and lipid recovery are reported in **Table 2**. As can be observed there is no consistency in the yield of the obtained raw extract. The main reason is most likely the high error in weighing small quantities in a vessel that is too large. However, it could also have been due to failed rotary drying, high losses during the quantitative transferring between vessels.
The sample obtained by Soxhlet extraction were not analysed for the final comparison due to practical reasons. The Folch method was proven more complete and provided more reproducible for comparative purposes.

Table 8. Summary of the weights crude extracts obtained by SFE.

Extraction parameters	Density (kgm^{-3})	Average weight (mg)	Relative standard deviation-%	Special remarks
40 °C, 281 bar	898.45	1.35 ± 0.61	45,05 %	Time limited: 15 minutes. No restrictor.
40 °C, 281 bar	898.45	1.26 ± 0.44	35,38 %	Time limited: 15 minutes.
40 °C, 281 bar	898.45	3.12 ± 1.21	38,81 %	Volume limited: 50 mL.
50 °C, 350 bar	899.40	1.11 ± 0.39	34,69 %	Time limited: 10 +10 minutes.
50 °C, 350 bar	899.40	2.14 ± 0.52	24,07 %	Volume limited: 50 mL
40 °C, 350 bar	934.90	1.41 ± 0.57	40,25 %	Volume limited: 50 mL

The weights of the SFE raw extracts obtained by different conditions have been summarized in **Table 8**. Each result represents average of three replicates. As can be observed, the standard deviation is high, indicating that the extraction the yield is not consistent. However, the error margin must be taken into account due to high tare, making the extract weights not decisive. The weighing has been done in 2.5 mL vial, which weight about 10 000 times more than the actual extract.
The weights of the raw extract obtained by Folch method were considerably more repeatable. The extracts by Folch method yielded 24.25 ± 0.06 mg (0.23 % relative standard deviation), which is also about 20 times more than the yield by SFE. The lipid fraction obtained with the

Folch method comprises about 1.2 % of the sample dry weight. The lipid fraction obtained by ethanol extraction yielded 1600 mg (from 100g sample), which was 1.6 % of the dry weight. For comparison, Glamočlija *et al*. used ethanol extraction to obtain 1-2.4 g of lipids from 25 g of dry *I. obliquus* powder, which was 4-9 % of the original sample (Glamočlija *et al*., 2015).

6.2 TLC

Table 9. Standard compounds of lipid classes used and concentrations.

TLC numbering	Lipid class	Concentration (g/l)
1	Free fatty acids 18:3 n-6 (FFA)	5.0
2	Phospholipid 19:0 (PL)	1.0
3	Triacylglycerol 17:0 (TAG)	1.0
4	Monoacylglycerol, 2-monoolein (MAG)	1.3
5	Diacylglycerol (DAG)	1.3
6	Cholesteryl ester, cholesteryl pentadecanoate 15:0	1.0
7	Steroid, cholesterol	1.1

The first plate was prepared by applying 20 µL of each standard on the plate using a syringe and then ran separately and as a mixture. The TLC run time was 1 hour and 4 minutes.

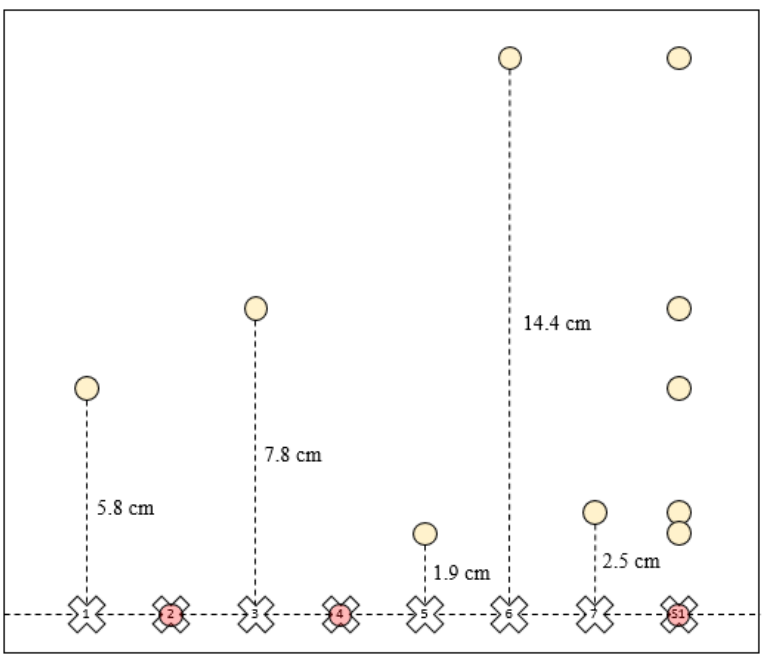

Figure 15. The results for the lipid standard TLC place, numbering is according to Table 3.

The results showed that the solvating power of this mobile phase is not applicable to phospholipids and 2-monoolein. Another TLC plate was prepared to determine the lipid classes in different extracted samples. The ethanol-extracted sample was extracted by Gabriele Beltrame

from 100 g sample of Chaga mushroom by soaking the milled mushroom sample in 500 mL ethanol.

Table 10. Second TLC plate.

TLC numbering	Sample name	Amount applied (µl)
8	SFE sample	50
9	Lipid standards mixture	20
10	SFE sample	20
11	SFE sample	10
12	Ethanol extract	20

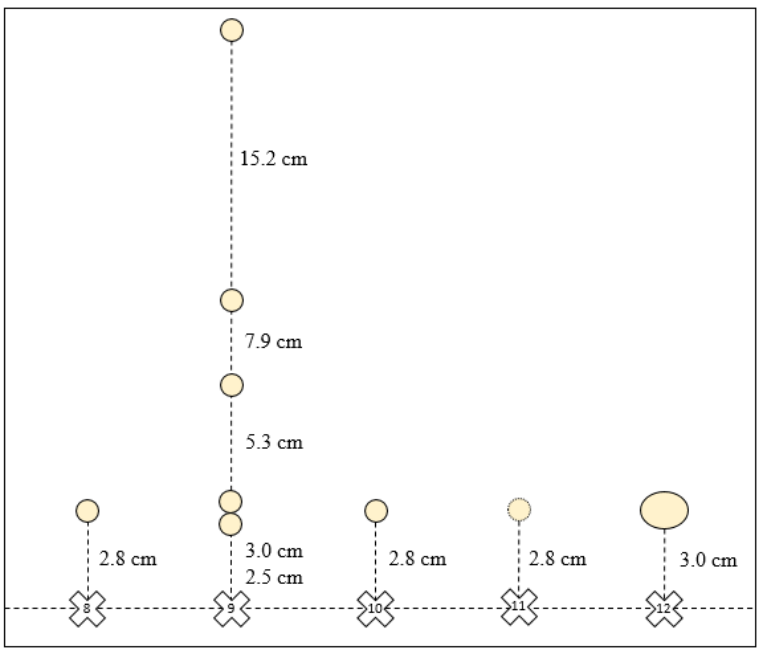

Figure 16. Results for second plate containing samples from SF, ethanol extractions and lipid standards mixture.

The total run time for the second TLC plate was 1 hour and 2 minutes. Both SF and ethanol extracted samples included compounds with similar properties to cholesterol and diacylglycerol lipids. Saponification was proven to be required.

Thin layer chromatography may also be used for preparative purposes. Once the sterols are isolated from the rest of the lipid, saponification step will not be needed. The mobile phase was proven sufficient for separating sterols, so a preparative TLC plate was tested by applying 200 µL of SFE extract to the plate with a semi-automated dispenser. Sufficient separation did not take place so the sample amount was increased to 1 mL. This time the separation was visible and the silica layer corresponding to the relative retention of cholesterol was scraped carefully into a test tube and soaked overnight in dichloromethane-methanol solvent (2:1). The sample was then centrifuged, separated from the silica solids and analyzed on GC-MS without diluting but showed no traces of sterols. The sample amount was possibly not sufficient, at this point of the thesis the

amount of sterol in SFE samples had not been quantified yet. The purpose of testing this preparative TLC step was to confirm the presence of sterols without the additional saponification step. The test was repeated but the result remained the same, thus the use of preparative TLC was discarded.

6.3 GC-MS

6.3.1 Mass fragmentation pathways

The overall structure and functional groups of the analytes determine how the molecules will resist or favour different fragmentation patterns (Middleditch, 1979). The fragment ions of the identified compounds and the relative abundancies are summarized in **Table 11**. Base ion (or base peak) is the fragment ion with the highest abundancy from which the relative abundancy of the other ions are calculated from.

The McLafferty rearrangement ion is prevalent in fragmentation of organic molecules referring to β-cleavage and specific migration of a γ-hydrogen atom to the functional group (McLafferty, 1959) The abundance of the ions by the McLafferty type of fragmentation is principally influenced by the location of the double bond and methylation pattern of the sterol rings (Rahier & Benveniste, 1989) The mass spectra of summarized TMS ethers showed high abundance in ions at m/z 73 for the trimethylsilyl group and McLafferty rearrangement ion at m/z 75 (Zaikin & Halket, 2009; Christie, 2013) The loss of TMSiOH at the third carbon (C-3) in **Figure 17** in the A ring, is a common fragmentation pathway for sterols as observed in most compounds summarized in **Table 11** (Zaikin & Halket, 2009).

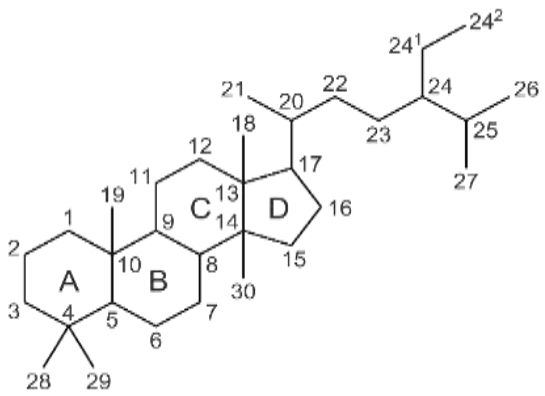

Figure 17. General sterol skeleton with carbon numbering.

Table 11. Summary of mass fragmentations with relative abundancies of corresponding ions.

