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Extraction possibilities of lipid fraction and authentication assessment of chaga (*Inonotus obliquus*)

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Abstract

Chaga (*Inonotus obliquus*) (Fr.) Pilát is a black perennial fungus that grows on adult birch trunk and traditionally been used as a health prevention remedy in different countries. The lipophilic compounds of chaga were isolated applying conventional (Soxhlet), non-conventional (ultra-sound assisted, accelerated solvent) and environmentally friendly (supercritical fluid) extraction methods utilizing both polar and non-polar solvents. For authentication of chaga samples, isotope ratio mass spectrometry (IRMS) and multi-elemental analysis was performed in this study. The yield and profile of lipids, sterols, and triterpenoids of various origins were discussed and potential application in terms of antimicrobial activity against pathogenic and opportunistic pathogenic microorganisms demonstrated. The results showed that CO_2 extraction delivered comparable to conventional extraction techniques amount of target compounds with a safer profile and therefore could represent the future for sustainable industrial-scale production of biologically active ingredients with antimicrobial activity. Authentication of chaga allowed to establish the quality parameters and properties of resources that will be useful for industries.

Keywords: supercritical extraction, triterpenoids, *Inonotus obliquus*, chaga, authenticity, extraction

1. Introduction

Chaga (*Inonotus obliquus*) (Fr.) Pilát is a black perennial fungus of the *Hymenochaetaceae* family that parasites on adult birch trunks as well as on other types of broadleaf tree species such as alder, mountain ash, maple, which distributed throughout the temperate zone of the northern hemisphere [1]. Since the 12_{th} century, chaga has traditionally been used in Russia, China, Poland, Baltic States as a remedy in the prevention and treatment of various ailments, including gastrointestinal disorders, cardiovascular diseases, cancer, tuberculosis, diabetes and skin problems. In ethnomedicine, chaga is mainly consumed in the form of tea or infusion [2-5]. The limited information on the phytochemical composition of the chaga fungus prompted the design of this study focusing on the evaluation of the composition of triterpenoids and phytosterols together with the antimicrobial potential of this unique but less studied plant material.

I. obliquus produces a wide range of bioactive metabolites, including triterpenoids, sesquiterpenes, sterols, benzoic acid derivatives, polysaccharides, and polyphenols. These compounds have broad clinically documented health benefits, including reactive oxygen (ROS) and nitrogen species (RNS) scavenging, antitumor and antiviral activity and the ability to boost the immune system during struggling with causative agents of infection diseases (*Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis*) [6-9]. The recent *in vivo* studies revealed that triterpenoids with no toxicity represent therapeutic and chemopreventive features for the treatment of inflammation and cancer, while *in vitro* trials demonstrated considerable suppression of Walker 256 carcinosarcoma, HeLa cervical cancer and MCF-7 human breast adenocarcinoma cells growth [10-12].

It is worth noting that *I. obliquus* can be found not only in natural environment, but it also be cultivated on different substrates using malt, birch saw dust, grain and potato dextrose agar [13], however, the composition of its metabolites and similarity to native chaga is not much studied.

Recent studies have reported that the chaga fungus has potential to be used in the production of therapeutic substances with a wide application in pharmaceutical and natural cosmetic industries [14]. In particular, the extracts of those obtained from Russian chaga fungus were reported to be rich in inotodiol and trametanolic acid the compounds responsible for cytotoxic effects on breast cancer cells [15]. Since the accumulation of health-promoting elements depends on geographical location, meteorological conditions, pollution and origin of raw material, more data need to be collected to fully understand the nature of this plant. Currently, there is almost no data available on phytochemical composition of the chaga fungus from Northern latitudes. The fingerprint of the most abundant elements building up living matter ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$) and multi-elemental analysis could be used as a method of geographical region and origin verification [16, 17]. Fatty acids, sterols and triterpenoids of *I. obliquus* can be used as biomarkers to discriminate geographical differences [18, 19].

The present study was designed to evaluate the lipophilic composition and antimicrobial activity of compounds recovered from chaga fungus using widely applied conventional extraction techniques. To compare the yield of target compounds environmentally friendly supercritical fluid extraction was used. Chaga samples of different origins were vitrificated using the lipid and elemental profile, light stable isotopes ratio and antimicrobial activity of fungus extracts.

Abbreviations:

ASE, Accelerated Solvent Extraction; BSTFA, N,O-Bis(trimethylsilyl) trifluoroacetamide; DMSO, Dimethyl Sulfoxide; GC-MS, Gas Chromatography-Mass Spectrometry; IOP, *Inonotus Obliquus* Powder; MIC, Minimum Inhibitory Concentration; Nitric acid, HNO_3; PCA, Principal Component Analysis; SE, Soxhlet Extraction; USE, Ultrasound Extraction; SFE, Supercritical Fluid Extraction.

2. Materials and methods:

Chemicals and reagents:

Commercial standard betulin (purity > 99.0%) was purchased from Extrasynthese (Genay, France), ergosterol (purity > 95.0%) and linoleic acid (purity > 99.0%) were obtained from Sigma Aldrich (St. Louis, MO, USA). MS grade ethyl acetate, isopropyl alcohol, benzene, toluene, cyclohexane, petroleum ether, iso-octane, hexane, N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), pyridine, nitric acid (HNO_3, concentration > 65%), sucrose obtained from Sigma Aldrich (St. Louis, MO, USA). MS grade chloroform, dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2) were purchased from Enola (Riga, Latvia). *L*-glutamic acid (USGS-40) was provided by Geological Survey (Reston, VA 20192, USA). Carrier gas (He), oxidizing gas (O_2), working gases (CO_2, N_2) were obtained from Linde Gas Ltd. (Riga, Latvia), Mueller-Hinton medium for bacteria and malt extract medium for yeast were purchased from ThermoFisher Scientific (Hampshire, UK).

Plant material:

To confirm the geographical differences, compare the amount of trace elements, evaluate the profile of lipophilic fractions, and validate the potential application of the compounds present in chaga fungus, the 21 types of *I. obliquus* fungi (Birch, Alder, Oats) were collected from different countries (**Table 1**). The **Figure 1** represents the data on the location of wild chaga samples picked from four regions of Latvia. Appearance of wild chaga: large, black, solid growths (≈1 cm^2). Inner part: brown, hard, closer to wood - softer and lighter. Samples were crushed to fine powder (0.25 mm) and dried before analyses (10% moisture). The remaining powder was frozen at -20 °C.

Table 1

Description of Chaga samples

Sample ID	Information (Country, origin, type)	Sample ID	Information (Country, origin, type)
1BF, 2BF, 3BF, 5BF	Finland, birch, commercial, natural*	**11BE, 12BE**	Estonia, birch, commercial, natural
4BF	Finland, birch, commercial, water extract	**13BL**	Latvia, birch, commercial natural
6BF	Finland, birch, wild*	**14BL, 15BL, 16BL, 17BL, 18BL**	Latvia, birch, wild
7BR	Russia, birch, commercial, extract	**19AL, 20AL**	Latvia, alder, wild
8BR, 9BR, 10BR	Russia, birch, commercial, natural	**21OA**	USA, oats, cultivated

**Natural – not cultivated; Wild – were collected from forest*

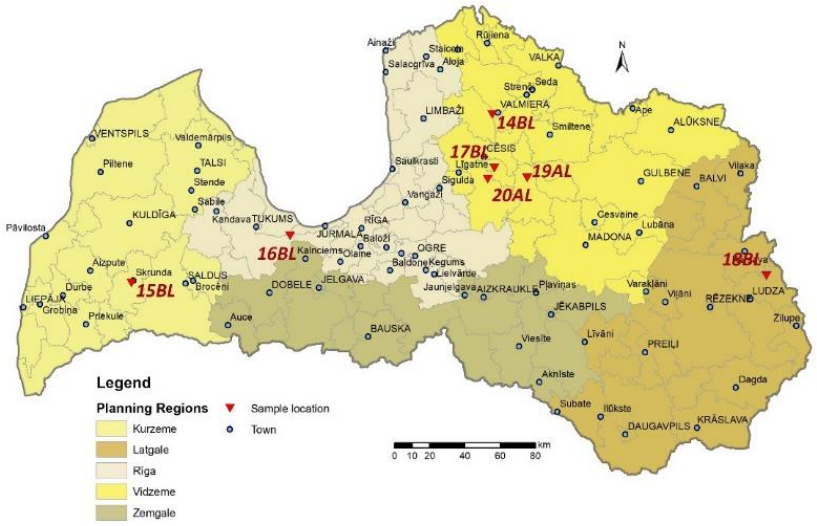

Fig. 1. Sampling sites of Latvian chaga samples

Isolation of lipophilic compounds from I. obliquus

Extraction of lipophilic compounds was done using both conventional and non-conventional extraction techniques (SE, USE, SFE, ASE) with different polarity solvents (ethanol, ethyl acetate, chloroform, isopropyl alcohol, benzene, toluene, petroleum ether, iso-octane, cyclohexane, hexane).

Soxhlet extraction:

Triplicate samples of 5 g of dried IOP were weighted with precision 0,1 mg in extraction thimbles (Behr extraction thimbles, 24 × 80 mm). Further, the extraction chambers were filled with 150 mL of relevant solvents and the thimbles placed inside. Soxhlet extractor (Behr Labor-Technik extractor EZ 30(H), Dusseldorf, Germany), which is fully automated was heated for 6 h at the boiling point of the respective solvent. Sufficient heat was used to give about 10 cycles of solvent per h.

Ultrasound extraction:

Triplicate samples of 10 g of dried IOP and 100 mL of the relevant solvent were mixed and subjected to ultrasonication in ultrasound bath (Cole-Parmer, Chicago, USA) at 50 Hz and 245 W output wattage for 20 min at 26 ± 1°C. The mixture was than filtered filtrated through 47 mm glass microfiber filter (Chmlab group, Barcelona, Spain). The used filter paper was then placed back into the extraction vessel and 100 mL of solvent were added to obtain maximum yield. The extraction process was repeated three times.

Supercritical Fluid Extraction:

SFE extraction was done using the method of Leal with slight modification [20]. Triplicate samples about of 500 mg of dried IOP were weighed in a 5 mL stainless-steel extraction cells (Shimadzu Corporation, Tokyo, Japan) with cellulose discs at the bottom and top. The extraction vessel was placed in the rack changer (Rack Changer II, Shimadzu Corporation) of a SFE apparatus (Nexera UC SFE-30A, Shimadzu) and the extraction was performed for 6 minutes (3 min − static mode; 3 min − dynamic mode) at 40 °C temperature. The control over the system during SFE extraction was carried out by SCL-40 system comptroller (Shimadzu Corporation).

