# Compositional and morphological analyses of wax in northern wild berry species

Priyanka Trivedi[a,*], Katja Karppinen[a], Linards Klavins[b], Jorens Kviesis[b], Petri Sundqvist[c], Nga Nguyen[a], Esa Heinonen[c], Maris Klavins[b], Laura Jaakola[d,e], Juha Väänänen[c], Janne Remes[c], Hely Häggman[a]

[a] Department of Ecology and Genetics, University of Oulu, FI-90014 Oulu, Finland

[b] Department of Environmental Science, University of Latvia, LV-1004, Riga, Latvia

[c] Centre of Microscopy and Nanotechnology, University of Oulu, FI-90014 Oulu, Finland

[d] NIBIO, Norwegian Institute of Bioeconomy Research, NO-1431 Ås, Norway

e Climate laboratory Holt, Department of Arctic and Marine Biology, UiT The Arctic University of Norway, NO-9037 Tromsø, Norway

Priyanka Trivedi: priyanka.priyanka@oulu.fi

Katja Karppinen: katja.karppinen@oulu.fi

Linards Klavins: linards.klavins@lu.lv

Jorens Kviesis: jorens.kviesis@lu.lv

Petri Sundqvist: petrisundq@gmail.com

Nga Nguyen: thi.nguyen@oulu.fi

Esa Heinonen: esa.heinonen@oulu.fi

Maris Klavins: maris.klavins@lu.lv

Laura Jaakola: laura.jaakola@uit.no

Juha Väänänen: juha.vaananen@oulu.fi

Janne Remes: janne.remes@oulu.fi

Hely Häggman: hely.haggman@oulu.fi

*Corresponding author

**Abstract**

Aerial surfaces of plants are covered by a waxy cuticle protecting plants from excessive water loss and UV light. In the present study, composition and morphology of cuticular waxes of northern wild berry species bilberry (*Vaccinium myrtillus* L.), lingonberry (*V. vitis-idaea* L.), bog bilberry (*V. uliginosum* L.) and crowberry (*Empetrum nigrum* L.) were investigated. Scanning electron microscopy (SEM) revealed differences in epicuticular wax morphologies and gas chromatography–mass spectrometry (GC–MS) analysis confirmed variation in chemical composition of cuticular waxes between the berry species. The dominant compounds in bilberry and lingonberry cuticular waxes were triterpenoids while fatty acids and alkanes were the dominant ones in bog bilberry and crowberry, respectively. Wax extracted by supercritical fluid extraction (SFE) from industrial press cakes of bilberry and lingonberry contained linoleic acid and γ-linolenic acid as the dominant compounds. Furthermore, *in vitro* sun protection factor (SPF) of berry waxes depicted good UV-B absorbing capacities.

*Keywords*: *Vaccinium*, *Empetrum*, fruits, cuticular wax, chemical composition, morphology, triterpenoids

## 1. Introduction

Cuticle acts as an interface between plant and environment covering the aerial parts of land plants, including leaves, stems and fruits. Plant cuticle evolved 450 million years ago as a protection against non-transpirational water loss but it also protects plants from UV light and pathogen attacks (Yeats & Rose, 2013). Solar radiation reaching the earth includes 10% UV light among which UV-B (280-320 nm) has the highest energy creating a need for protection not only for plants but also for humans due to risk of skin cancer.

The plant cuticle is composed of a polyester polymer called cutin and cuticular wax. Cuticular wax is a complex mixture of very-long-chain fatty acids and their derivatives such as alkanes, ketones, primary and secondary alcohols, aldehydes and esters but also includes secondary metabolites such as triterpenoids, sterols, tocopherols and phenolic compounds (Yeats & Rose, 2013). Cuticular wax composition can vary greatly depending on species, organ and developmental stage (Lara, Belge, & Goulao, 2014). The cuticular wax is present as intracuticular wax, an amorphous mixture of lipids embedded in the cutin, and outermost epicuticular wax (Barthlott, Mail, Bhushan, & Koch, 2017). The epicuticular wax forms various morphologies such as films or different types of three-dimensional crystallized structures on plant surfaces (Jeffree, Riederer, & Müller, 2006). The epicuticular wax can be visible to the naked eye either as whitish, dull or glossy coating.