Triterpenoids	M^+ m/z	Base ion m/z	Prominent fragment ions m/z (Relative abundance, %)
Cholesterol (ISTD)	458	129(100)	329(95), 368(71), 73(45), 75(41), 353(33), 458(31), 255(17), 147(17)
Ergosterol	468	363(100)	337(65), 69(48), 73(41), 131(20), 468(19), 378(19), 143(19), 129(18)
Lanosterol	498	393(100)	69(68), 73(33), 498(31), 75(21), 483(21), 129(20), 147(16), 229(11), 255(10)
β-sitosterol	486	129(100)	357(72), 396(67), 73(51), 75(33)
Stigmastanol	489	69(100)	393(46), 73(40), 75(25)
Inotodiol	586	337(100)	73(88), 297(85), 75(36), 517(31), 129(27), 69(27), 229(23), 255(17)
Trametenolic acid	600	73(100)	158(39), 129(33), 585(31), 69(31), 281(31), 189(23), 187(21), 147(19)
Betulin	587	73(100)	189(71), 203(60), 75(42), 129(36), 147(25)

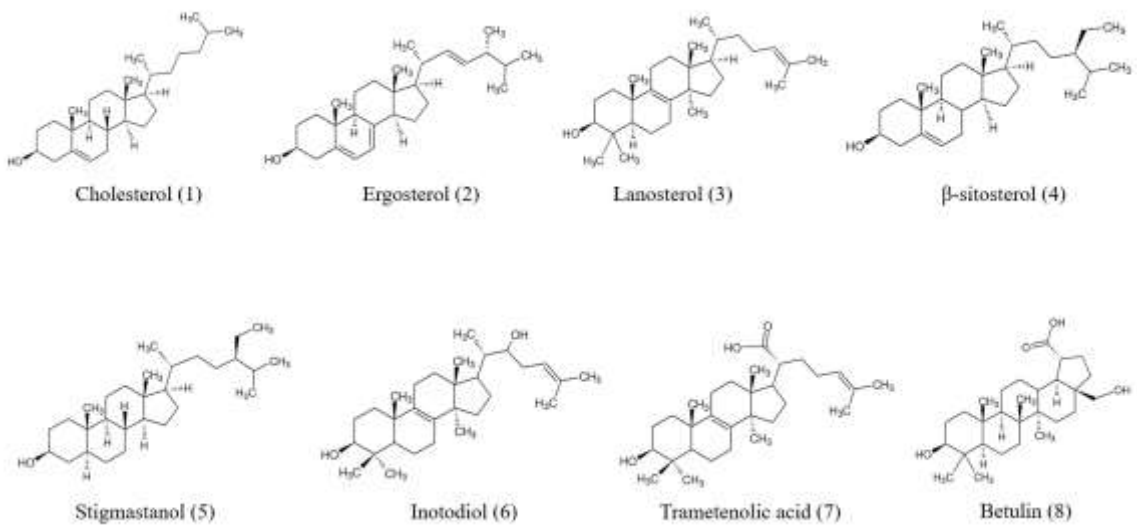

Figure 18. The chemical structures of investigated triterpenoids comprising the cholesterol (1), ergosterol (2), lanosterol (3), β-sitosterol (4), stigmastanol (5), inotodiol (6), trametenolic acid (7) and betulin (8) structures.

Silylated trimethylsilyl ethers occasionally display the molecular [M^+] ion by acquisition of TMS group [m/z 73]; the occurrence depends on the stability of the compound. For instance, double bonds, cyclic structures and aromatic rings can stabilize or destabilize the molecular ion affecting the probability of the appearance (Thevis & Schänzer, 2005) The trimethylsiliconium at m/z 73 and polydimethylsiloxane (PDMS) at m/z 147 ions are formed in multi-step fragmentations,

which require high internal energy and show a strong and characteristic dependence on electron energy opposite to that of the McLafferty rearrangement ions. The ion at m/z 147 is indicative of aerosolized polydimethylsiloxane, a prevalent contaminant in SIMS analysis and is prominent in majority of the identified compounds in **Table 11** (Piehowski, 2008).

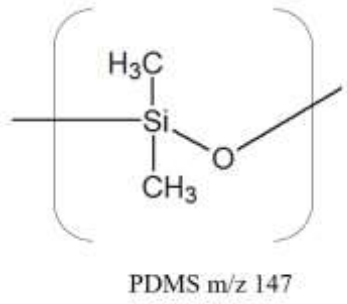

Figure 19. Mass fragment ion at m/z 147.

6.3.2 Characteristic fragments

The mass spectra of cholesterol TMS ether showed the molecular ion at m/z 458. The base ion at m/z 129 is characteristic for sterols (**Figure 20**). Cholesterol mass spectra also showed relatively intense peaks at m/z 353 [M^+ - TMS - CH_3] and m/z 255 [M^+ - SC - ROH - CH_3], owing to the cleavage of trimethylsilyl-, methyl-, hydroxyl group and the side chain (SC) (Goad & Akihisa, 1997; Teichmann *et al*., 2006).

The observed ions in mass spectra of cholesterol at m/z 329 and m/z 368 results from the fragmentation mechanism of free Δ^5 -sterols including losses of carbons from the sterol A- and B –rings by retro-Diels-Alder reaction (Knights, 1967) The retro-Diels-Alder Reaction occurs with intensive heat in a one-step reaction (Thevis & Schänzer, 2005). The Diels-Alder reaction takes a diene (two double bonds separated by one single bond in the cis conformation) and a dienophile (compound with an alkene) creating a six membered ring with one double bond. This leads to the breaking of three double bonds, and creates one new double bond and two single bonds (Pretsch *et al*., 2000).

Cholesterol, ergosterol, lanosterol and inotodiol showed relatively intense ions at m/z 129, which is typical for silylated Δ^5 -sterols but also other sterols (Teichmann *et al*., 2006). The ion comprises carbons 1-3 along with the trimethylsilanol (TMSiOH) group at the C3 (CH_3=CH-CH=O^+-TMS). However, the complementary fragment [M^+ - 129] is less commonly observed (Goad & Akihisa, 1997).

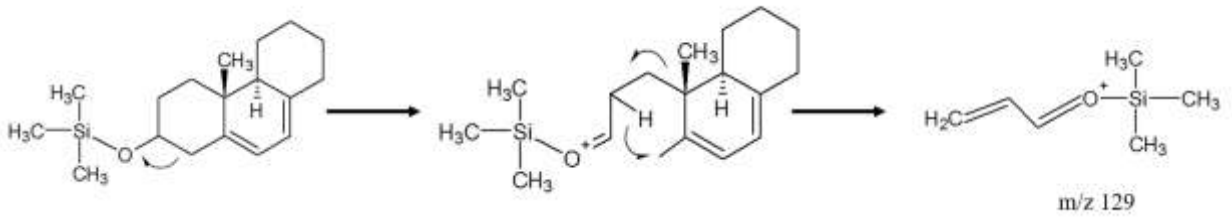

Figure 20. Formation of the m/z 129 ion by the loss of TMSi-group with C-1, C-2 and C-3 in the sterol A-ring (Brooks et al., 1968).

Ergosterol showed a base peak at m/z 363, resulting from the breakdown of TMSiOH and a methyl group from the C-ring. The observed fragmentation pattern of ergosterol corresponds to the previously published results regarding ergosterol mass fragmentation (Goad, 2013 & Honda *et al.*, 1996) The second most prevalent ion in ergosterol mass spectra is the A ring fragment shown in **Figure 21**, seeing an intense peak at m/z 337. The loss of carbon atoms 1 and 3 is substituted with a hydrogen from C-9, the mechanism also includes loss of 131 mass units from the molecular ion (Kenny & Welzel, 1995) Fragment ions at m/z 131 and 143 are characteristic fragment ion for $\Delta^{5,8}$ -sterols (Goad & Akihisa, 1997).

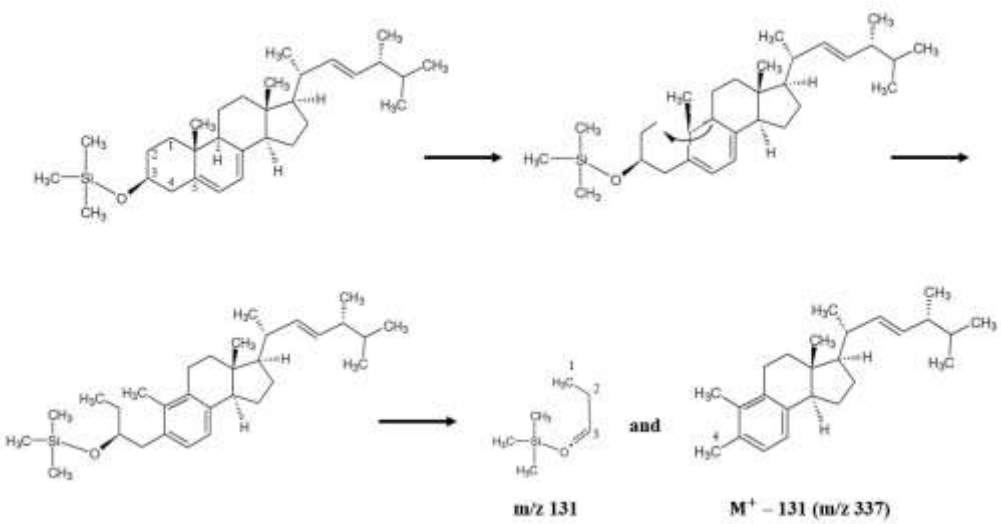

Figure 21. The fragmentation of the A ring of ergosterol m/z 337.

Lanosterol mass spectra typically give a strong molecular ion, which can also be the base peak. The molecular ion M^+ is present at m/z 498 with 31% relative abundancy. In this case, the base ion at m/z 393, this is produced by the cleavage of the hydroxyl group and a methyl group (Son *et al.*, 2014) The fragmentation ions at m/z 255 and 229 are typical for Δ^8 -sterols; these are shown in **Figure 22** (Goad & Akihisa, 1997).

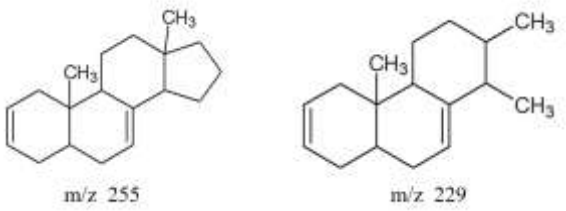

Figure 22. Mass fragment ions at m/z 255 and m/z 229.

The mass spectra of inotodiol and trametenolic acid showed an abundant ion at m/z 69, which is typical for the fragmentation of the $\Delta^{24(25)}$ -side chain (Goad & Akihisa, 1997) The base ion for inotodiol is present at m/z 337, which suspected to be derived from the A-ring fragment similar to ergosterol (**Figure 21**).

The mass spectra of betulin exhibit an intense peak at m/z 189, which is characteristic for the fragmentation of triterpenoid molecules with a lupane skeleton with a hydroxy group in position 3. Betulin, lupeol, betulinic acid and lupenone are considered as representative of triterpenes with a lupane structure (Modugno *et al.*, 2006) This ion arises from the fragmentation of the C ring system by cleavage of the 9–11 and 8–14 bonds followed by the loss of H_2O molecule. Another abundant fragment ion is at m/z 203, which is related to the retention of an additional methylene group from the C ring with respect to the fragment ion at m/z 189 (Prokes & Hlozek, 2007; Ahmad *et al.*, 2010).

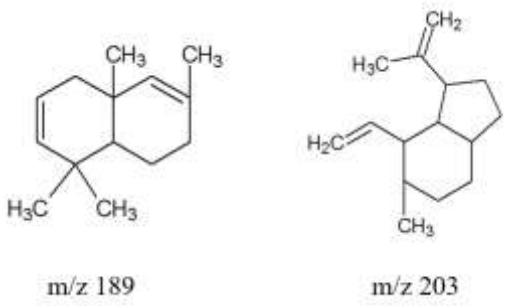

Figure 23. Structures of ions at m/z 189 and m/z 203 characteristic for betulin mass fragmentation.

The quantities of β-sitosterol and stigmastanol were the lowest, which also showed as lesser fragments making the identifying by GC-MS more challenging especially in the case of stigmastanol. The base peak for β-sitosterol derives from the loss of the loss of TMSi-group with C-1, C-2 and C-3 in the sterol A-ring shown in **Figure 20**. The second most abundant ion at m/z 357 derived from [M^+ - 129] with 72% abundancy, and the third most abundant ion is due to loss of TMSiO. The trimethylsiliconium ion at m/z 73 was also present with relatively high abundancy (51%).

The base peak of stigmastanol at m/z 69 is not due to the fragmentation of the $\Delta^{24(25)}$ -side chain as for lanosterol and trametenolic acid because stigmastanol does not have this double bond. Many low m/z ions can be seen (m/z 69) in sterol mass spectrum because of contaminants (Goad & Akihisa, 1997). The presence of stigmastanol was confirmed by comparison of retention time against the stigmastanol standard.