The conditions used for the extraction compounds of interest: mobile phase consisted of solvent A – supercritical fluid of CO_2 (Linde Gas Ltd., Latvia, purity > 99.0%); and solvent B (modifier) – ethanol (purity 96.8%); ratio of CO_2 to EtOH 85:15 (%); the flow rate of mobile phase – 3.0 mL min^{-1}; extraction vessel and rack changer temperature – 40 °C; co-solvent consumption – up to 9.4 mL.

To compare the yield of target compounds, two extraction pressures either with 28 MPa or 38 MPa were used in this experiment. The required pressure was ensured by two back pressure regulators (SFC-30A, Shimadzu Corporation) which have been operated under 50 °C. The extracts were collected in plastic 15 mL Falcon tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany).

Accelerated Solvent Extraction:

ASE was done according to the procedure proposed by Tang with slight modification [21], i.e., triplicate samples of 4 g of dried IOP were weighted and placed into the stainless-steel extraction cell with cellulose filter at the bottom. The extractions were carried out using an ASE 350 Dionex extractor (ThermoFisher Scientific, Hampshire, UK) at 90 °C temperature, 10.3 MPa pressure, for 5 minutes. 4 cycles for each sample extraction have been given. Solvent consumption - up to 21 mL. All the obtained extracts were loaded into a round-bottom flask for evaporation. Solvents were evaporated using a rotary evaporator (Rota-Vap, Schwabach, Germany) until approximately 5 mL lipid/100 mL water extract fraction was left in the flask.

The lipophilic extracts were placed in a glass tube and flushed with liquid N_2 (AGA, Latvia). The aqueous extract was frozen and freeze-dried using a VirTis BenchTop Pro (SP Industries, Warminster, PA, USA) at −50 ± 1 °C temperature and 0.080 mbar pressure for 48 h. Samples were kept under −20 ± 1 °C until the analysis.

Preparation of silyl (TMS) derivatives for GC-MS analysis:

Briefly, 1300 µL of pyridine and 200 µL of BSTFA reagent was added to each 5 mg of extracted sample in 1.5 mL glass chromatographic vials (Waters, Milford, USA) and heated for a 1 h at 60 ± 1 °C. Tubes were then allowed to cool to room temperature (22 ± 1 °C).

Qualitative and quantitative analyses of phytosterol and triterpenoid derivatives were carried out using a GC-2010 plus equipped with a GC-MS QP-2010 Ultra mass detector (Shimadzu Corporation, Kyoto, Japan). All analyses of TMS derivatives were done using Restek Rxi®-5MS (30 m × 0.25 mm × 0.25 µm) column with a stationary phase of low polarity (Crossbond 5% diphenyl + 95% dimethyl polysiloxane (Restek, Bellefonte, PA, USA). Helium m (ultra-high purity 5.0 grade − 99.999%) was used as carrier gas with a total flow rate of 10.8 mL min^{-1} and a column flow rate of 0.71 mL min^{-1} (Linde Gas Ltd., Latvia) with the split ratio 1:10. The injector temperature was set to 290 °C. The initial oven temperature was maintained at 200 °C for 2 min, then raised to 250 °C at the rate of 30 °C min^{-1} and held for 7 min then increased to 310 °C at the rate of 10 °C min^{-1} and kept for 14 min. Injection of 1.0 µL sample was performed using an autosampler. Mass selective detector with quadrupole mass analyzer was used with electron impact (EI) ionization, with an ionization voltage of 70 eV. The ion source temperature was 230 °C and the interface temperature was 290 °C. Identification of the compounds separated in the GC was performed using Shimadzu LabSolutions 4.30 software, coupled with the NIST017 spectral library (NIST, Gaithersburg, MD, USA) and literature.

For the calculation of triterpenoids calibration curve of betulin in the range of 0.0008−1.35 mg mL^{-1} was constructed. For the calculation of phytosterols, the ergosterol standard in the range 0.0008−1.35 mg mL^{-1} was used. For the quantitative analysis of fatty acids γ-linoleic acid in the range 0.006−0.40 mg mL^{-1} was used.

Light stable isotope ratio analysis ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$)

For determination of the ratio of C and N stable isotopes, 5 mg IOP were weighed into tin capsules (EuroVector, Pavia, Italy) on an analytical balance (KERN ALJ 220-4, ± 0.01 mg). All samples were prepared in triplicate. Glutamic acid standard were prepared for the construction of calibration curve in the range of 0.2−1.5 mg to analyze total amount of C and N. To monitor the stability of the obtained values, one glutamic acid control sample (1.0 mg) was analyzed for every 10 samples.

For determination of the ratio of O stable isotopes 1.0 mg IOP was weighed into silver capsules (EuroVector, Pavia, Italy). Sucrose standard were prepared for calibration – 0.2, 0.5, 0.8, 1.0 and, 1.5 mg to analyze total amount of O. The one sucrose control sample (1.0 mg) was analyzed for every 10 samples to monitor the IRMS system stability.

The ratio of C, N and O stable isotopes of samples were measured on the isotope ratio mass spectrometer (Nu Horizon, Wrexham, United Kingdom). Analysis was run under acceleration voltage of 5kV, mass range 2–100 Da, mass dispersion up to 30 cm. Elemental analyzer (EuroVector Euro EA3000, Pavia, Italy) filled with chromium (III) oxide (Cr_2O_3), silvered cobaltous oxide (CO_3O_4) (1030 °C). A quartz reduction tube filled with copper shards (650 °C) was utilized for carbon and nitrogen isotopes analysis. Outer tube made of aluminum alloys and filled with vitreous carbon particles and silver wool was used for oxygen isotopes analysis. The results were processed by the Nu Stable Control Software v1.69. Mass concentration of C, N, O was calculated from glutamic acid and sucrose calibration curves.

Quantification of trace elements

For the analysis of trace elements, to each 2 g of IOP 8 mL 65% HNO_3 and 2 mL 30% H_2O_2 were added in Teflon tubes. The tubes were than closed to ensure high pressure. The prepared samples were digested using a microwave system (Milestone Advanced Microwave digestion system, ETHOS™ EASY, Bergamo, Italy) at 200 °C for 30 min. The resulting samples diluted to a volume of 50 mL with deionized water (MilliPore, 7.4 µS cm^{-1}). The concentrations of inorganic elements were determined by inductively coupled plasma spectrometry with optical emission detection (Thermo Scientific iCAP 7000 series ICP spectrometer, Waltham, MA, USA) with 7 pm at 200 nm optical resolution, 167.021 nm - 852.145 nm wavelength resolution, 0.55 L min^{-1} nebulizer gas flow rate, 12 L min^{-1} plasma gas flow rate, 27.12 MHz solid state RF source.

Antimicrobial assay of the lipophilic extracts

The antimicrobial activity of obtained extracts was evaluated according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 2003) with slight modification. Four standard strains of test microorganisms were used for antimicrobial activity testing included Gram-positive (*Staphylococcus aureus* MSCL 334) and Gram-negative bacteria (*Pseudomonas aeruginosa* MSCL 331; *Escherichia coli* MSCL 332) and yeast (*Candida albicans* MSCL 378). Bacteria were grown on Mueller-Hinton agar medium (Oxoid, CM0337, ThermoFisher Scientific, Hampshire, UK) at 37 °C for 20–24 h, while yeast on malt extract agar medium (Oxoid, CM0059, ThermoFisher Scientific) at 37 ± 1 °C for 48 h. After cultivation, a suspension of microorganisms in sterile water was prepared by determining the optical density (OD) with a spectrophotometer until OD value at 540 nm 0.16 was obtained. Afterward, the prepared suspension was evenly distributed over the surface of the agar medium. When the surface of the medium was dry (~ 5 min), round holes were made manually in the agar with a sterile metal cylinder with a diameter 7 mm and the medium was removed. Under aseptic conditions, the tested chaga extracts (30 mg each) were solubilized in 1 mL 50% DMSO. 70 µL of the prepared solution was placed in the wells prepared and the plates were incubated at 37 ± 1 °C for 24 h and 48 h for bacteria and yeast, respectively. The negative control consisted of 50% DMSO. The results were read by measuring the diameter of the growth inhibition zone of the microorganisms.

Statistical Analyses

Statistic data analyses that indicate the variation of extraction yield of chaga by different extraction methods, lipid extracts of chaga and its antimicrobial activity of different origin were performed with ANOVA post-hoc Tukeys HSD test using SAS JMP 16 software. The significance of the origin of *I. obliquus* was done by principal component analysis (PCA) of lipid content, stable isotope ratios and metal concentration with SAS JMP 16 software.

3. Results and discussion

Extraction results

The interactions between intracellular phytochemicals and cell walls have a significant influence on extractability of compounds of interest which are present in plant matrices [22], including chaga fungus [23]. Recently, a number of extractants were investigated to recover the non-polar constituents, and in most cases, such solvents or their mixtures as acetone, methanol, chloroform, hexane, isopropanol, butanol, dichloromethane had been favored even though they may be harmful to operators and environment. Among the extraction methods documented, superior extractability of phytochemicals from oil samples was reported applying SFE coupled to SFC. Besides, through the extraction process, which takes place in a closed loop reduction of qualitative and quantitative losses of analytes could be achieved and up to 95-100% of sterols recovered [24, 25]. To date, very little information has been provided regarding the extraction of phytosterols and triterpenoids from chaga fungus, therefore a preliminary study on the extractability of target compounds from Red alder (*Alnus rubra*) chaga sample (19AL) with the use of ethanol (Et), ethyl acetate (Ea), chloroform (Chl), isopropanol (Ipo), benzene (Bz), toluene (Tl), cyclohexane (Chex), isooctane (Ioct), petroleum ether (Pe), hexane (Hex) was done. To identify the most efficient extraction method/s, such approaches as accelerated solvent (ASE), Soxhlet (SE), ultrasound-assisted (USE) and supercritical fluid extraction (SFE) were employed.

From **Figure 2**, can be seen that the extraction yield increases with the increase of solvent polarity that could be explained by the presence of more polar compounds in chaga matrix than non-polar. Experimental data revealed that the highest yield of the extract could be obtained using either SE or USE and Et as a sole solvent. The extractability yield for these approaches was ranged from 2.9 to 2.1%, respectively. Despite the extraction technique applied, a notably lower yield of extracts was obtained using Ioct, Pe, and Hex. The extraction yield fluctuated from 0.4 to 0.5%. Comparable to SE yield of extracts were recovered using non-conventional SFE in a combination of CO_2 and Et as a co-solvent at ratio 85:15 (*v*/*v*). The extraction yield is significantly lower than that of SE+Et and USE+Et. However, it worth noting that due to improved selectivity, expeditiousness, automation, and environmental safety this approach could represent the future for sustainable industrial-scale production compounds of interest significantly reducing the costs of solvents and other materials necessary for traditional liquid-solvent-based extraction.