The studies on plant cuticular waxes have largely focused on vegetative parts such as leaves while surfaces of fruits and berries have been less studied (Trivedi et al., 2019). Berries are important component of healthy diet and it is well established that the dietary intake of berries has a positive and profound impact on human health (Seeram, 2008). The health effects are mainly due to bioactive compounds such as polyphenols, flavonoids, carotenoids and vitamins (Jimenez-Garcia et al., 2013). However, berries also include other types of bioactive components such as compounds present in wax. For example, triterpenoids have various health beneficial properties such as anticancer, anti-inflammatory, antimicrobial and cardioprotective (Szakiel et al., 2012b). Juice industry is one of the

major users of berries and the industrial leftovers, berry press cakes, form a potential source for bioactive compounds and berry wax fractions to be utilized in commercial products.

Bilberry (*Vaccinium myrtillus* L.) and lingonberry (*V. vitis-idaea* L.) are economically the most important wild berries of Northern Europe widely utilized by food industry including juice industry. Crowberry (*Empetrum nigrum* L.) and bog bilberry (*V. uliginosum* L.) are less utilized nevertheless widely distributed wild berries in northern areas. These berry species have been studied extensively for secondary metabolites (Jurikova et al., 2016; Karppinen, Zoratti, Nguyenquynh, Häggman, Jaakola, 2016). However, they have not been investigated for their cuticular wax composition, although triterpenoid profile of bilberry cuticular wax has been reported earlier (Szakiel, Pączkowski, & Huttunen 2012a).

The objective of the present study was to investigate the amount, chemical composition as well as morphology of cuticular wax in important northern wild berries, including bilberry, lingonberry, bog bilberry and crowberry. Also, the berry press cakes (residues of juice industry) of bilberry and lingonberry were extracted by supercritical fluid extraction (SFE), and the composition analyzed. In addition, *in vitro* sun protection factor (SPF) of the waxes is reported and the potential commercial use of berry waxes discussed.

## 2. Materials and methods

### *2.1. Plant material*

Berries of four different wild species were utilized in the present study, namely bilberry, lingonberry, bog bilberry and crowberry. Ripe fruits of the berry species were collected carefully using forceps in August 2017 from natural forest stands in Oulu region, Finland. Industrial press cakes of bilberry and lingonberry were obtained from Polarica Ltd., Tornio, Finland.

### *2.2. Scanning electron microscopy (SEM)*

For SEM analysis, the fresh berries were immediately dried after collection by using a vacuum freeze-drier (Edwards High Vacuum International, West Sussex, England) before fixing on aluminium stubs. The berry surfaces were then sputter-coated with 20 nm layer of platinum by using a sputter coater (Agar High Resolution Sputter Coater, Agar Scientific Ltd, Essex, UK) and then investigated for the three-dimensional surface micromorphology by using SEM (Helios Nanolab 600, Oregon, USA). SEM was operated at 5 kV with a current value of 86 pA at secondary electron mode. Images were taken at 2500X and 10000X magnification.

*2.3. Cuticular wax extraction and determination of wax amount*

The cuticular wax from the ripe berries of different berry species was separately extracted with chloroform (Sigma-Aldrich, St. Louis, USA) immediately after collection and transportation to the laboratory at ambient temperature. One hundred berries per species were individually dipped twice in 10 ml chloroform for 30 seconds. The two extracts were combined, evaporated to dryness under nitrogen flow at room temperature and the dry weight was measured. The cuticular wax extraction was performed in triplicates for each berry species. The amount of wax was expressed as weight per unit surface area ($\mu g/cm^2$). For calculating the surface areas, images of the dipped berries on a white surface were taken immediately after extraction. Image J software v1.50i (NIH, Maryland, USA) was used to calculate the total surface area of the berries as $S = 4\ \pi r^2$, where r is the radius of berry (assuming that the berries are spherical).

*2.4. Wax extraction from industrial berry press cakes*

The berry press cakes of bilberry and lingonberry were dried in an oven at 60 °C and milled to fine powder by using a handheld grinder before wax extraction. Supercritical fluid extraction (SFE) was performed by using Xtractor (Chematur Ecoplanning Pvt Ltd, Tampere, Finland). The operating parameters used for extraction were 350 bar at 60 °C with a $CO_2$ flow rate of 0.4-0.5 L/min for 10-L extraction. The yield of the wax was expressed as mg/g dry weight of starting material.