The observed fragmentation ions are generally in agreement with the previously published results concerning mass fragmentation of triterpenes and sterols (Kahlos and Hiltunen 1988-1989). The reference data (1) is represented in **Table 12**, where it is compared to the obtained fragmentation ions (2).

Table 12. GC-MS spectrometric data of the TMS ether of the main triterpenoids (Kahlos & Hiltunen 1988-1989)

Triterpenoids	**Fragment ions (m/z)**						
Lanosterol							
1	393(100)	69(78)	498(52,M+)	483(40)	109(32)	187(12)	227(11)
2	393(100)	69(98)	498(31,M+)	483(21)	109(38)	187(16)	227(14)
Inotodiol							
1	297(85)	517(73)	571(65)	427(57)	387(33)	337(10)	586(7,M+)
2	297(85)	517(31)	571(5)	427(17)	387(5)	337(100)	-
Trametenolic acid							
1	281(24)	187(24)	213(17)	585(7)	495(7)	405(3)	600(3,M+)
2	281(31)	187(21)	213(9)	585(31)	495(19)	-	600(13,M+)
Ergosterol							
1	363(100)	69(85)	337(58)	468(56,M+)	253(55)	131(48)	378(28)
2	363(100)	69(48)	337(65)	468(19,M+)	-	131(20)	378(19)
Cholesterol							
1	129(100)	229(44)	107(40)	121(36)	368(25)	353(15)	255(15)
2	129(100)	-	107(29)	-	368(71)	353(33)	255(17)

1. *Reference (Kahlos & Hiltunen 1988-1989)*
2. *Observed fragmentations (summarized based on GC-MS data)*

6.4 Calibration curves

The reagent compounds for all seven identified triterpenoids were not available. Inotodiol was quantified by using the betulin standard curve. Trametenolic acid, β-sitosterol and stigmastanol were quantified by using the ergosterol standard curve. Ergosterol and lanosterol were quantified with the equations showed in **Figures 24 – 25**. Each analysis contained a known concentration of analyte (different to draw a linear response) and constant amount of ISTD. The concentration of each triterpenoid standard is summarized in **Table 13**. The data for standard curves are shown

as ratio of analyte peak area and ISTD peak area ratio (A/A_{istd}). The concentration is similarly shown as ratio of analyte concentration and ISTD concentration (c/c_{istd}).

Table 13. The concentrations of the ergosterol, lanosterol and betulin standards (µg / mL).

Standard number	Ergosterol	Lanosterol	Betulin	Cholesterol (ISTD)
1 (blank)	0	0	0	3,79
2	1,01	3,13	5,39	3,79
3	2,01	6,25	10,77	3,79
4	3,02	9,38	16,16	3,79
5	4,03	12,50	21,54	3,79
6	5,03	15,63	26,93	3,79
7	6,04	18,75	32,32	3,79
8	7,04	-	37,70	3,79
9	8,05	-	43,09	3,79
10	9,06	-	48,47	3,79

6.5 Standards

Table 14. Ergosterol standard curve data.

Standard number	A/A_{istd}	SD-% (area ratio)	c/c_{istd}
1 (blank)	0.00	-	0.00
2	0.18	2.40 %	0.27
3	0.41	2.57 %	0.53
4	0.63	0.60 %	0.80
5	0.83	2.31 %	1.06
6	1.07	5.40 %	1.33
7	1.25	1.56 %	1.59
8	1.48	0.05 %	1.86
9	1.74	2.60 %	2.12
10	2.26	6.01 %	2.66

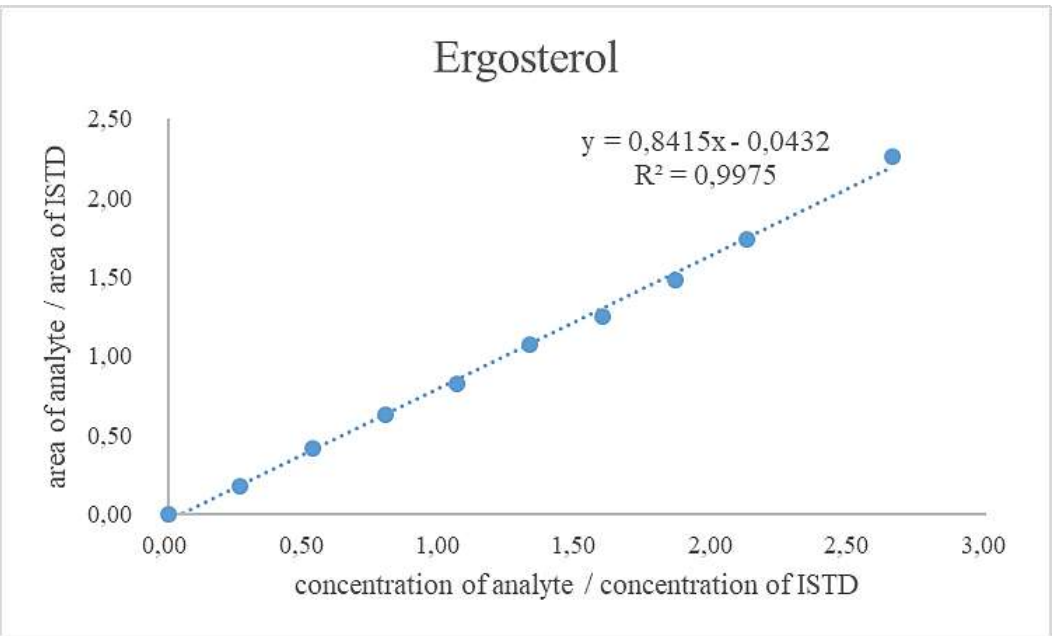

Figure 24. Ergosterol standard curve. The ratio of the area of analytes to the area of ISTD are plotted on the x-axis. The ratio of the concentration of analytes to the concentration of ISTD is plotted on the y-axis.

Table 15. Lanosterol standard curve data.

Standard number	**A/A_{istd}**	**SD-% (area ratio)**	**c/c_{istd}**
1 (blank)	0,00	0,00 %	0,00
2	0,44	6,97 %	0,82
3	0,92	2,96 %	1,65
4	1,48	7,36 %	2,47
5	1,83	5,00 %	3,30
6	2,28	7,44 %	4,12
7	2,64	3,48 %	4,95

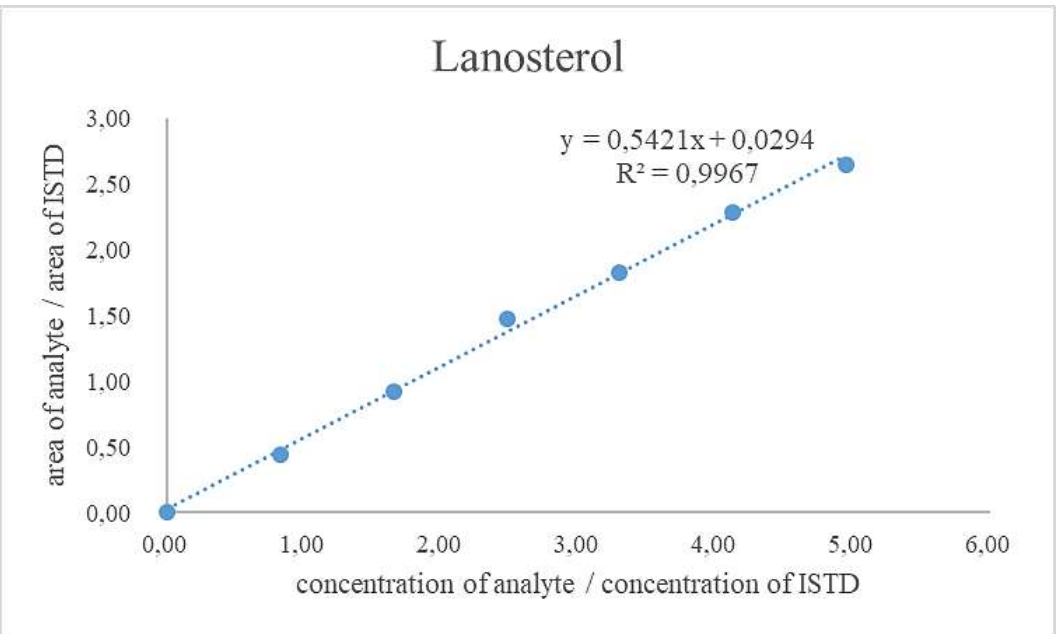

Figure 25. Lanosterol standard curve. The ratio of the area of analytes to the area of ISTD are plotted on the x-axis. The ratio of the concentration of analytes to the concentration of ISTD is plotted on the y-axis.

Table 16. Betulin standard curve data.

Standard number	A/A_{istd}	SD-% (area ratio)	c/c_{istd}
1 (blank)	0,00	0,00 %	0,00
2	1,32	4,98 %	1,42
3	2,52	2,24 %	2,84
4	3,91	3,10 %	4,26
5	5,23	2,74 %	5,38
6	6,62	6,15 %	6,37
7	9,23	0,48 %	9,95
8	10,92	0,41 %	11,37
9	12,48	2,49 %	12,79
10	13,99	3,16 %	14,33

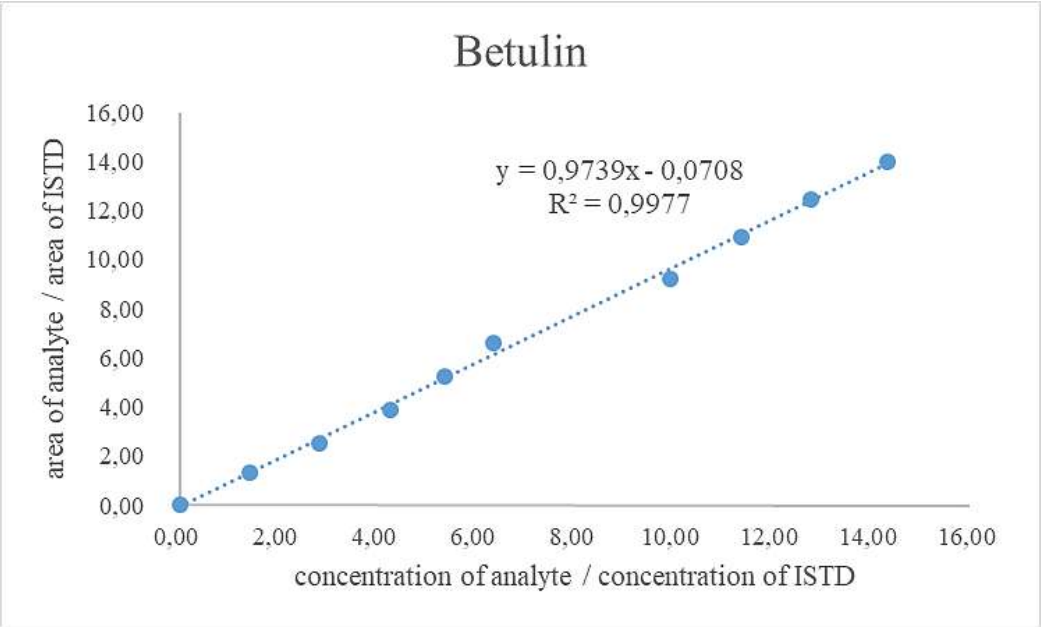

Figure 26. Betulin standard curve. The ratio of the area of analytes to the area of ISTD are plotted on the x-axis. The ratio of the concentration of analytes to the concentration of ISTD is plotted on the y-axis.