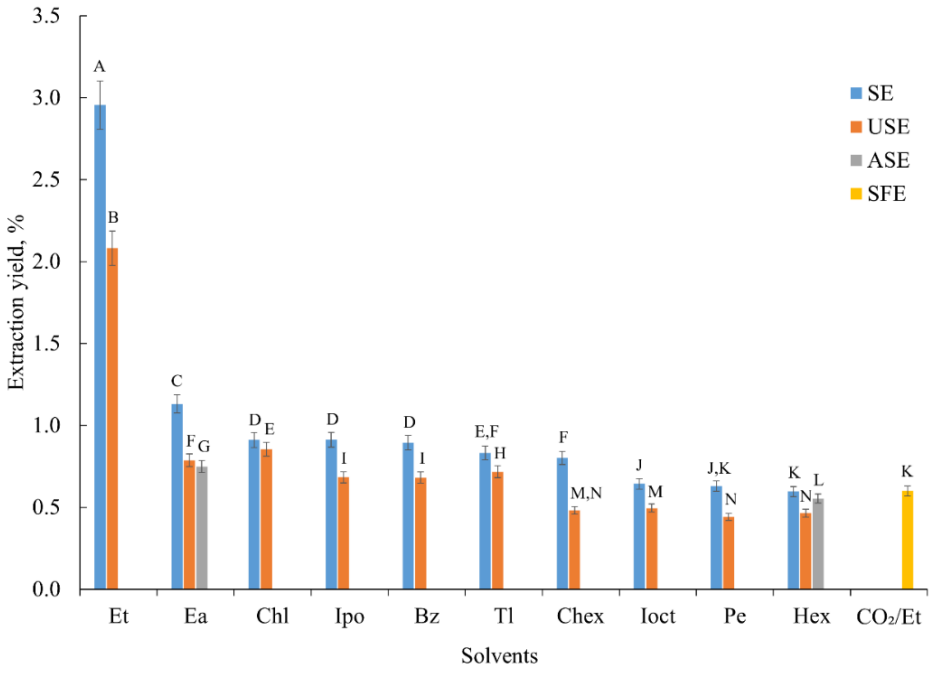

Fig. 2. Extraction yield with different polarity solvents (Et – ethanol; Ea – ethyl acetate; Chl – chloroform; Ipo – isopropanol; Bz – benzene; Tl – toluene; Chex – cyclohexane; Ioct – isooctane; Pe – petrol ether; Hex – hexane)

The extraction method does not significantly affect the extraction result, but optimal results were obtained using Soxhlet with all solvents. Although it is time consuming.

GC-MS composition profile

Chromatograms with a mixture of phytosterols and triterpenoids were obtained by analyzing chaga-derived extracts. Among the phytosterols and triterpenoids analyzed, ergosterol, lanosterol, inotodiol, tramethenolic acid, and betulin were found to be dominant compounds of *I. obliquus* extracts. The inotodiol fraction prevailed in the extracts of chaga obtained using ethyl acetate as a solvent (**Fig. 3.**). A similar result was reported previously by Kahlos, pointing out the presence of inotodiol exclusively in the sclerotia of *I. obliquus* [26]. While the fraction of betulin appears in the lipid extract of the alder chaga, which is more specific for birch trunks. The report of Chen reveals that red alder (*Alnus rubra*) also represents some amount of botulin [27], which presumably could be absorbed by the fungus itself from the tree trunk on which its parasitizes. The formation/accumulation of betulin in chaga depends on its body size.

Trametenolic acid is another biologically active triterpenoid, which is due to its anti-inflammatory, anticancer, and anti-ischemic activities received tremendous interest among scientists and medicines producers [28, 29]. The presence of this compound in *Leiotrametes lactinea* (previously *Trametes lactinea*) was initially found and successfully isolated by Zhang [30]. However, due to the extinction threat caused by the loss of symbiotic hosts, pollution, and climate change this species recently has been included in the list of the Global Red List (GFRL) [31]. Therefore, new sources of this promising compound must be identified to allow the production of it on an industrial scale. However, the presence of this compound in sclerotia of *I. obliquus* was recently reported by Sagayama [32], indicating the potential effectiveness of this species as proliferative agents on human follicle dermal papilla cells.

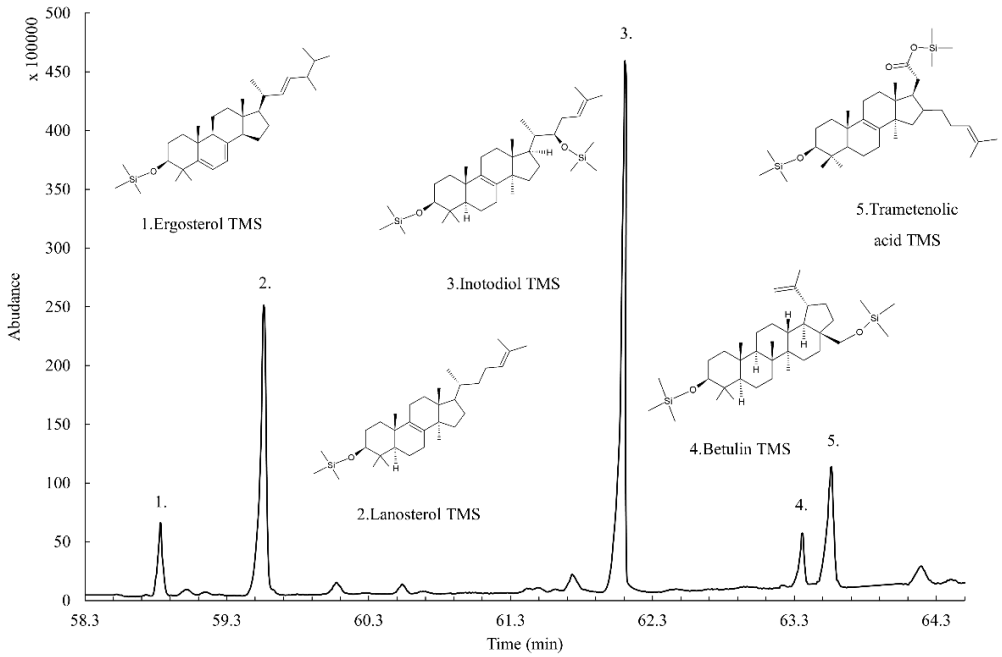

Fig. 3. Chromatographic separation of triterpenoids and phytosterols profile of *I. obliquus* lipid fraction using GC-MS. Extraction of triterpenoids was done utilizing ASE and ethyl acetate as a solvent.

In a more recent study, the presence of betulin, inotodiol, lanosterol, and trametenolic acid in *I. obliquus* was reported by Kim [33]. The authors observed that the vast majority of inotodiol and trametenolic acid were located in the outer parts of chaga fungus, while betulin in the inner part. It has been speculated that due to the presence of lanosterol a precursor of ergosterol the older chaga fungus may contain notably higher amount of this compound. Moreover, the anti-proliferative activity of the triterpenoid fraction from the outer part against AGS, MCF-7, and PC3 cell lines has been demonstrated by the authors.

Based on the spectral data and literature available [26], appearance of inotodiol and trametenolic acid has been also confirmed in the present study. The fragmentation patterns of inotodiol and trametenolic acid are provided in supplement.

It should be noted that due to some limitations in analytical tools, in particular the NIST library and scarcity in literature data, no additional triterpenoids in chaga samples were identified. However, according to Taji, Handa, Liu such lanostane-type triterpenoids as nonotsuoxodiol B, inonotsuoxodiol C, epoxyinonotsudiol, and methoxyinonotsutriol could also be extracted using chloroform as a solvent and further identified utilizing either LC-MS or NMR approaches [34-36].

The concentration of lipophilic compounds of the same material varies depending on the solvent and the extraction method used. All the results of compounds are reflected in the supplement. The yields of inotodiol as the main representative of triterpenoids recovered from *I. obliquus*, depending on the solvent type and the extraction technique used are given in **Figure 4**. As seen, USE and SE delivered a significantly higher amount of inotodiol as a solvent using Chl, Chex, Pe, and Hex. The percentage ratio of inotodiol to other triterpenoids presenting in the lipophilic fraction has amounted to 40:60%. The highest yield of lanosterol comparable to inotodiol was achieved when SE and USE techniques were used. The percentage abundance of this compound in the whole mixture has amounted to 50%. However, in the case of recovery of betulin and ergosterol, significant differences between SE and USE extraction techniques could be seen. The absence of these two compounds in the extracts obtained by SE could be associated with the intensity of extraction.

During the experiments, it was observed that Ioct has a low solvation capability of triterpenoids especially inotodiol, therefore the lowest yield of target compounds was obtained using this solvent. The USE extraction yielded significantly higher amount of phytosterols and triterpenoids as compared to the yield of the compounds when SE was used. It has been concluded that USE as a treatment type could be used to effective recovery of non-phenolic compounds *I. obliquus* significantly reducing the costs of solvents and energy necessary for SE extraction. Similar results were obtained by Géry, indicating good extractability of betulin, betulinic acid, and inotodiol during 96 h maceration process and utilizing ethyl acetate as a sole solvent [37]. However, the authors observed considerable fluctuations in the release of these compounds depending upon the origin of plant. It has been highlighted that chaga fungus originated in French could be utilized for the production of betulin and betulinic acid, while the Canadian fungus superb candidate for inotodiol manufacturing. The obtained results are in a good agreement with the reports of [38-41].

In the last several years, diverse attempts have been made to replace conventional extraction techniques, i.e., SE, USE, microwave dissolution, and SFE and to effectively recover compounds of interest in a shorter time and the lowest costs. As an example of efficient and relatively rapid extraction method that is so far has been used for extracting various chemicals from a complex solid or semisolid sample matrix, incl., seaweed [42], green tea (*Camellia sinensis*) [43], and aged flue-cured tobacco leaves (*Nicotiana tabacum*) [44] is ASE or sometimes called pressurized solvent extraction. Taking advantage of solvent high pressure and elevated extraction temperature, this technique allows increase the solubility of target analytes, assist in breaking down interactions between micro and macromolecules, encourage the penetration of solvent into the cells, and increase the mass transfer rate of organic compounds to the solvent.