*2.5. GC-MS analysis*

Derivatization of fatty acids was performed as previously described (Dobson, Shrestha, Hilz, Karjalainen, McDougall & Stewart 2012). Extracted berry wax was dissolved in 0.5 mL toluene (Sigma-Aldrich). Then, 3 mL of 14% boron trifluoride-methanol solution (Sigma-Aldrich) was added and the mixture heated at 60 °C for 180 min. Resulting fatty acid methyl esters were dissolved in hexane and used for GC-MS analysis.

GC-MS analysis was performed using PerkinElmer Clarus 580 system equipped with Clarus SQ 8 C mass-selective detector (Waltham, MA, USA) and Omegawax 250 column (30 m × 0.25 mm, 0.25 µm, Darmstadt, Germany). Analysis of FAME's and polyfunctional compounds as trimethylsilyl derivatives was performed on ELITE 5MS column (30 m × 0.25 mm, 0.25 mm, PerkinElmer, Waltham, MA, USA) after derivatization of hexane fraction with 60 µL *N*,*O*-Bis (trimethylsilyl)trifluoroacetamide (Sigma-Aldrich). Analysis in both columns was initiated at 75 °C for 2 min and then increased from 75 °C to 150 °C at a rate of 20 °C/min. For Omegawax 250 column, further temperature was increased from 150 °C to 270 °C and for Elite 5MS the increase was from 150 °C to 310 °C at 4 °C/min. In the final isothermal step, temperature was held for 5 min at 270 °C for Omegawax 250 and 310 °C for Elite 5MS. The total run time was 39.50 min and 54.75 min for Omegawax 250 and Elite 5MS, respectively. Injection volume was 0.5 µL with injection and interface temperatures kept at 290 °C. Helium (AGA, Riga, Latvia) was used as a carrier gas at the flow rate of 1.0 mL/min and split flow of 10.0 mL/min. Electron impact was set to 70 eV and scan range from 42 to 750 m/z. Identification of compounds was done using NIST MS 2.2 library (Gaithersburg, MD, USA). The analysis was performed in triplicate. Quantification of compounds was done using standard solutions of methyl heptadecanoate (≥99.0%), ergosterol (≥99%), hexadecanol (≥99%), 1-dodecanal (≥98.0%), (±)-α-tocopherol (99%), 1-octadecanol (99%), and *n*-tetracosane (≥99.5%) obtained from Sigma-Aldrich in the concentration range of 1.5–500 µg/mL.

145 *2.5 Determination of in vitro sun protection factor (SPF)*

146 Extracted wax was dissolved in methanol (Fisher Scientific, Waltham, USA) for bilberry and
147 lingonberry waxes, and hexane (Fisher Scientific) for bog bilberry and crowberry waxes. The choice
148 of solvent was based on the maximal solubility of wax in the respective solvents. The absorption
149 spectra of the wax solutions in quartz cuvette were obtained in the range of 290 to 320 nm every 1
150 nm by using UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific, Vantaa, Finland).
151 Measurements were done in triplicates. The SPF was calculated by the following equation (Mansur
152 et al., 1986):

153 $$SPF = CF \times \sum_{290}^{320} EE\ (\lambda) \times I\ (\lambda) \times Abs\ (\lambda)$$

154 In the equation, EE (λ) is erythemal efficiency spectrum, I (λ) is solar simulator intensity spectrum,
155 Abs (λ) is the absorbance of the measured sample, and CF is a correction factor (= 10). The constant
156 values of normalized product function (EE × I) used in the calculations as determined by Sayre et
157 al. (1979) can be found in Supplementary Table 1.

158

159 *2.6. Statistical analysis*

160 One-way analysis of variance (ANOVA) with Duncan's multiple range test at $p < 0.05$ was
161 performed using SPSS statistical program version 25.0 (IBM, Chicago, USA). Principal component
162 analysis was performed by using SAS JMP®, Version 13 (SAS Institute Inc., Cary, NC, USA).