The linearity of the standard curves were relatively good within the applied region of the results (R^2= 0.9967-0.9975).

6.6 Folch extraction result

Table 17 Quantitative results by Folch extraction (mg/100g dry weight) for identified triterpenoids comprising the ergosterol (2), lanosterol (3), β-sitosterol (4), stigmastanol (5), inotodiol (6), trametenolic acid (7) and betulin (8) in Chaga.

	2	**3**	**4**	**5**	**6**	**8**	**7**
Folch extraction	36.74 ± 3.8 %	74.11 ± 1.2 %	5.02 ± 6.9 %	1.76 ± 8.7 %	127.16 ± 2.4 %	34.72 ± 10.2 %	44.84 ± 2.1 %

The Folch extracts showed high amounts in inotodiol, which comprised nearly 40 % of the total triterpenoid content. Relatively high quantities of lanosterol was also found, followed by trametenolic acid, ergosterol and betulin. β-sitosterol and stigmastanol were found in significantly lower quantities compared to the other triterpenoids comprising less than 2 % of the total triterpenoid content in Folch extracts.

6.7 SFE and ethanol results

Table 18. Quantitative results by SFE and ethanol extraction (mg/100g dry weight) for identified triterpenoids comprising the ergosterol (2), lanosterol (3), β-sitosterol (4), stigmastanol (5), inotodiol (6), trametenolic acid (7) and betulin (8) in Chaga.

	2	**3**	**4**	**5**	**6**	**8**	**7**
40 °C, 281 bar (no restrictor)	20.01 ± 1.1 %	74.32 ± 0.3 %	3.41 ± 2.0 %	1.4 ± 8.7 %	101.53 ± 0.5 %	14.62 ± 5.7 %	0
40°C, 281 bar (15 minutes)	17.91 ± 5.3 %	78.27 ± 0.6 %	4.43 ± 4.9 %	1.42 ± 6.9 %	104.84 ± 0.7 %	11.03 ± 6.6 %	0
40 °C, 281 bar (50 mL)	16.74 ± 2.6 %	59.87 ± 0.5 %	3.47 ± 4.8 %	1.33 ± 7.5 %	91.35 ± 0.3 %	12.12 ± 7.0 %	0
50 °C, 350 bar (10+10min)	18.5 ± 1.9 %	58.98 ± 0.3 %	3.58 ± 5.8 %	1.34 ± 5.6 %	101.09 ± 0.3 %	15.03 ± 13.8 %	0
50, °C, 350 bar (50 mL)	17.19 ± 0.9 %	62.66 ± 0.4 %	3.69 ± 3.5 %	1.45 ± 1.7 %	99.64 ± 1.6 %	14.59 ± 3.6 %	0
40 °C, 350 bar (50 mL)	17.05 ± 3.5 %	58.83 ± 0.5 %	3.19 ± 6.6 %	1.25 ± 4.6 %	87.69 ± 0.7 %	11.8 ± 5.8 %	0
Ethanol extract	19.28 ± 1.3 %	62.01 ± 1.3 %	4.13 ± 1.2 %	1.73 ± 11.7 %	107.01 ± 1.0 %	27.72 ± 12.4 %	28.44 ± 3.2 %

Inotodiol was found to be the most abundant triterpenoid also in SFE extracts. Similar to Folch results, the lanosterol concentration was the second highest also in SFE extracts. The significant difference in Folch and SFE results was the trametenolic acid, which could not be extracted by SFE.

The standard deviation of betulin was relatively high in both methods. The betulin peak showed a co-eluting peak (**Figure 27**), which could cause errors in peak area determination explaining the high standard deviation. Stigmastanol also showed high standard deviation in the results, which is most likely due to the low concentration.

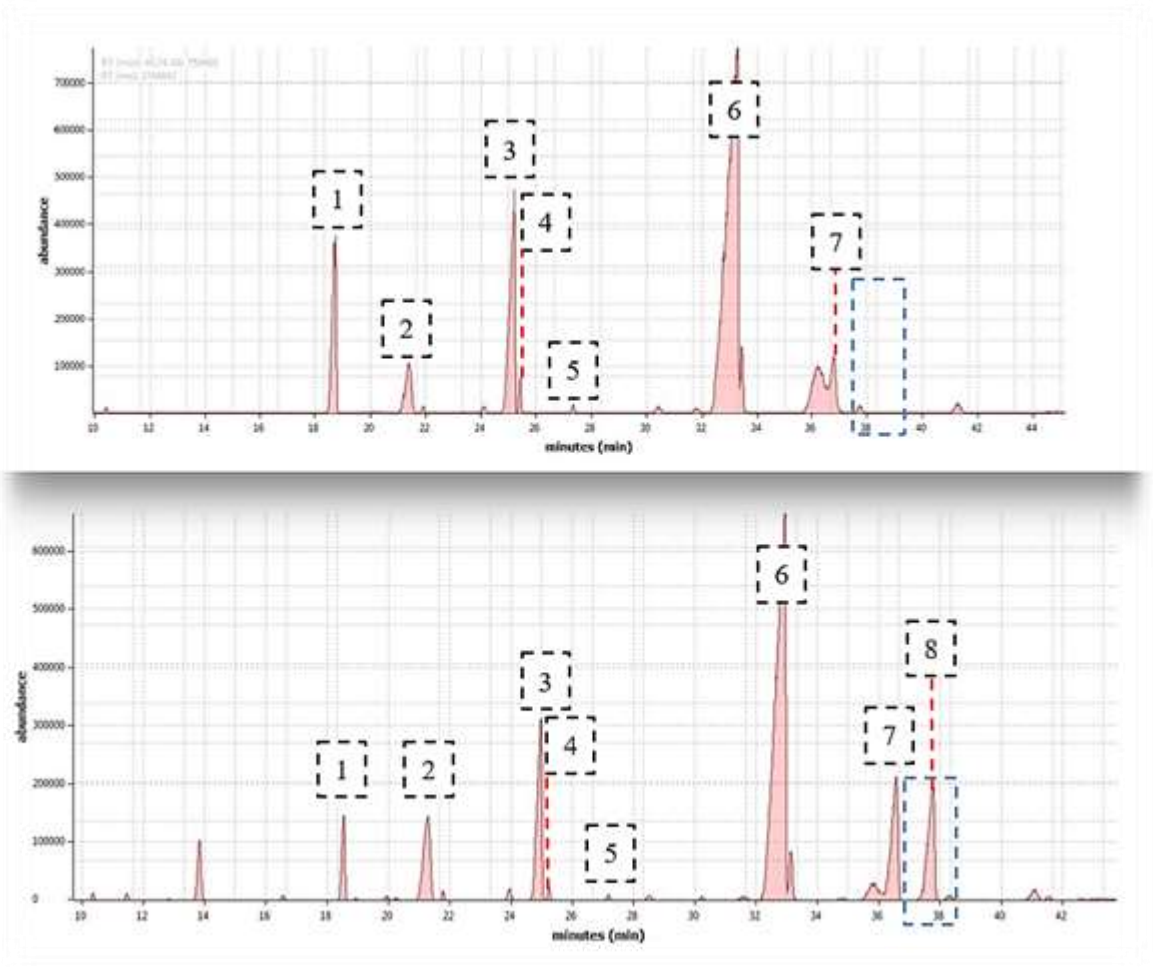

Figure 27. Comparison between chromatograms of GC-FID obtained by Folch method (lower) and SFE (upper).

Table 19. Peaks identified and numbered for Figure 24.

Numbering	Triterpenoid
1	Cholesterol (ISTD)
2	Ergosterol
3	Lanosterol
4	β-sitosterol
5	Stigmastanol
6	Inotodiol
7	Betulin
8	Trametenolic acid

6.8 Compositional profile

Table 20 summarizes the seven identified and quantified sterol and triterpenoid constituents from SF extracts: ergosterol (17.9±1.3 mg / 100 g dry weight), lanosterol (65.5±7.2 mg / 100 g), β-sitosterol (3.6±0.5 mg / 100 g), stigmastanol (1.4±0.1 mg / 100 g), betulin (13.2±4.3 mg / 100 g) and inotodiol (97.7±11.5 mg / 100 g). Folch extracts produced higher yield in ergosterol (36.7±1.5 mg / 100 g), betulin (34.7±4.2 mg / 100 g) and inotodiol (127.7±5.5 mg / 100 g) but lanosterol (74.1±1.0 mg / 100 g), β-sitosterol (5.0±0.4 mg / 100 g), stigmastanol (1.8±0.2 mg / 100 g) levels were similar or slightly higher compared to SF extracts. Relatively high amounts of trametenolic acid (44.8±1.0 mg / 100 g) was also found from Folch extracts but not in SF extracts, possibly due to the difference in solvent polarity. The quantification results with different extraction parameters and methods are presented in **Table 20**.

Table 20. Quantitative results (mg/100g dry weight of Chaga) for identified triterpenoids comprising the ergosterol (2), lanosterol (3), β-sitosterol (4), stigmastanol (5), inotodiol (6), trametenolic acid (7) and betulin (8) in Chaga.

	2	**3**	**4**	**5**	**6**	**8**	**7**
40 °C, 281 bar (no restrictor)	20.01 ± 1.1 %	74.32 ± 0.3 %	3.41 ± 2.0 %	1.4 ± 8.7 %	101.53 ± 0.5 %	14.62 ± 5.7 %	0
40 °C, 281 bar (15 minutes)	17.91 ± 5.3 %	78.27 ± 0.6 %	4.43 ± 4.9 %	1.42 ± 6.9 %	104.84 ± 0.7 %	11.03 ± 6.6 %	0
40 °C, 281 bar (50 mL)	16.74 ± 2.6 %	59.87 ± 0.5 %	3.47 ± 4.8 %	1.33 ± 7.5 %	91.35 ± 0.3 %	12.12 ± 7.0 %	0
50 °C, 350 bar (10+10min)	18.5 ± 1.9 %	58.98 ± 0.3 %	3.58 ± 5.8 %	1.34 ± 5.6 %	101.09 ± 0.3 %	15.03 ± 13.8 %	0
50, °C, 350 bar (50 mL)	17.19 ± 0.9 %	62.66 ± 0.4 %	3.69 ± 3.5 %	1.45 ± 1.7 %	99.64 ± 1.6 %	14.59 ± 3.6 %	0
40 °C, 350 bar (50 mL)	17.05 ± 3.5 %	58.83 ± 0.5 %	3.19 ± 6.6 %	1.25 ± 4.6 %	87.69 ± 0.7 %	11.8 ± 5.8 %	0
Folch extract	36.74 ± 3.8 %	74.11 ± 1.2 %	5.02 ± 6.9 %	1.76 ± 8.7 %	127.16 ± 2.4 %	34.72 ± 10.2 %	44.84 ± 2.1 %
Ethanol extract	19.28 ± 1.3 %	62.01 ± 1.3 %	4.13 ± 1.2 %	1.73 ± 11.7 %	107.01 ± 1.0 %	27.72 ± 12.4 %	28.44 ± 3.2 %