Thus far, this is the first study aimed at extracting non-phenolic biologically active compounds from *I. obliquus* and to compare the yield of target compounds with other conventional solid-liquid extractions. Despite the fast-growing popularity of ASE this approach has demonstrated notably fewer efficiency in extracting phytosterols and triterpenoids from *I. obliquus* matrix than that of SE and USE. This observation makes it possible to conclude that due to high temperature (90 °C) that the plant material underwent during the extraction, possible decomposition of target compounds took place. A similar observation has been made by Rudzinska [45], pointing to thermo-oxidative degradation of some phytosterols as a function of temperature and time with the simultaneous formation of aroma volatiles. Meanwhile, Mohammad [46] found ASE useful in isolation of thermally stable organic contaminants (POPs) from environmental matrices.

For the purpose of extracting non-phenolic biologically active compounds from *I. obliquus* in a safer to environment and human way, at the same time to reduce the likely decomposition of thermolabile compounds, the SFE technique has been used in this study. Among the techniques used over the past decade, the SFE has established itself as a technology effective in extracting both polar and non-polar compounds from various plant matrices such as tomato (*Solanum lycopersicum*) by-products [47], grape (*Vitis vinifera*) seeds [48], and olive (*Olea* spp.) processing by-products [49]. Recently, the effectiveness of SFC in extracting phytosterols and triterpenoids from *I. obliquus* has been demonstrated by Huynh in his Master thesis (Supercritical CO_2 extraction of triterpenoids from *Inonotus obliquus*) [50], however, this is only the one report available so far regarding this species. As is seen from Fig. 4, the concentration of inotodiol isolated using CO_2 in the presence of modifier is equal to the yield obtained using USE and toluene as a sole extractant. It has been observed that the increase of extraction pressure from 28 to 38 MPa increases the number of lipophilic compounds in the final extracts (phytosterols specifically) due to improved the mass transfer rate (enhanced solvent permeability in cells) (S**upplement**), however, the yields were significantly lower than those obtained using USE.

The highest extractability was obtained using Chl, followed by Chex, and Hex. Similar results were obtained by Kim [41], pointing out that due to the higher polarity of inotodiol which contains more hydroxyl and aldehyde groups than cholesterol, these solvents (Chl in particular) are considered as the optimal for inotodiol and 3β-hydroxylanosta-8,24-dien-21-al extraction. In the research of Wei [51], the superiority of USE over other extraction methods used was confirmed.

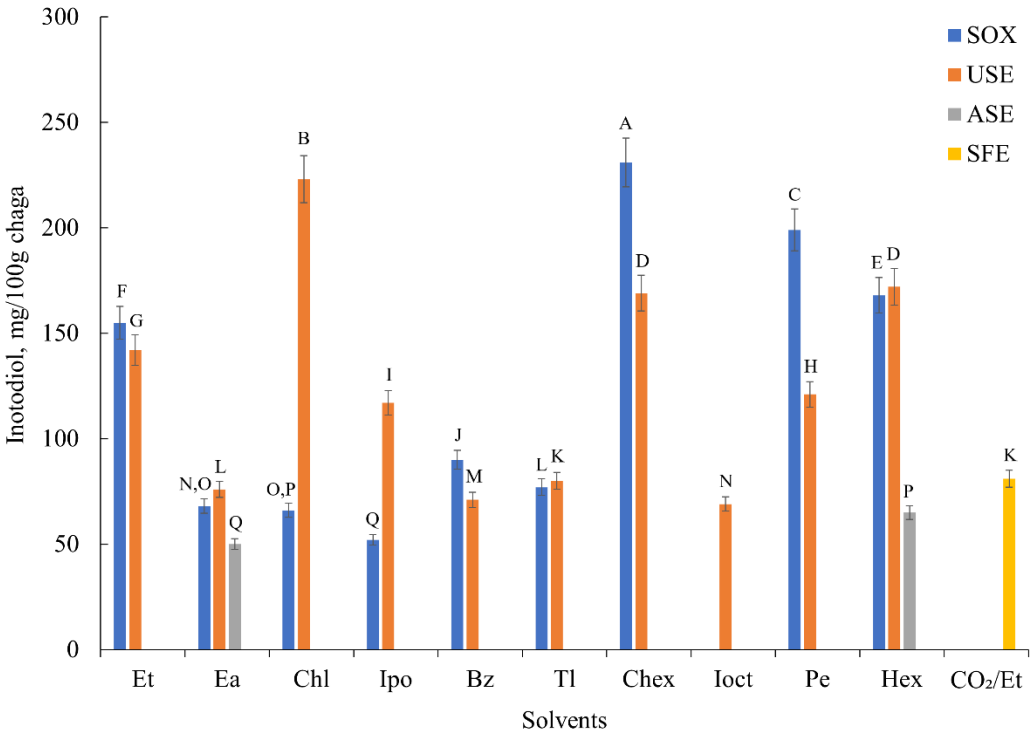

Fig. 4. The recovery of inotodiol using commonly applied conventional and non-conventional extraction techniques and industrial extractants (Et – ethanol; Ea – ethyl acetate; Chl – chloroform; Ipo – isopropanol; Bz – benzene; Tl – toluene; Chex – cyclohexane; Ioct – isooctane; Pe – petrol ether; Hex – hexane)

It is worth noting, though, that despite the yield of triterpenoids was significantly lower than using cavitation, the SFE approach due to easy operation, low production costs, and environmental benefits could represent further sustainable production of pharmaceuticals and other ingredients.

The lipid content of analyzed samples

Food authentication is necessary due to increased public awareness of food quality and safety. In connection with the globalization of food markets and, as a consequence, the increase in the availability and variety of food from other countries, there is an interest in elucidation the origin and quality of products, which may further contribute to consumers' health. To identify the profile of constituents present in different food stuff and verify the origin of this food various analytical methods have been developed and tested: molecular techniques, genomics – proteomics, chromatographic, isotopic, elemental and immunological techniques, vibrational and fluorescence spectroscopy, mass spectrometry (MS) and nuclear magnetic resonance (NMR) [52].

To distinguish differences between the selected plant materials, in this study 21 samples of different I. obliquus origins were used. For the extraction of bioactive constituents the USE and Ea as a sole solvent were applied in this experiment. As it is seen from **Figure 5**, the amount of lipid for almost all samples fluctuated in the range from 0.5 to 1.0%. Comparable results were reported by Gery [37] where wild species of *I. obliquus* originated from Canada, France, and Ukraine were subjected to conventional extraction with cyclohexane as a sole solvent. The authors no observed significant differences between the samples, the amount of lipids fluctuated in the range from 1.0 to 1.2%. Furthermore, similar yields of lipids were obtained from Chinese chaga samples applaying Ea as a solvent [23].

The lows yield of lipids has been obtained when the commercial chaga fungi (4BF) sample was subjected to extraction with an aqueous solvent, corresponding to 0.07%. The cultured (domesticated) chaga grown on oat substrate and originated from the USA (21OA) contained five times higher lipids than other samples. Approximately two times higher lipids content was obtained from *I. obliquus* Russian chaga sample (7BR) that has been cultivated on birch substrate. It is assumed that the high content of lipids found in commercial samples could be associated with availability of nutrients, moisture and additional supplementation which the plants receive during the entire growth period. Another credible explanation is with biotic and abiotic factors that may have a direct impact on accumulation of macromolecules, including lipids. No significant differences were found between wild types of *I. obliquus* in terms of lipid content.

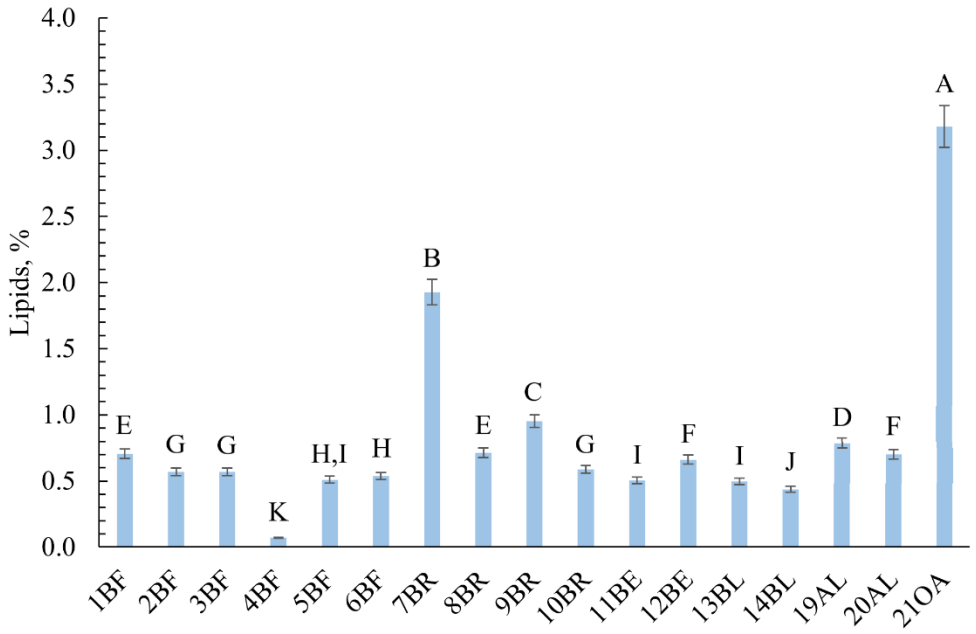

Fig. 5. The lipid content of different types of chaga

The composition of the lipid fraction of chaga samples was studied by GC-MS and results were represented in **Table 3** and **4**. Analysis of lipids by gas chromatography is a routine that is readily available in many laboratories and is more sensitive than ^{13}C NMR. Almost all chaga of birch origin has a similar composition of triterpenoids and sterols (**Table 3**). They contained ergosterol, lanosterol, inotodiol, betulin, trametenolic acid and stigmasterol in low concentration (<LOQ). Some compounds such as stigmasterol have not been identified due to the abundance of inotodiol and lanosterol. It is concluded that preparative chromatography as a separation technique will allow fractionation and concentration the constituents found in chaga samples and therefore facilitate quantitative analysis that could be done in the future.

The concentration of ergosterol varied from 0.04 to 22.0 mg 100 g^{-1}, lanosterol: 1.05 – 112 mg 100 g^{-1}, inotodiol: 1.5 – 112 mg 100 g^{-1}, betulin: 0.60 – 6.82 mg 100 g^{-1}, trametenolic acid: 0.42 – 7.10 mg 100 g^{-1}. In general, the concentration of betulin in Latvian samples is higher than that of other origins. The highest concentration of triterpenoids and sterols found in samples of alder origin (19AL, 20AL). A commercial aqueous extract of chaga (4BF) contained such compounds as lanosterol, inotodiol and trametenolic acid, but in small amounts.

Table 2

The composition of sterols and triterpenoids in chaga samples (mg 100 g^{-1} chaga)

Sample/ compound	Ergosterol	Lanosterol	Stigmasterol	Inotodiol	Betulin	Trametenolic acid
1BF	ND*	24	<LOQ*	12	1.18	0.74
2BF	ND	46	ND	40	1.74	3.99
3BF	0.98	47	ND	51	0.60	2.38
4BF	ND	1.05	ND	1.05	<LOQ	0.03
5BF	3.15	40	<LOQ	30	5.47	1.71
6BF	5.21	84	ND	54	0.71	2.41
7BR	ND	ND	ND	<LOQ	ND	ND
8BR	<LOQ	45	<LOQ	30	5.46	1.17
9BR	4.14	64	ND	49	1.49	2.94
10BR	ND	53	1.57	36	6.69	2.98
11BE	0.73	39	ND	26	0.37	<LOQ
12BE	0.04	48	ND	38	1.63	1.77
13BL	ND	40	ND	33	6.82	1.67
14BL	22	73	1.42	38	6.47	0.42
19AL	1.00	112	<LOQ	76	4.41	7.10
20AL	ND	179	8.67	43	17	15
21OA	ND	ND	ND	ND	ND	ND

**Limit of quantification (LOQ) – 0.01 mg 100 g^{-1} of chaga; ND – not detected*

As it is seen, there are two samples those contained no sterols and triterpenoids. The 21OA sample which has been grown using oat substrate with high lipid content has only fatty acids (**Table 4**). It was found that palmitic, linoleic and oleic acids dominated in this sample. Thus, specific triterpenoids of *I. obliquus* cannot be synthesized by fungal spores on artificial conditions (*in vitro*), especially on oats. The composition of lipid fraction of 7BR also differed from others and contained only organic acids. The most common are levulinic acid, protocatechuic acid, syringic acid, 3,4-dihydroxyhydrocinnamic acid. Consequently, 7BR sample is artificially grown chaga with absence of triterpenoids.

Table 3

The composition of organic acids in chaga sample (mg 100 g^{-1} chaga)

Sample/ compound	Levulinic acid	Lactic Acid	Protocatechoic acid	Syringic acid	3,4-Dihydroxyhydrocinnamic acid	Palmitic Acid	Leinoleic acid	Oleic acid	11-Octadecenoic acid	Stearic acid
1BF	0.07	0.06	0.35	0.35	ND	0.19	0.36	ND	0.26	ND
2BF	ND	ND	ND	ND	ND	ND	0.14	ND	ND	ND
3BF	ND	ND	ND	ND	ND	ND	0.21	ND	ND	ND
4BF	ND	ND	ND	0.30	ND	0.30	ND	ND	ND	ND
5BF	ND	ND	ND	ND	ND	0.16	1.32	0.25	0.08	ND
6BF	ND	ND	ND	ND	ND	ND	0.33	ND	ND	ND
7BR	1.70	0.76	4.82	21	1.65	ND	ND	ND	ND	ND
8BR	ND	ND	ND	ND	ND	ND	0.35	ND	ND	ND
9BR	ND	ND	ND	ND	ND	ND	0.54	ND	ND	ND
10BR	ND	ND	ND	ND	ND	0.25	0.98	ND	ND	ND
11BE	ND	ND	ND	ND	ND	ND	0.59	ND	ND	ND
12BE	ND	ND	ND	ND	ND	ND	0.69	ND	ND	ND
13BL	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14BL	ND	ND	ND	ND	ND	ND	0.69	ND	ND	ND
19AL	ND	ND	ND	ND	ND	2.78	6.58	ND	1.79	1.35
20AL	ND	ND	ND	ND	ND	1.99	25	ND	2.82	ND
21OA	ND	0.34	ND	ND	ND	23	63	73	2.15	1.90

ND – not detected

The principal component analysis (PCA) data (**Figure 6**) showed a similar conclusion that lipid profile of chaga can be used as an authentication method to distinguish cultivated or wild types of chaga, grown on birch or alder, water extract of chaga or natural chaga.

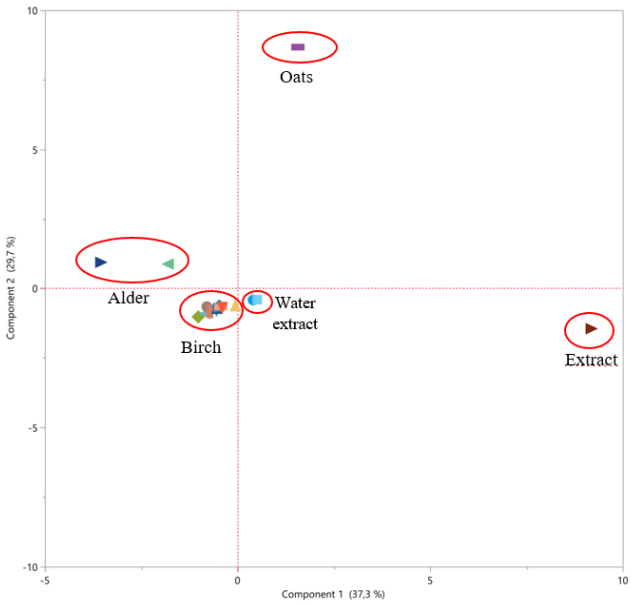

Fig. 6. Principal component analysis (PCA) of lipid profile within different origin of chaga samples.

The amounts of inotodiol described in the other studies [37,41] are similar to the results found in the current research. However, its variation of concentration is confusing. It can be assumed that the inotodiol concentration depends on the age of the chaga and on which part of the body of the fungus was sampled (upper or inner part).

The identification of betulin in all extracts can be considered as an artifact characterizing the link between the fungus and its host. It can be argued that the concentration of betulin in the chaga depends on its body size. In agreement with this study [10], cultivated chaga does not contain specific *I. obliquus* triterpenoids. Experimental results demonstrate that under conditions of cultivation, chaga are not able to form complex triterpenoids such as inotodiol and trametenolic acid within 2 months. Based on the literature and the data obtained, it can be stated that the geographical origin of chaga does not affect the concentration of lipid metabolites.

Stable isotopes ratios of carbon, nitrogen, oxygen in chaga

Isotope ratios are useful for food authentication because their ratios vary with climatic conditions, land cover, geographic origin, and geology of food ingredients [52]. In the group of light isotopes, the most studied ratios are ^{2}H /^{1}H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{18}O/^{16}O$, while $^{34}S/^{32}S$ are used less frequently [53]. Stable isotope analysis has been used to detect the tampering of honey, fruit juice, maple syrup or wine with cheaper fillers such as water or corn sugar syrup [54].

The data of light stable isotopes ratio of chaga were represented at **Figure 7**. The analysis of carbon isotopes ratios show the type of fixation of carbon dioxide in plants [55]. Almost all samples have similar values, based on this, chaga belongs to the C_3 type with the Calvin-Benson cycle. It is typical for plants of which the first product of photosynthesis is two molecules of 3-phosphoglyceric acid (3-PGA) are formed at temperate zones [56]. But Finnish chaga was found to have the lowest $^{13}C/^{12}C$ isotope ratio, indicating that they are less degraded than others. These low values can be explained by the location in Finland, where is a cold

temperature and less sunny days (photosynthesis is slow). The carbon content of the chaga samples ranges from 41% to 49% (**supplement**). No correlation was found between chaga origin (birch, alder, oats) and location. In contrast, the 7BR sample, which also differed from other analyzes, showed the lowest carbon content, indicating that the chaga product was counterfeit.

The nitrogen isotopes ratio depends on air temperature, the degree of soil mineralization and on the absorbed protein [57, 58]. Nitrogen isotope data of samples collected in the territory of Latvia range from -4.0 to 0.4 ‰. Its isotopes ratio is independent of temperature, as all chaga samples grew in the temperate zone at similar temperatures. The main factor influencing the data is the degree of mineralization of the soil on which the samples grew. $\delta^{15}N$ values of chaga varied from -5 ‰ to 4 ‰. A wide range of $^{15}N/^{14}N$ isotopes ratio was found in Russian chaga samples, ranging from positive to negative, indicating that the samples were collected in different regions of Siberia with different habitats and flora. Nitrogen isotope data of samples collected in the territory of Latvia range from -4.0 to 0.4 ‰. Similar values of nitrogen isotopes ratio were determined in the alder samples collected in Latvia (19AL, 20AL), which indicate the same degree of soil mineralization. As can be seen from the map (**Figure 1**), the locations of their samples are close to each other. Commercial chaga collected in Latvia (13 BL) have similar low values (-4.0 ‰) as those collected in Kemeri (16 BL). It can be argued that the commercial sample was collected in one area. However, additional samples are needed to confirm this. According to the data obtained from Latvian samples, it is possible to determine in which area the chaga grew. However, additional samples are needed to confirm it. The nitrogen content of different types of chaga is about 0.5 %, the obtained data are in supplement. Results confirmed by literature [59]. However, the 21OA sample has a high amount of nitrogen which means that a lot of nitrogen compounds from oats were assimilated during the growth process. That influenced the values of the nitrogen isotope ratios, increasing them in the positive side. However, a positive nitrogen isotope ratio value was also found for the 7BR sample of Russian origin. It can be assumed that the manufacturer added additional supplements to increase the value of the product.

The ratio of ^{18}O and ^{16}O depends on different factors, such as the precipitation and evoparation of water (dilution and distillation), the distance from the ocean or the elevation over sea level. The rainwater closer to the ocean presents a higher concentration of the ^{18}O isotope since it precipitates faster than the lighter isotope [60]. Chaga is grown in the temperate climate zone, it is not exposed to high temperatures – hence the evopatranspiration of water is not intense. Natural chaga is characterized by values of $\delta^{18}O$ from 9 to 16‰. Differences in oxygen isotopes were not found in alder and birch origin of chaga. It was also found that artificially grown chaga on oats from America (21OA) has the highest results – 17‰. It can be assumed that this sample was grown in a different climate zone, possibly in an incubator.