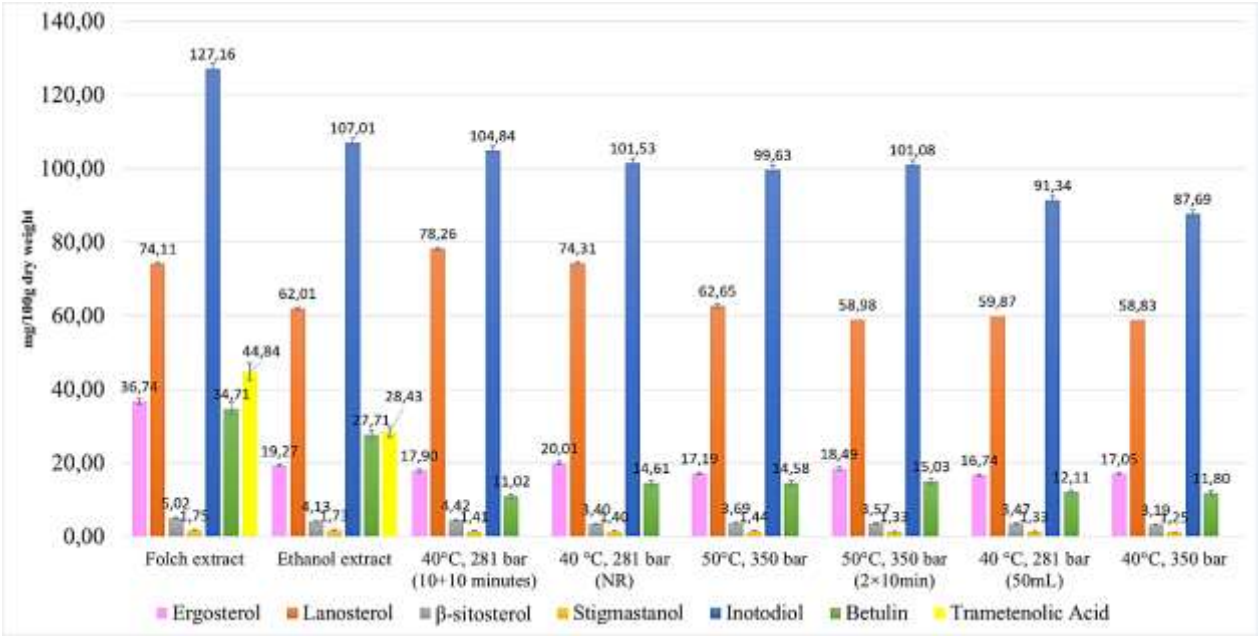

Figure 28. Summarized triterpenoid content in I. obliquus obtained by different extraction methods.

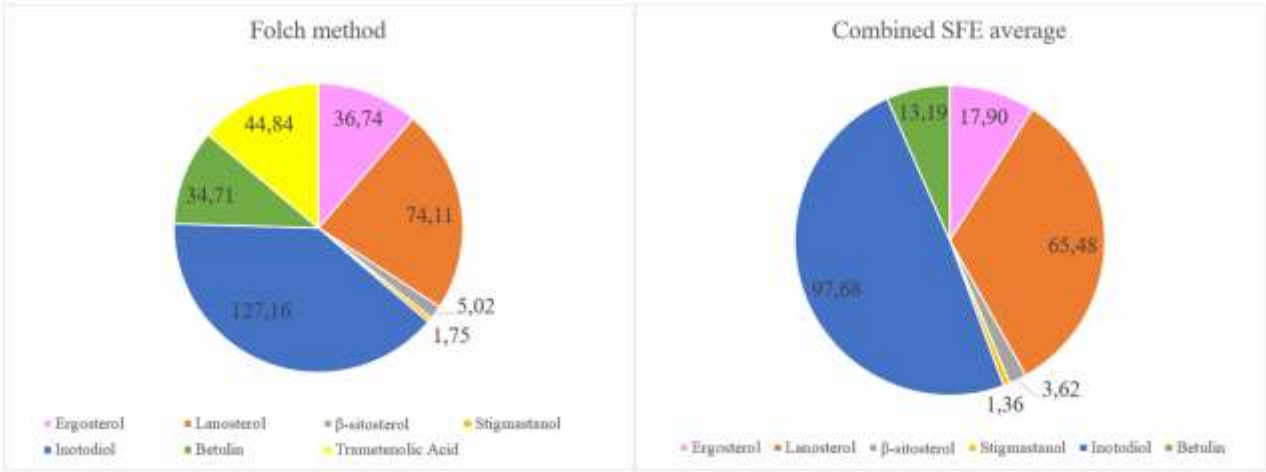

Figure 29. Compositional profile of the obtained triterpenoids (mg / 100g dry weight) by Folch method and SFE average.

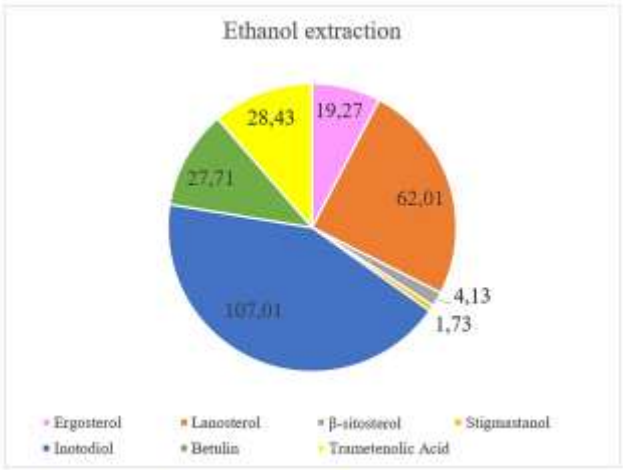

Figure 30. Compositional profile of the obtained sterols (mg / 100g) by ethanol extraction.

The results obtained by Folch method, combined SFE and ethanol extraction are presented in **Figures 29 – 30**. The representative portions of each triterpenoid in different extraction methods

are relatively similar except for trametenolic acid, which was not present in SFE samples. The values of triterpenoids in Chaga mostly followed the same order: inotodiol, lanosterol, ergosterol, betulin (higher than ergosterol by ethanol extraction), β-sitosterol and stigmastanol. Inotodiol comprises nearly 50 % of the total triterpenoid content in all methods. The similar triterpenoid profiles could signify that SFE can be utilized for triterpenoid extraction from Chaga in laboratory scale nearly as well as conventional methods.

6.8.1 Comparison of means

Among the eight different extraction conditions investigated, the highest total triterpenoid content was expectedly obtained by Folch extraction. As observed in **Figures 31-37**, the difference was statistically significant between Folch method and SFE, except for lanosterol extracted at 40 °C, with 281 bar and no restrictor (40.281 NR) (**Figure 32**). Ethanol extraction and SFE showed statistical equivalence in all quantified sterols except for stigmastanol, which was only comparable to Folch method ($p < 0.05$). Data are expressed as means ± standard deviation. The different letters (a-e) indicates a significant difference between quantified triterpenoids by one-way Anova followed by T-test ($p < 0.05$). The SFE conditions are marked in the figures as temperature and pressure combinations. The "T" is assigned to the extractions that were limited by time and the others were limited by the solvent volume.

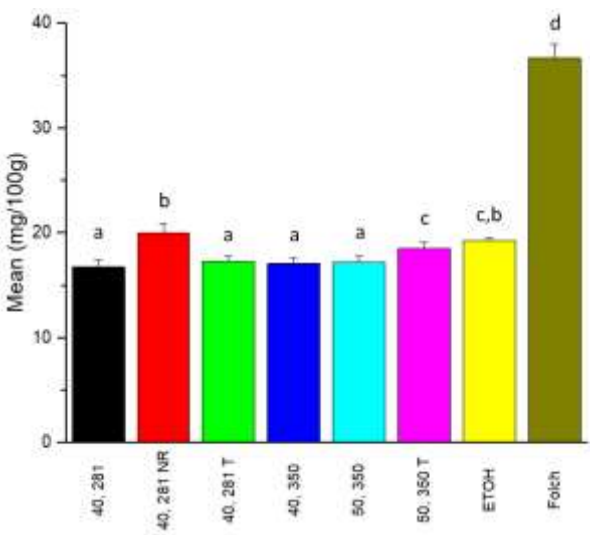

Figure 31. The obtained ergosterol quantities by different extraction conditions. The different letters within this figure indicate that means are significantly different ($p < 0.05$).

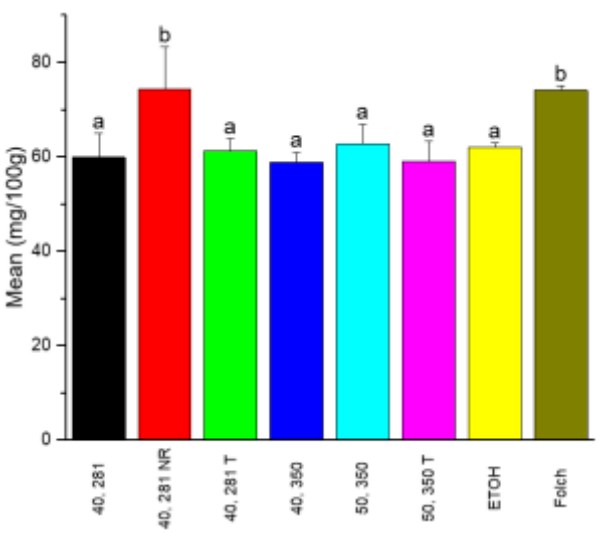

Figure 32. The obtained lanosterol quantities by different extraction conditions. The different letters within this figure indicate that means are significantly different ($p < 0.05$).

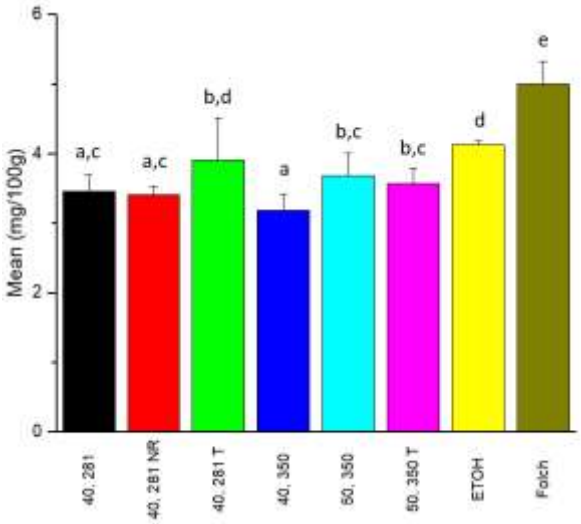

Figure 33. The obtained β-sitosterol quantities by different extraction conditions. The different letters within this figure indicate that means are significantly different ($p < 0.05$).

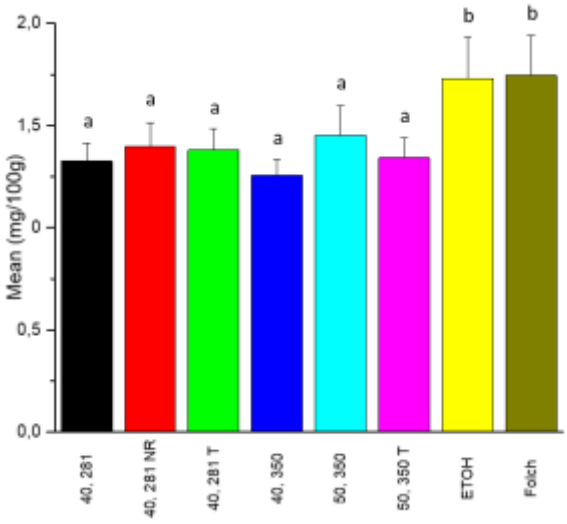

Figure 34. The obtained stigmastanol quantities by different extraction conditions. The different letters within this figure indicate that means are significantly different ($p < 0.05$).