Thus, IRMS of nitrogen and oxygen isotopes ratios can be used to determine whether chaga is naturally or artificially obtained. The same conclusion was drawn from the analysis of the main components - PCA (**Figure 8**).

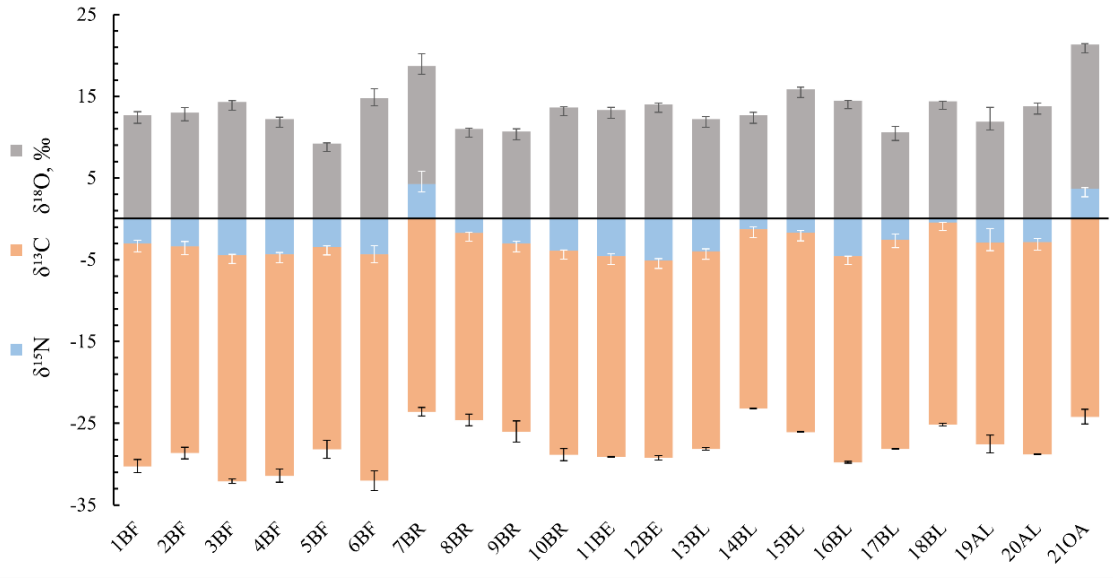

Fig. 7. Ratios of light stable isotopes in chaga samples of different origin (means and standard deviations, n=3)

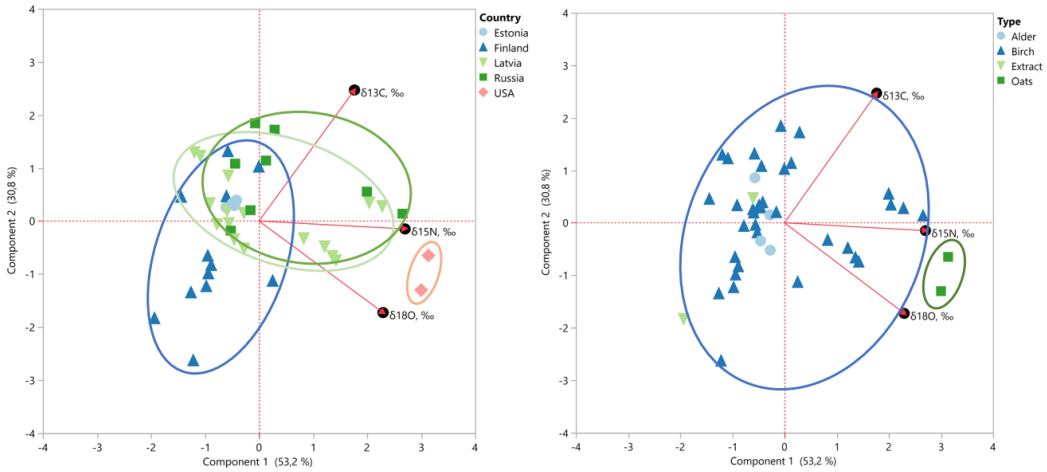

Fig. 8. Principal component analysis (PCA) of carbon isotope ratios ($\delta^{13}C$), nitrogen isotope ratios ($\delta^{15}N$) and carbon isotope ratios ($\delta^{18}O$) parameters within different origin chaga samples

Trace elements of chaga of various origins

Chaga mainly consumed in the form of tea. Therefore, determination of the macro- and microelement composition of chaga is of urgent importance because some elements can easily transfer into the liquid phase. Toxic elements such as arsenic, cobalt, cadmium and lead were detected in samples at low concentrations or below the detection limit as well as B, Cr, Li and Mo. There were Be, Sb, Se, Tl, V under the detection limit in all samples (**Table 5**). Products made from chaga are not toxic and acceptable for human consumption.

Fertilization, harvesting, botanical origin, soil type and pollution all cause fluctuations in element concentrations that also depend on geographic origin [61]. Potassium is the most abundant element in all natural chaga samples. Its concentration ranges from 3.2 mg/g to 94.4 mg/g of chaga (about 3-9%). The concentration of sodium and manganese in *I. obliquus* is approximately 0.02%. The composition of commercial chaga water extract does not differ from the natural chaga. However, the amount of calcium in it is much lower (15 times). It can be assumed that most of the calcium in the chaga is in the form of an organic compound or salt that cannot be dissolved in water. Elevated concentrations of nitrogen (1.7%), phosphorus (0.3%) and sulphur (0.2%), which are grain-specific nutrients, were found in cultivated chaga (21OA). Natural chaga contains about 0.5% nitrogen, 0.03% sulphur and 0.02% phosphorus. This also confirmed by data from the literature [59]. Another source has studied the cultivation of *I. obliquus* mycelium on rice grains and their quality characteristics. The content of potassium, calcium and magnesium was lower than in the samples obtained in the work. Elements such as Zn and Mn were not detected as same as the heavy metals As, Pd, Cd and Hg [62]. Thus, cultivation is a reflection of the composition of nutrients present in the medium on which *I. obliquus* mycelium was grown. It can be concluded that natural chaga has a high concentration of potassium due to the tree trunks on which it parasites and the wild habitat. Chaga grown under sterile conditions will not be toxic. The content of all elements of the chaga samples is available in supplement.

Table 4
Elemental composition of chaga samples (μg/g chaga)

Elements	MAX	MIN	Median	Elements	MAX	MIN	Median
Al	238	<2	91	**Mn**	341	32	240
B	27	<11	<11	**Mo**	1.0	<0.3	<0.3
Ba	81	2	52	**Na**	1503	<19	26
Ca	4154	74	2408	**Ni**	25	<0.2	0.4
Cd	1.9	0.3	<0.2	**P**	3101	76	208
Co	0.5	<0.3	<0.3	**Pb**	2.3	<1.4	<1.4
Cr	60	<0.8	3.1	**S**	1567	146	334
Cu	12	1.6	5.3	**Si**	240	<3	120
Fe	275	13	108	**Sr**	56	1.3	12
K	94369	4660	41175	**Ti**	8.7	<0.3	4.3
Mg	2125	814	1447	**Zn**	84	17	60

** MAX - the highest concentration detected; MIN - the lowest concentration found.*

Principal component analysis was applied to determine the difference between the origins of chaga from alder and birch, as well as from different countries (**Figure 9**). Finnish natural birch chaga is characterized by high concentrations of elements such as chromium and nickel. Some samples from Russian have elevated concentrations of aluminium, barium, iron and titanium. It can be assumed that they were collected near to the Ural Mountains. The chromium content in Latvian samples is below the detection level. Interestingly, the 17BL sample collected at the roadside also has a heavy metal content below the LOD. The composition of elements of the samples is similar to the samples collected in the forest. Thus, *I. obliquus* does not absorb heavy metals from the polluted environment. The Latvian commercial chaga (13BL) does not differ significantly from the wild samples in terms of content. The PCA analysis of microelements in chaga samples of different origins shows the specific characteristics of the sampling site - natural birch chaga does not form clusters or accumulate together. Metals of chaga depend on environmental influences (geographical origin) and pollution.

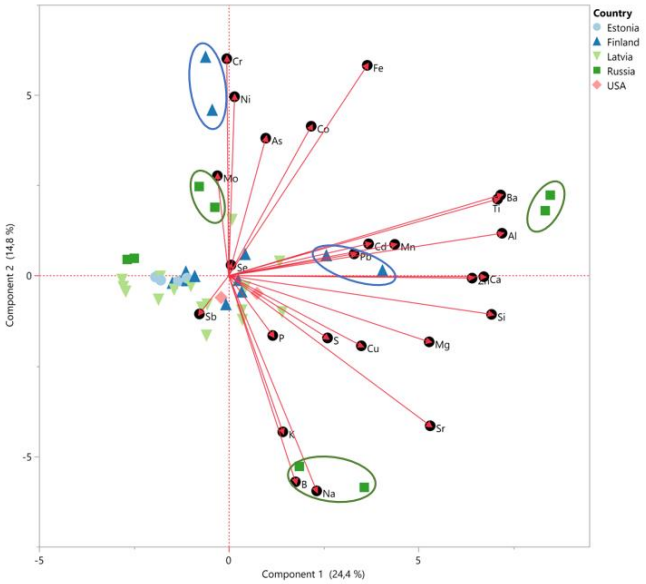

Fig. 9. Principal component analysis (PCA) of multielement parameters within different origin chaga samples

Antimicrobial activity of chaga extracts

Sensitivity of three bacteria and yeast was determined by agar diffusion method to ethyl acetate and water extracts of chaga of different origins. The inhibitory zone was measured and the test data are summarized in **Figure 10**. The results show that *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are resistant to chaga triterpenoids. In turn, all extracts act as good inhibitors of pathogenic *Candida albicans* yeasts. The antifungal activity of aqueous extracts of chaga samples was reduced. Glamočlija tested chaga water and ethanol extracts of various origins with MIC tests to pathogenic bacteria and pathogenic fungi. According to data, ethanol extract has a greater effect than aqueous extract on pathogenic bacteria, but similar to fungi [8].