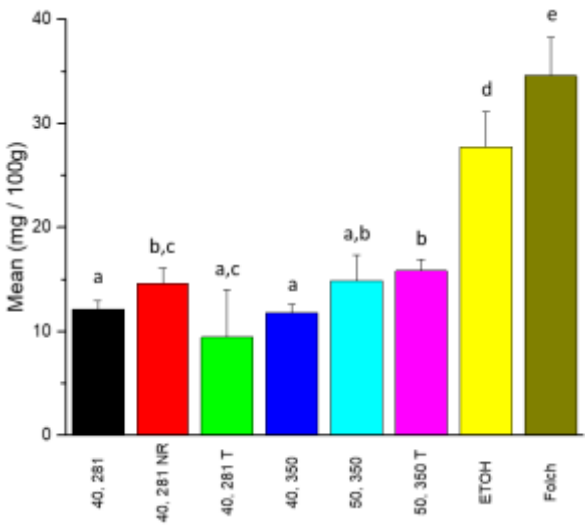

Figure 35. The obtained betulin quantities by different extraction conditions. The different letters within this figure indicate that means are significantly different ($p < 0.05$).

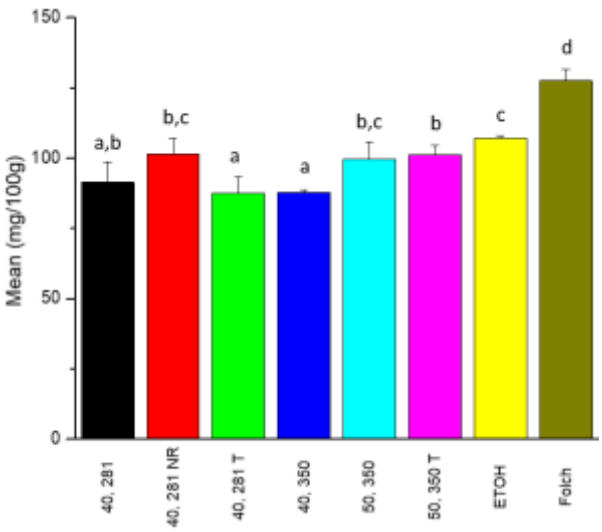

Figure 36. The obtained inotodiol quantities by different extraction conditions. The different letters within this figure indicate that means are significantly different ($p < 0.05$).

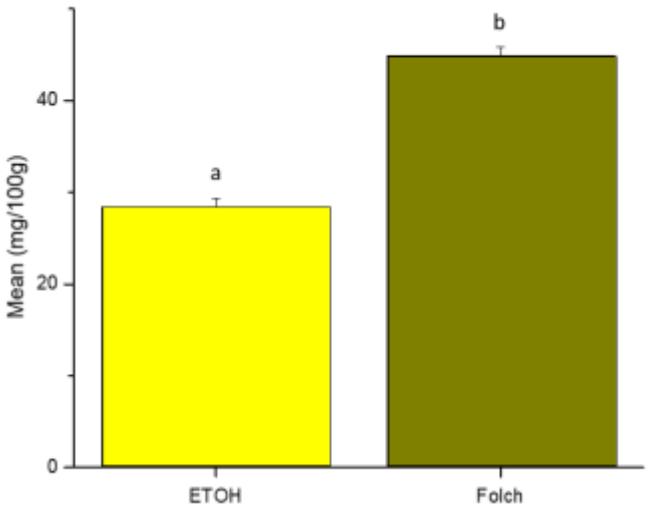

Figure 37. The obtained trametenolic acid quantities by Folch and ethanol extraction. The different letters within this figure indicate that means are significantly different ($p < 0.05$).

Folch extraction yielded the highest amount of inotodiol (7.33-58.85 mg/100 g higher than SFE, $p < 0.05$), lanosterol (5.94-27.33 mg/100 g higher, $p < 0.05$), ergosterol (1.89-24.57 mg/100 g higher, $p < 0.05$), betulin (1.81-33.7mg/100 g higher, $p < 0.05$), β-sitosterol (0.16-2.63 mg/100 g higher, $p < 0.05$), and stigmastanol (0.13-0.79 mg/100 g higher, $p < 0.05$). Folch method also yielded higher amount of trametenolic acid compared to ethanol extraction (19.36-21.07 mg/100 g higher, $p < 0.05$). Clear differences were seen in the contents of ergosterol and betulin in Folch samples, which were significantly higher than the SFE samples.

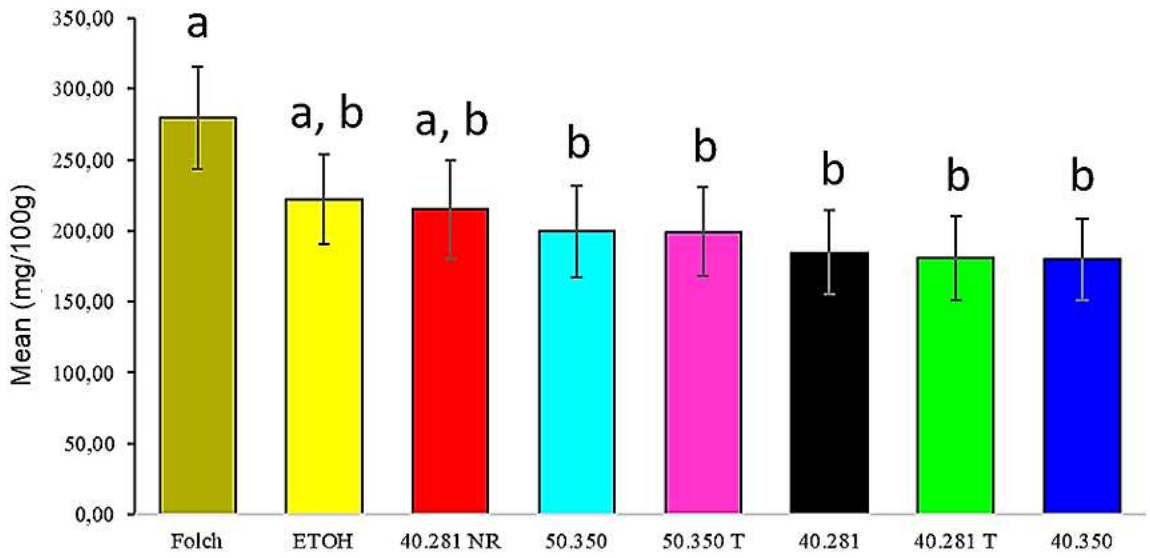

Figure 38. Comparison of total triterpenoid quantity in different extraction conditions. Sum of inotodiol, lanosterol, ergosterol, betulin, β-sitosterol and stigmastanol. The different letters within this figure indicate that means are significantly different ($p < 0.05$).

Trametenolic acid has been excluded from the sum of sterols in **Figure 38** in order to get equal comparison. As can be observed, the relative composition of triterpenoids by different SFE conditions also varied to some extent. The highest total sterol quantity by SFE was obtained by 40 °C degrees and 281 bar resulting 898.45 kgm^{-3} fluid density and without restrictor (NR). However, extractions without the restrictor made the fluid flow less stable affecting the flowrate and occasional clogging. The second highest total sterol quantity was obtained by using 50 °C degrees and 350 bar (899.40 kgm^{-3}), there was no significant difference between the time (15 minutes) and volume (50 ml) limited extraction. Whereas the numerically lowest total sterol content was obtained with the highest density, 934.90 kgm^{-3} (40 °C and 350 bar). However, the differences in results between different SFE conditions and ethanol extraction were not statistically significant ($p = 0.05$).

An increasing trend in sterol concentration was detected when the temperature increases and fluid density decreases. These findings indicate that the elevation of vapor pressure (due to higher temperature) increase the solvating effect despite the lower density of fluid. This observation could be verified by extending amount of replicate extractions or another extraction condition with higher temperature e.g. 55 °C and 385 bar (899.19 kgm^{-3}), which should result higher yield.

The PCA biplot of the extraction conditions and the samples is shown in **Figures 39 - 40**. The PCA was conducted to give an overview on the compositional response of triterpenoid compositions to different extraction methods and SFE parameters. As can be observed in **Figure 39**, the biplot in planes Dim1-Dim2 is heavily influenced by trametenolic acid, which was not obtained by SFE. Trametenolic was removed from the biplot in **Figure 40** in order to observe the correlation of more comparable components.

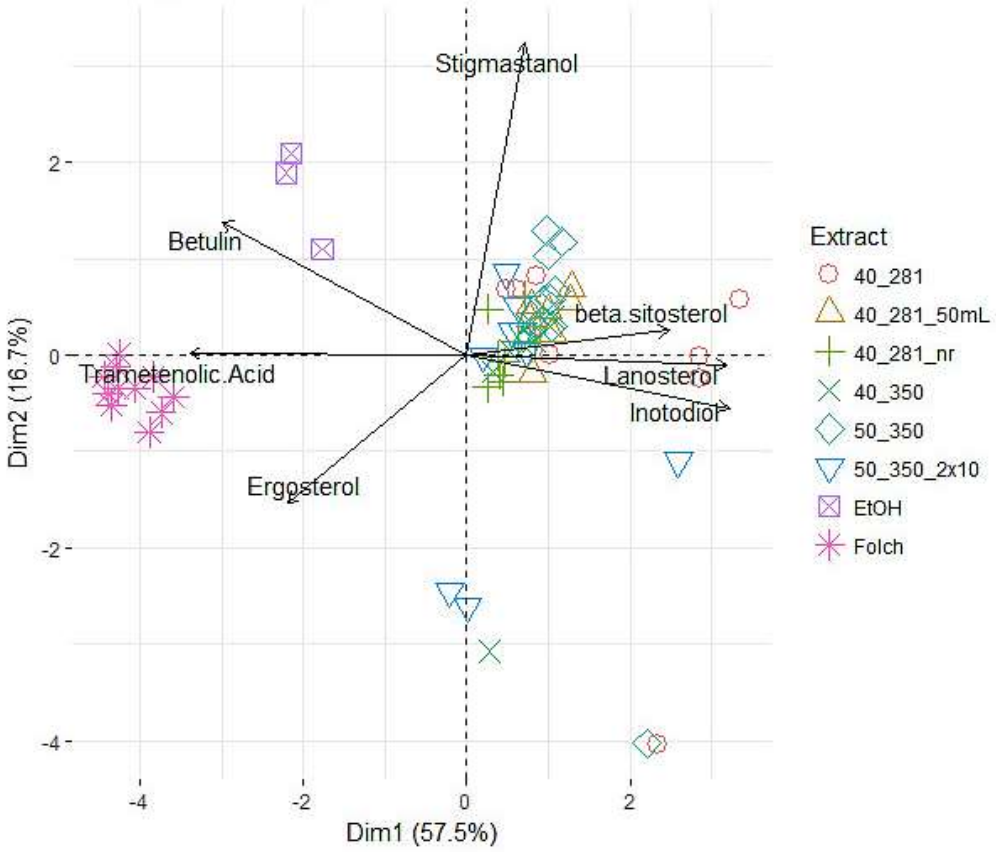

Figure 39. PCA biplot of compositional response of total triterpenoids in Chaga extracts to different extraction methods and SFE parameters.