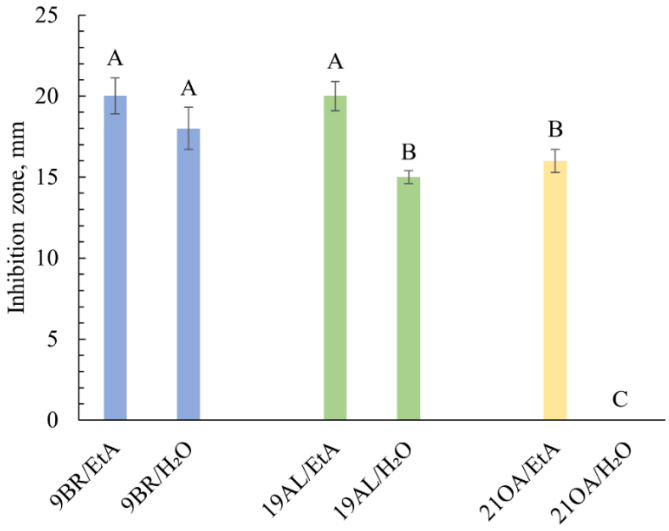

Fig. 10. Antimicrobial activity of chaga extracts against *Candida albicans* yeast (means and standard deviations, n=3)

The aqueous extract of cultured chaga did not show inhibitory activity against *Candida albicans*. Thus, it can be concluded that artificially grown chaga on oats have a different composition of the aqueous extract, which does not contain compounds that inhibit the growth of yeast. But water and lipid extracts of birch and alder chaga have a similar effect against *C. albicans.* The study should be extended to determine the minimum inhibitory concentration or MIC for the extracts. Using antimicrobial tests of chaga water extract, it is possible to distinguish whether the chaga was grown artificially or naturally.

4. Conclusions

Chaga is a rich source of unique lipids. The polarity of the solvent affects the lipid extraction efficiency from the chaga. Maximal lipid yields can be obtained using intensive sample treatment during extraction, for example, treatment with ultrasound or using accelerated solvent extraction and optimal yield provides use of solvents such as chloroform. Extraction using CO_2 in the supercritical state in the presence of a cosolvent can be considered as prospective.

High content of fatty acids was found in cultivated chaga in the absence of sterols and triterpenoids. As well as increased concentration of nitrogen, phosphorus and sulphur, which are typical nutrients for grain. The lipid profile can be used to distinguish between cultured/wild, birch/alder chaga. Almost all studied samples have similar values of stable carbon isotope ratios indicating that the chaga belongs to C_3 plants growing at moderate temperatures. Ratio of stable nitrogen isotopes in chaga samples do not depend on temperature, but depend on degree of mineralization of the soil and captured protein. Using IRMS analysis, it is not possible to determine the country of origin of the chaga sample. However, the values of stable nitrogen to oxygen isotope ratios, allows to identify natural or cultured chaga. The elemental composition of the chaga characterizes the specific sampling site – natural birch chaga does not cluster together. Chaga lipids and aqueous extracts have antifungal properties. Artificial/natural chaga can be distinguished by antimicrobial assays.

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5. References

1. Shikov, A. N., Pozharitskaya, O. N., Makarov, V. G., Wagner, H., Verpoorte, R., Heinrich, M. (2014). Medicinal plants of the Russian pharmacopoeia; their history and applications. Journal of Ethnopharmacology, 154(3), 481–536.
2. Saar, M. (1991). Fungi in khanty folk medicine. Journal of Ethnopharmacology, 31(2), 175–179. doi:10.1016/0378-8741(91)90003-v
3. Park, Y.-M., Won, J.-H., Kim, Y.-H., Choi, J.-W., Park, H.-J., Lee, K.-T. (2005). In vivo and in vitro anti-inflammatory and anti-nociceptive effects of the methanol extract of Inonotus obliquus. Journal of Ethnopharmacology, 101(1-3), 120–128. doi:10.1016/j.jep.2005.04.003
4. Ma, L., Chen, H., Dong, P., Lu, X. (2013). Anti-inflammatory and anticancer activities of extracts and compounds from the mushroom Inonotus obliquus. Food Chemistry, 139(1-4), 503-508. doi:10.1016/j.foodchem.2013.01.030
5. Szychowski, K. A., Bartosz, S., Tadeusz, P., Jan, G. (2020). Inonotus obliquus–from folk medicine to clinical use. Journal of Traditional and Complementary Medicine. doi:10.1016/j.jtcme.2020.08.003
6. Van Q., Nayak B.N., Reimer M., Jones P.J.H., Fulcher R.G., C.B. Rempel (2009). Anti-inflammatory effect of Inonotus obliquus, Polygala senega L., and Viburnum trilobum in a cell screening assay. Journal of Ethnopharmacol, 125 (3) (2009), pp. 487-493.
7. Lee, S. H., Hwang, H. S., Yun, J. W. (2009). Antitumor activity of water extract of a mushroom, Inonotus obliquus, against HT-29 human colon cancer cells. Phytotherapy Research, 23(12), 1784–1789. doi:10.1002/ptr.2836
8. Glamočlija, J., Ćirić, A., Nikolić, M., Fernandes, Â., Barros, L., Calhelha, R. C., Ferreira I., Soković M., Van Griensven, L. (2015). Chemical characterization and biological activity of Chaga (Inonotus obliquus), a medicinal "mushroom." Journal of Ethnopharmacology, 162, 323–332. doi:10.1016/j.jep.2014.12.069
9. Nagajyothi, P. C., Sreekanth, T. V. M., Lee, J., Lee, K. D. (2014). Mycosynthesis: Antibacterial, antioxidant and antiproliferative activities of silver nanoparticles synthesized from Inonotus obliquus (Chaga mushroom) extract. Journal of Photochemistry and Photobiology B: Biology, 130, 299–304. doi:10.1016/j.jphotobiol.2013.11.022

10. Shin, Y., Tamai, Y., Terazawa, M. (2000). Chemical Constituents of Inonotus obliquus I.: A new triterpene, 3β-hydroxy-8, 24-dien-lanosta-21, 23-lactone from sclerotium. Eurasian Journal of Forest Research, 1, 43-50.
11. Saleem, M. (2009). Lupeol, a novel anti-inflammatory and anti-cancer dietary triterpene. Cancer Letters, 285(2), 109–115. doi:10.1016/j.canlet.2009.04.033
12. Zhao, L. W., Zhong, X. H., Yang, S. Y., Zhang, Y. Z., & Yang, N. J. (2014). Inotodiol inhibits proliferation and induces apoptosis through modulating expression of cyclinE, p27, bcl-2, and bax in human cervical cancer HeLa cells. Asian Pacific Journal of Cancer Prevention, 15, 3195-3199.
13. Nikitina, S. A., Habibrakhmanova, V. R., Sysoeva, M. A. (2016). Chemical composition and biological activity of triterpenes and steroids of chaga mushroom. Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 10(1), 63–69. doi:10.1134/s1990750816010108
14. Wold, C. W., Gerwick, W. H., Wangensteen, H., & Inngjerdingen, K. T. (2020). Bioactive triterpenoids and water-soluble melanin from Inonotus obliquus (Chaga) with immunomodulatory activity. Journal of Functional Foods, 71, 104025. doi:10.1016/j.jff.2020.104025
15. Lee, M. G., Kwon, Y. S., Nam, K. S., Kim, S. Y., Hwang, I. H., Kim, S., Jang, H. (2021). Chaga mushroom extract induces autophagy via the AMPK-mTOR signaling pathway in breast cancer cells. Journal of Ethnopharmacology, 274, 114081.
16. Potočnik, D., Nečemer, M., Perišić, I., Jagodic, M., Mazej, D., Camin, F., Ogrinc, N. (2020). Geographical verification of Slovenian milk using stable isotope ratio, multi-element and multivariate modelling approaches. Food Chemistry, 326, 126958. doi:10.1016/j.foodchem.2020.126958
17. Mahne Opatić, A., Nečemer, M., Lojen, S., Vidrih, R. (2017). Stable isotope ratio and elemental composition parameters in combination with discriminant analysis classification model to assign country of origin to commercial vegetables – A preliminary study. Food Control, 80, 252–258. doi:10.1016/j.foodcont.2017.05.010
18. Rogowska, A., Styczyński, M., Pączkowski, C., Szakiel, A., Pinheiro de Carvalho, M. Â. A. (2019). GC-MS analysis of steroids and triterpenoids occurring in leaves and tubers of Tamus edulis Lowe. Phytochemistry Letters. doi:10.1016/j.phytol.2019.01.012
19. Becker, R., Dashbaldan, S., Pączkowski, C., Golis, T., Szakiel, A. (2019). Comparison of steroids and triterpenoids in leaf cuticular waxes of selected Polish and Russian cultivars and genotypes of edible honeysuckle. Phytochemistry Letters. doi:10.1016/j.phytol.2019.01.009
20. Leal, P. F., Maia, N. B., Carmello, Q. A. C., Catharino, R. R., Eberlin, M. N., Meireles, M. A. A. (2007). Sweet basil (Ocimum basilicum) extracts obtained by supercritical fluid extraction (SFE): Global yields, chemical composition, antioxidant activity, and estimation of the cost of manufacturing. Food and Bioprocess Technology, 1(4), 326–338. doi:10.1007/s11947-007-00301.
21. Tang, Y., Zhang, Y., Rosenberg, J. N., Sharif, N., Betenbaugh, M. J., Wang, F. (2016). Efficient lipid extraction and quantification of fatty acids from algal biomass using accelerated solvent extraction (ASE). RSC advances, 6(35), 29127-29134.
22. Radenkovs, V., Püssa, T., Juhnevica-Radenkova, K., Kviesis, J., Salar, F. J., Moreno, D. A., Drudze, I. (2020). Wild apple (Malus spp.) by-products as a source of phenolic compounds and vitamin C for food applications. Food Bioscience, 100744. doi:10.1016/j.fbio.2020.100744
23. Zhang, Y., Zhao, Y., Cui, H., Cao, C., Guo, J., Liu, S. (2011). Comparison of Hypoglycemic Activity of Fermented Mushroom of Inonotus obliquus Rich in Vanadium and Wild-Growing I. obliquus. Biological Trace Element Research, 144(1-3), 1351–1357. doi:10.1007/s12011-011-9043-8
24. Snyder, J. M., King, J. W., Taylor, S. L., Neese, A. L. (1999). Concentration of phytosterols for analysis by supercritical fluid extraction. Journal of the American Oil Chemists' Society, 76(6), 717–721. doi:10.1007/s11746-999-0165-5
25. Taylor, S. L., King, J. W. (2000). Enrichment of ferulate phytosterol esters from corn fiber oil using supercritical fluid extraction and chromatography. Journal of the American Oil Chemists' Society, 77(6), 687–688. doi:10.1007/s11746-000-0110-9
26. Kahlos, K. (1994). Inonotus obliquus (Chaga Fungus): in vitro culture and the production of inotodiol, sterols, and other secondary metabolites. In Medicinal and Aromatic Plants VI (pp. 179-198). Springer, Berlin, Heidelberg. doi: 10.1007/978-3-642-57970-7_12