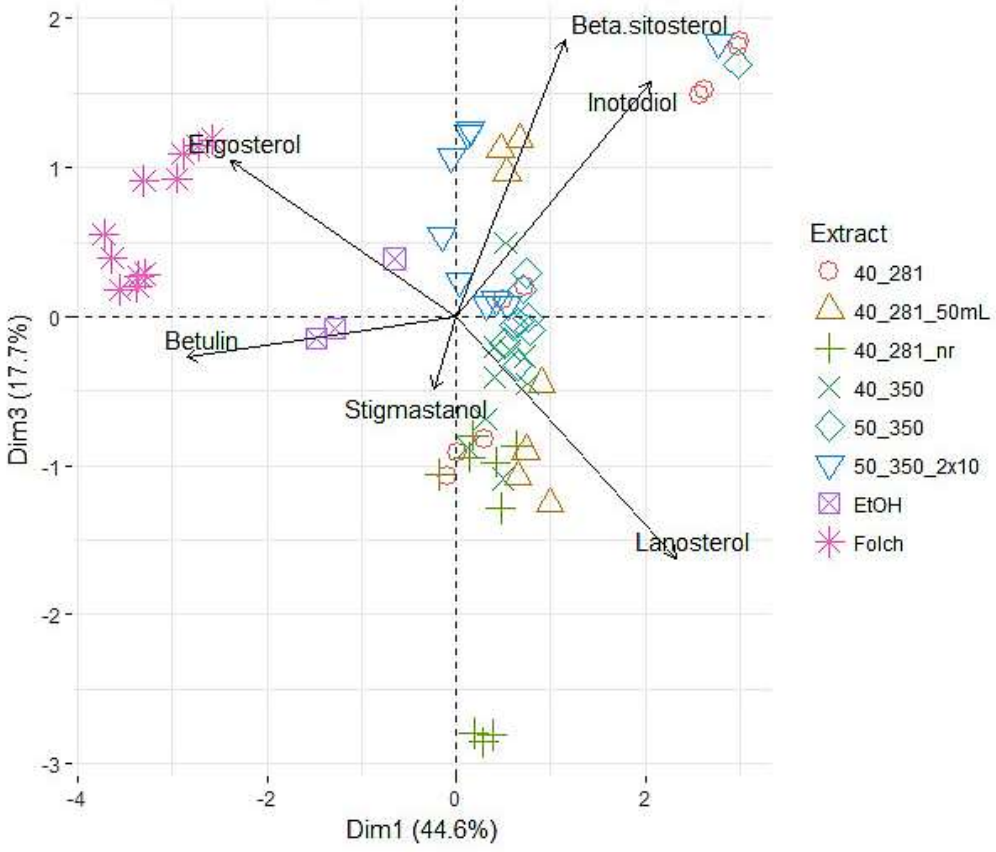

Figure 40. The fixed PCA biplot (without trametenolic) of compositional response of triterpenoids in Chaga extracts to different extraction methods and SFE parameters.

The biplot in planes Dim1-Dim3 explained 62.3 % of the variance of the data. Majority of the SFE samples were explained by Dim1 as having relatively high contents of inotodiol, lanosterol and β-sitosterol. The separation of these components indicates that inotodiol, lanosterol and β-sitosterol are more feasible to extract by SFE. In contrast, components obtained by SFE showed low contents of betulin and ergosterol. High quantities of ergosterol and betulin was found in Folch method, relatively good results were also obtained by ethanol extraction for these components.

The stabilizing effect of the restrictor can be observed in the presented biplots. The samples by the extractions without restrictor (40_281_nr) are more scattered and showed little correlation to any major components, most likely due to an unstable solvent flow.

7 Discussion

The increasing usage of vegetable oils and extracts in food, cosmetic, and pharmaceutical industries have made the extraction of oils by using SC-CO_2 an attractive alternative compared to conventional processes. Several large-scale (industrial) SFE units are operated worldwide and are economically competitive compared to conventional extractions. Industrial SFE processes are mainly used for the extraction of solid natural materials, for food ingredients and phytopharmaceuticals (Sovova & Stateva, 2011). Extracts by SFE from different plants have also shown higher antioxidant activity than extracts obtained by using conventional solvent extraction with organic solvents. The reason behind, is most likely due to a difference in composition deriving from the extraction conditions applied (Daukšas *et al*., 2001). SFE has been long introduced as a greener alternative for conventional extraction methods. Combined with the long history of use of Chaga, this work presented an interesting intersection of a traditional folk remedy and modern scientific research. There are numerous studies regarding the role of *I. obliquus* in cancer treatment and easing symptoms of various diseases. The triterpenoids found in Chaga have an important role in prevention of oxidative cell damage and antitumor, cytotoxic and hypocholesterolemic activity. In this study, seven major triterpenoids including betulin, ergosterol, inotodiol, lanosterol, stigmastanol, trametenolic acid, and β-sitosterol, have been extracted and quantified from the Chaga extracts. These findings are in agreement with the previously published results regarding triterpenoids extracted from *I. obliquus* (Wang *et al*., 2014; Glamočlija *et al*., 2015). The literature study briefly summarizes the current understanding of biological activity of mushroom *I. obliquus* for food and drug applications. This is the first reported work on SFE of sterols from Chaga is being compared quantitatively to a conventional extraction method.

Quantification and full characterization of SF extracts have seen great advances in the last decades. In addition, diverse statistical tools have been developed for the optimization of the operating conditions. The available extraction data have been actively used to improve mathematical models in order to evaluate the economic feasibility of the SFE process. However, the new patents and SFE works have been mainly focused on the extracts production and their subsequent characterization. Important weaknesses regarding studies on the SFE of natural samples are still found. For example, the solute-matrix interactions are not yet fully understood and require more profound research and in order to correctly taken into account by reliable predictive models (Reverchon, 1997). In addition, extensive studies regarding the scale-up is still needed, in order to stimulate a wider implementation of this green technology. The relatively large capital costs linked to high-pressure operation and struggles with the cost-effectiveness for

low volume products hinder the interest from the industrial point of view. To the date, difficulties are inevitable when transferring a validated and optimized method from one extractor to another. There is not enough knowledge on how to overcome scale-up, analyte-matrix interactions issues, and which are the most appropriate modifiers for specific compounds and matrices (de Melo *et al.*, 2014).

Sample preparation and pre-treatment steps are typically one of the most time consuming and laborious steps of the analytical extraction processes, especially when solid samples are involved. Similar to conventional extraction methods, the sample preparation steps in SFE are crucial and must not be neglected. For lipid extraction, the alkaline hydrolysis or acid pre-treatment could be required for materials with strong interactions between the lipids and sample matrix in order to release the conjugated forms of lipids (Anklam *et al*., 1998) For example, pre-treatment by hot water has shown to increase the yield for some lipid compounds in SFE (Rizhikovs *et al*., 2015). The only applied sample pre-treatment in this work was milling, which is a relatively simple and fast process.

8 Conclusions

Conventional extraction procedures, especially in the laboratory scale, are time consuming and produce solvent waste. SFE offered clear advantages in different steps of the extraction including shorter extraction time and no additional cleaning or solvent removal steps were required. Typically, conventional liquid extractions require multiple steps and concentration of the final product, which can result in loss or degradation of the target analytes. SFE process can be, on the other hand, nearly fully automated in both laboratory and industrial scale.

In this work, it was found that the total extraction time of a single Folch sample compared to SFE was nearly four times longer. The triterpenoid yield obtained by Folch method was higher compared to SFE ($p < 0.05$). In addition, trametenolic acid was found only in Folch and ethanol extracts most likely due to higher solvent polarities. Folch method yielded statistically the highest concentration in each extracted triterpenoid, including betulin, ergosterol, inotodiol, lanosterol, stigmastanol, trametenolic acid, and β-sitosterol. The statistical evaluation also showed that inotodiol, lanosterol and β-sitosterol are more feasible to extract by SFE. Meanwhile, components obtained by SFE showed low contents of betulin and ergosterol.

The SFE temperatures applied in this work were between 40-50 °C, close to the most studied temperature region for the SFE of natural matrices (de Melo *et al*., 2014). At lower temperatures, there is a lower risk of thermal degradation of vulnerable compounds, which is expected to enhance the extract quality. The choice of extensively tested extraction parameters is the most practical approach at the starting point in order to test the extraction system. The chosen temperature and pressure combinations resulted fluid densities that were relatively similar and the obtained triterpenoid concentrations by SFE were similar. There was no statistically significant difference in the total triterpenoid content among the six applied SFE conditions, which was partly expected based on the differences. The obtained results indicate that SFE of Chaga at the used temperature-pressure combinations can provide reproducible results in triterpenoids extraction with relatively time- and cost-effective results in the laboratory scale. It must also be kept in mind that the SF extractions in this work were carried out without any cosolvents, which could have enhanced the extraction efficiency. The main target was to test SFE at its purest (greenest) form and compare it to conventional methods, which is why cosolvents were not deployed.

The ergosterol concentration obtained by Folch method in this work is similar with the results reported by Zheng *et al*., who extracted the mycelium of wild Chaga (Zheng *et al*., 2010). Yusoo *et al*. also extracted Chaga mycelium and obtained higher concentration of ergosterol (Yusoo *et al*., 2001). The average ergosterol concentration obtained by SFE in this work was nearly twice

lower compared to the previously published results. A significant difference was found in the inotodiol concentration, which was found to be higher in this work. Du *et al*., reported concentrations of 13.0 mg/100 g of inotodiol in Chaga (Du *et al*., 2011). Meanwhile, SFE extract contained 97.68 mg/100 g of inotodiol and Folch extracts contained 127.16 mg/100 of inotodiol, comprising nearly 40 % of the total sterol content. Zheng *et al.* found the inotodiol content to be 25.4 % of the total triterpenoid content (Zheng *et al*., 2010). The growth and storage conditions are known to have a direct impact on the composition of bioactive compounds. In addition, the compositional profile is most likely to be different depending on the sampling.

Despite the current challenges including lower efficiency and instrument costs, it is clear that many crucial SFE equipment related technical restrictions have already been overcome over the years, thanks to new technological advantages. For example, the new SFE extraction systems in both laboratory and industrial scale have shown to be more rigid by design and produce precise results with high efficiency. The cost of SFE apparatuses and associated maintenance work have also decreased significantly due to designs that are more durable. The sustained development of technology and growing understanding of supercritical fluids are expected to improve SFE technology to reach efficiencies similar to conventional extraction methods (Gil-Ramírez *et al*, 2013).

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Appendix

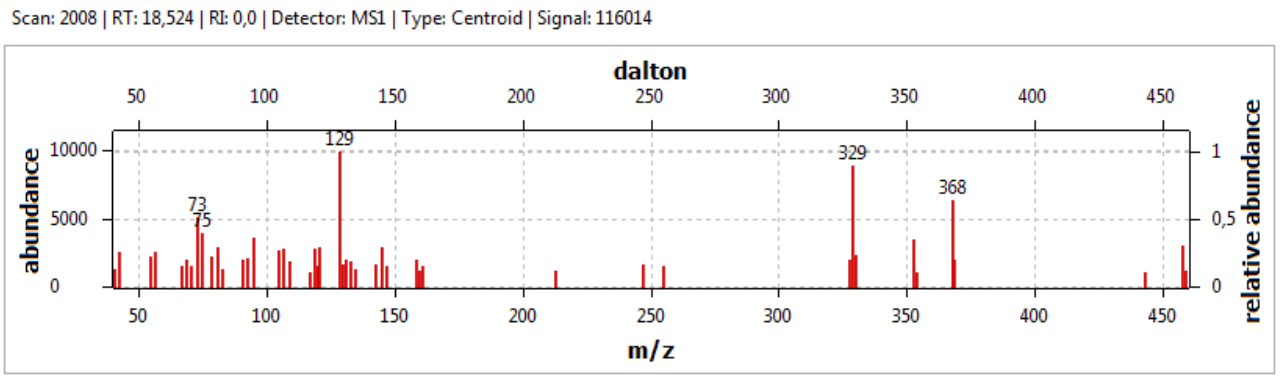

Figure 41. Obtained mass spectrum for cholesterol (OpenChrom).

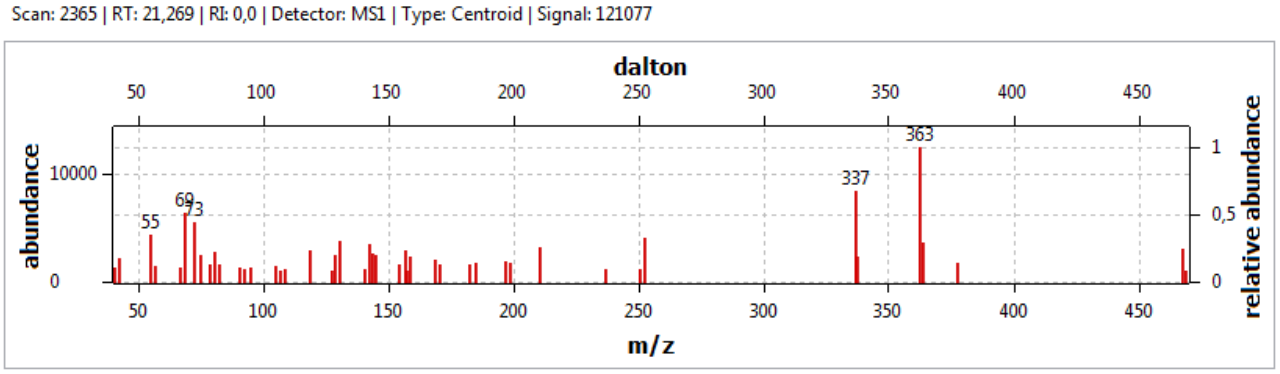

Figure 42. Obtained mass spectrum for ergosterol (OpenChrom).

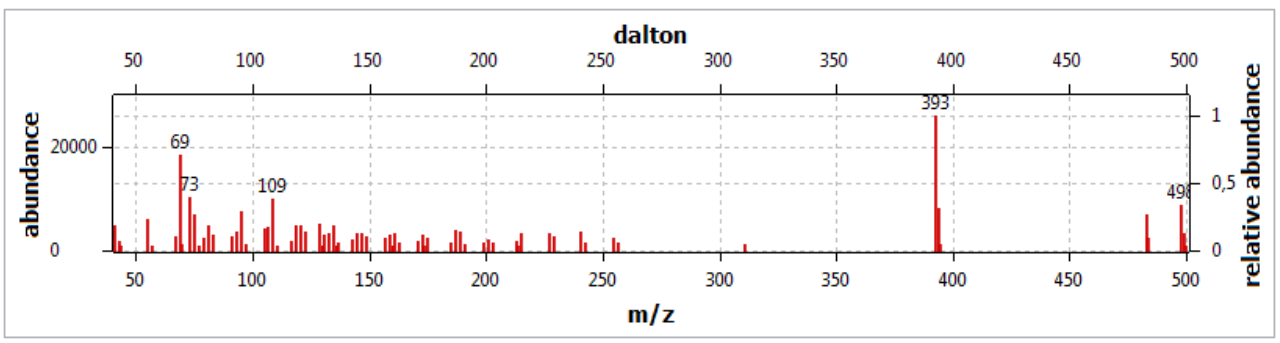

Figure 43. Obtained mass spectrum for lanosterol (OpenChrom).

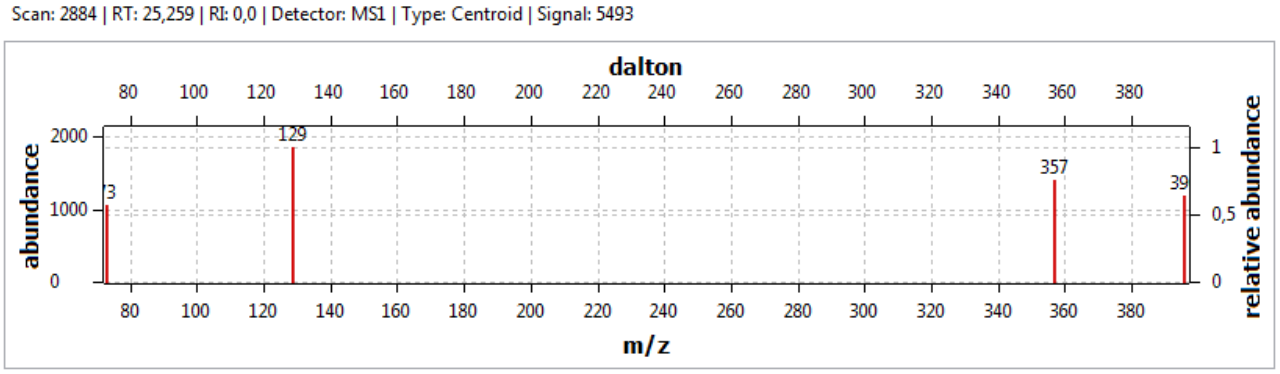

Figure 44. Obtained mass spectrum for β-sitosterol (OpenChrom).

Stigmastanol:

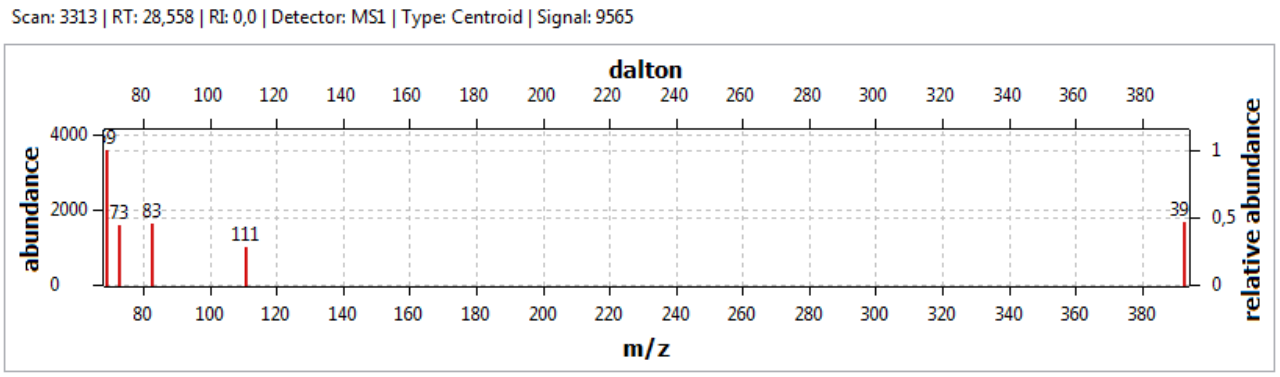

Figure 45. Obtained mass spectrum for stigmastanol (OpenChrom).

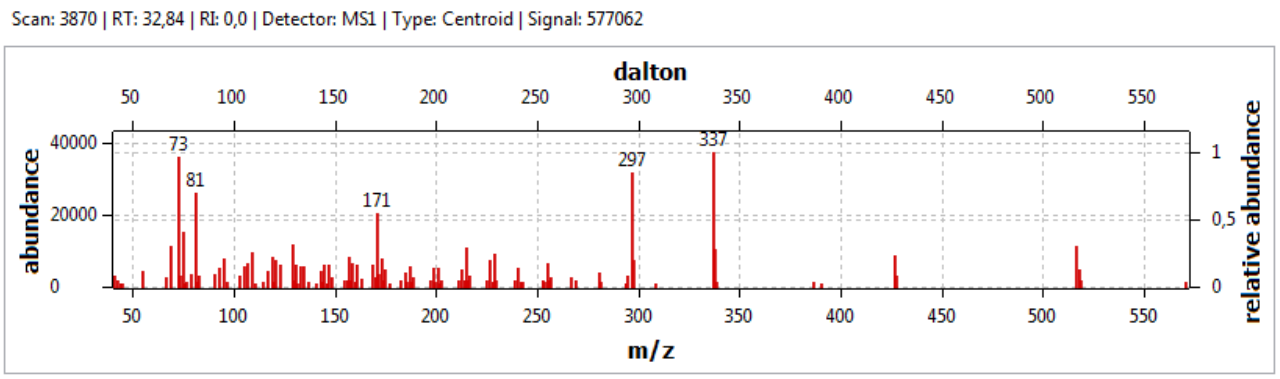

Figure 46. Obtained mass spectrum for inotodiol (OpenChrom).

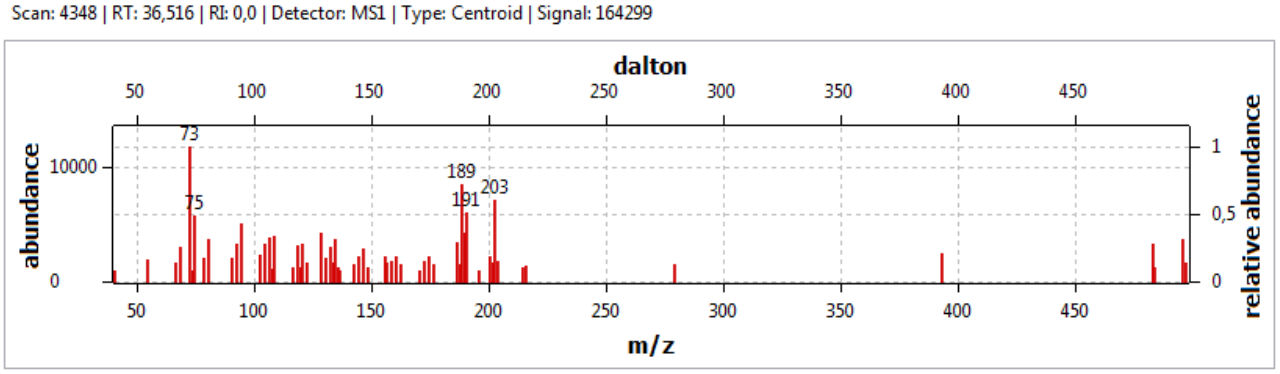

Figure 47. Obtained mass spectrum for trametenolic acid (OpenChrom).

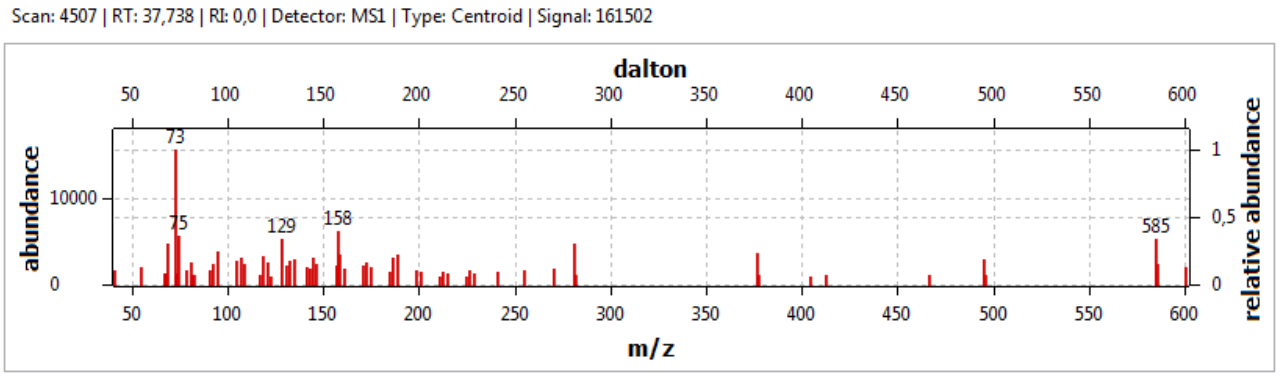

Figure 48. Obtained mass spectrum for betulin (OpenChrom).