27. Chen, J. (1996). Diarylheptanoid glycosides from red alder bark.
28. Zhang, Q., Wang, J., He, H., Liu, H., Yan, X., Zou, K. (2014). Trametenolic Acid B Reverses Multidrug Resistance in Breast Cancer Cells Through Regulating the Expression Level of P-Glycoprotein. Phytotherapy Research, 28(7), 1037-1044.
29. Wang, J., Wang, A., He, H., She, X., He, Y., Li, S., Zou, K. (2019). Trametenolic acid B protects against cerebral ischemia and reperfusion injury through modulation of microRNA-10a and PI3K/Akt/mTOR signaling pathways. Biomedicine & Pharmacotherapy, 112, 108692.
30. Zhang, Q., Huang, N., Wang, J., Luo, H., He, H., Ding, M., Zou, K. (2013). The H+/K+-ATPase inhibitory activities of Trametenolic acid B from Trametes lactinea (Berk.) Pat, and its effects on gastric cancer cells. Fitoterapia, 89, 210-217.
31. The Global Fungal Red List, http://iucn.ekoo.se/en/iucn/welcome
32. Sagayama, K., Tanaka, N., Fukumoto, T., Kashiwada, Y. (2019). Lanostane-type triterpenes from the sclerotium of Inonotus obliquus (Chaga mushrooms) as proproliferative agents on human follicle dermal papilla cells. Journal of Natural Medicines, 73(3), 597-601.
33. Kim, J., Yang, S. C., Hwang, A. Y., Cho, H., Hwang, K. T. (2020). Composition of triterpenoids in Inonotus obliquus and their anti-proliferative activity on cancer cell lines. Molecules, 25(18), 4066.
34. Taji, S., Yamada, T., In, Y., Wada, S. I., Usami, Y., Sakuma, K., Tanaka, R. (2007). Three new lanostane triterpenoids from Inonotus obliquus. Helvetica Chimica Acta, 90(11), 2047-2057.
35. Handa, N., Yamada, T., Tanaka, R. (2012). Four new lanostane-type triterpenoids from Inonotus obliquus. Phytochemistry Letters, 5(3), 480-485. DOI: 10.1016/j.phytol.2012.04.010
36. Liu, C., Zhao, C., Pan, H. H., Kang, J., Yu, X. T., Wang, H. Q., Chen, R. Y. (2014). Chemical constituents from Inonotus obliquus and their biological activities. Journal of Natural Products, 77(1), 35-41. doi: 10.1021/np400552w
37. Géry, A., Dubreule, C., André, V., Rioult, J. P., Bouchart, V., Heutte, N., Garon, D. (2018). Chaga (Inonotus obliquus), a future potential medicinal fungus in oncology? A chemical study and a comparison of the cytotoxicity against human lung adenocarcinoma cells (A549) and human bronchial epithelial cells (BEAS-2B). Integrative cancer therapies, 17(3), 832-843. doi: 10.1177/1534735418757912
38. Zheng, W. F., Liu, T., Xiang, X. Y., Gu, Q. (2007). Sterol composition in field-grown and cultured mycelia of Inonotus obliquus. Acta pharmaceutica Sinica, 42(7), 750.
39. Du, D., Zhu, F., Chen, X., Ju, X., Feng, Y., Qi, L. W., Jiang, J. (2011). Rapid Isolation and Purification of Inotodiol and Trametenolic Acid from Inonotus obliquus by High-speed Counter-current Chromatography with Evaporative Light Scatting Detection. Phytochemical Analysis, 22(5), 419-423. doi: 10.1002/pca.1297
40. Sun, Y., Yin, T., Chen, X.-H., Zhang, G., Curtis, R. B., Lu, Z.-H., Jiang, J.-H. (2011). In Vitro Antitumor Activity and Structure Characterization of Ethanol Extracts from Wild and Cultivated Chaga Medicinal Mushroom, Inonotus obliquus (Pers.:Fr.) Pilát (Aphyllophoromycetideae). International Journal of Medicinal Mushrooms, 13(2), 121–130. doi:10.1615/intjmedmushr.v13.i2.40
41. Kim, J.H., Park, S.K., Cho, C.W. et al. Quality control and evaluation of Inonotus obliquus using HPLC method with novel marker compounds. Journal of Analytical Science Technology 11, 52 (2020). doi: 10.1186/s40543-020-00249-z
42. Gallagher, P. A., Shoemaker, J. A., Wei, X., Brockhoff-Schwegel, C. A., Creed, J. T. (2001). Extraction and detection of arsenicals in seaweed via accelerated solvent extraction with ion chromatographic separation and ICP-MS detection. Fresenius' Journal of Analytical Chemistry, 369(1), 71–80. doi:10.1007/s002160000585
43. Kellogg, J. J., Wallace, E. D., Graf, T. N., Oberlies, N. H., Cech, N. B. (2017). Conventional and accelerated-solvent extractions of green tea (Camellia sinensis) for metabolomics-based chemometrics. Journal of Pharmaceutical and Biomedical Analysis, 145, 604–610. doi:10.1016/j.jpba.2017.07.027
44. Shen, J., Shao, X. (2005). A comparison of accelerated solvent extraction, Soxhlet extraction, and ultrasonic-assisted extraction for analysis of terpenoids and sterols in tobacco. Analytical and Bioanalytical Chemistry, 383(6), 1003–1008. doi:10.1007/s00216-005-0078-6

45. Rudzińska, M., Przybylski, R., Wąsowicz, E. (2009). Products Formed During Thermo-oxidative Degradation of Phytosterols. Journal of the American Oil Chemists' Society, 86(7), 651–662. doi:10.1007/s11746-009-1397-0
46. Mohammad Azmin, S. N. H., Abdul Manan, Z., Wan Alwi, S. R., Chua, L. S., Mustaffa, A. A., Yunus, N. A. (2016). Herbal Processing and Extraction Technologies. Separation & Purification Reviews, 45(4), 305–320. doi:10.1080/15422119.2016.1145395
47. Rozzi, N. L., Singh, R. K., Vierling, R. A., Watkins, B. A. (2002). Supercritical Fluid Extraction of Lycopene from Tomato Processing Byproducts. Journal of Agricultural and Food Chemistry, 50(9), 2638–2643. doi:10.1021/jf011001t
48. Murga, R., Ruiz, R., Beltrán, S., Cabezas, J.L., 2000. Extraction of natural complex phenols and tannins from grape seeds by using supercritical mixtures of carbon dioxide and alcohol. Journal of Agricultural and Food Chemistry 48 (8), 3408– 3412
49. Ibáñez, E., Palacios, J., Señoráns, F. J., Santa-María, G., Tabera, J., Reglero, G. (2000). Isolation and separation of tocopherols from olive by-products with supercritical fluids. Journal of the American Oil Chemists' Society, 77(2), 187–190. doi:10.1007/s11746-000-0030-8
50. Huynh, N. (2019). Supercritical CO2 extraction of triterpenoids from Inonotus obliquus. Master thesis.
51. Wei, L., Zhang, W., Yin, L., Yan, F., Xu, Y., Chen, F. (2015). Extraction optimization of total triterpenoids from Jatropha curcas leaves using response surface methodology and evaluations of their antimicrobial and antioxidant capacities. Electronic Journal of Biotechnology, 18(2), 88–95. doi:10.1016/j.ejbt.2014.12.005
52. Danezis, G. P., Tsagkaris, A. S., Camin, F., Brusic, V., Georgiou, C. A. (2016). Food authentication: Techniques, trends & emerging approaches. TrAC Trends in Analytical Chemistry, 85, 123-132.
53. Kelly, S. D. (2003). Using stable isotope ratio mass spectrometry (IRMS) in food authentication and traceability. Food authenticity and traceability, 156-183.
54. Kelly, S., Heaton, K., Hoogewerff, J. (2005). Tracing the geographical origin of food: The application of multi-element and multi-isotope analysis. Trends in Food Science & Technology, 16(12), 555–567. doi:10.1016/j.tifs.2005.08.008
55. Krull, E., Sachse, D., Mügler, I., Thiele, A., Gleixner, G. (2006). Compound-specific δ13C and δ2H analyses of plant and soil organic matter: A preliminary assessment of the effects of vegetation change on ecosystem hydrology. Soil Biology and Biochemistry, 38(11), 3211-3221. doi: 10.1016/j.soilbio.2006.04.008
56. Schwab, V. F., Garcin, Y., Sachse, D., Todou, G., Séné, O., Onana, J. M., Gleixner, G. (2015). Effect of aridity on $\delta^{13}C$ and $\delta^{2}D$ values of C_3 plant-and C_4 graminoid-derived leaf wax lipids from soils along an environmental gradient in Cameroon (Western Central Africa). Organic Geochemistry, 78, 99-109.
57. Cui, J., Lamade, E., Fourel, F., Tcherkez, G. (2020). $\delta^{15}N$ values in plants are determined by both nitrate assimilation and circulation. New Phytologist, 226(6), 1696-1707. doi: 10.1111/nph.16480.
58. Yang, Y., Siegwolf, R. T., Körner, C. (2015). Species specific and environment induced variation of δ13C and δ15N in alpine plants. Frontiers in Plant Science, 6, 423. doi: doi.org/10.3389/fpls.2015.00423
59. Shashkina, M. Y., Shashkin, P. N., Sergeev, A. V. (2006). Chemical and medicobiological properties of chaga. Pharmaceutical Chemistry Journal, 40(10), 560-568. doi: 10.1007/s11094-006-0194-4.
60. Rossier, J. S., Maury, V., de Voogd, B., Pfammatter, E. (2014). Use of Isotope Ratio Mass Spectrometry (IRMS) Determination ($^{18}O/^{16}O$) to Assess the Local Origin of Fish and Asparagus in Western Switzerland. CHIMIA International Journal for Chemistry, 68(10), 696–700. doi: 10.2533/chimia.2014.696.
61. Drivelos, S. A., Georgiou, C. A. (2012). Multi-element and multi-isotope-ratio analysis to determine the geographical origin of foods in the European Union. TrAC Trends in Analytical Chemistry, 40, 38–51. doi:10.1016/j.trac.2012.08.003
62. Son, J. H., Kim, I. D., Kim, M. O., Gaveh, E. A., Shin, D. H. (2013). Quality characteristics of rice inoculated with Inonotus obliquus mycelia and incubated under different cultivating conditions. African Journal of Biotechnology, 12(33). doi: 10.5897/AJB12.2387.