163

164 **3. Results and discussion**

165 *3.1 Morphology of epicuticular wax*

166 The epicuticular wax on the surface of berries appeared as a whitish wax on ripe fruits of bilberry
167 (Fig. 1a) and bog bilberry (Fig. 1b), while glossy wax was present on the ripe fruits of lingonberry
168 (Fig. 1c) and crowberry (Fig. 1d). Despite of the similar appearance of bilberry and bog bilberry wax
169 by the naked eye, SEM analysis revealed different type of surface morphology between the waxes.

Syntopism, a phenomenon of occurrence of more than one type of crystalloids on one cell surface was visible on bilberry and lingonberry fruits. According to the previously made classification (Barthlott et al.,1998; Jeffree, Riederer, & Müller, 2006), platelets along with rodlet-like structures were observed on bilberry surface (Fig. 1a). Instead, bog bilberry surface showed only coiled rodlets in SEM analysis (Fig. 1b). On the other hand, lingonberry fruit surface showed a thick crust of wax with a syntopism of plates and platelets (Fig. 1c). On crowberry surface, plate like morphology was observed on smooth layer of wax (Fig. 1d).

From the SEM analysis it can be seen that in lingonberry and crowberry fruits the density of epicuticular wax crystals is lower compared to the fruits of bilberry and bog bilberry. Less dense epicuticular wax crystals have earlier been associated with glossy phenotype in orange fruits (Liu, Zeng, Ji, Liu, Liu & Liu, 2012). Since the wax chemical composition highly affects the crystal formation on the plant surface (Koch & Ensikat, 2008), our SEM analysis indicates differences in cuticular wax composition between the studied berry species and, thus, it was studied in more detail.

*3.2. Amount and chemical composition of cuticular wax*

The cuticular wax from the surface of berries was extracted with chloroform. High variability was observed in the cuticular wax load between the different berry species. The amount of wax was 108.5, 331.3, 921.8 and 871.1 μg/cm$^2$ in fruits of bilberry, bog bilberry, crowberry and lingonberry, respectively (Fig. 2a). The higher quantities of wax observed in glossy berries (lingonberry and crowberry), where the wax crystallization level is lower, means that the epicuticular wax crusts on lingonberry and films on crowberry is thick. The amount of cuticular wax in bilberry was comparable to the amount reported for different blueberry (*Vaccinium* spp.) varieties (from 48 μg/cm$^2$ to 172 μg/cm$^2$) in earlier studies (Chu, Gao, Cao, Fang, Chen & Xiao, 2017). Generally, highly variable amount of cuticular wax has been reported earlier for different fruits species and cultivars, for example, 337–770 μg/cm$^2$ in persimmon fruits (Tsubaki, Ozaki, Yonemori & Azuma, 2012) and 366–1038 μg/cm$^2$ in apple fruit cultivars (Belding, Blankenship, Young & Leidy, 1998).

196 The GC-MS analysis shows that the chemical composition of cuticular wax varies markedly
197 between the berry species. The differences in chemical composition are likely due to genetic
198 differences in different berry species. The major classes of compounds in berry cuticular waxes were
199 found to be triterpenoids, alkanes, fatty acids, aldehydes, primary alcohols and ketones (Fig. 2b).
200 Secondary alcohols and esters were not found in this study. The major compounds found in berry
201 cuticular waxes are presented in Table 1. The cuticular wax constituents were subjected to the
202 principal component analysis (PCA) and the composition of the cuticular wax of the four examined
203 berry species show clear differences forming distinct clusters (Fig. 2c). Variance of the first two
204 components PC1 (57.9%) and PC2 (32.8%) accounts for 90.7% of the data variability (Fig. 2c). The
205 chemotaxonomic significance of cuticular wax composition for classifying family, genus or species
206 in plants is well established (Medina et al.,2006; Maffei et al., 2004). Our study shows that the
207 composition of cuticular wax is characteristic to different berry species and, therefore can be used to
208 distinguish between the investigated species.
209
210 *3.2.1 Triterpenoids*
211 Triterpenoids are commonly found in cuticular waxes of fruits (Szakiel et al., 2012b).
212 Triterpenoids represented the most abundant class of compounds in bilberry and lingonberry wax
213 accounting for 39.6% and 69.6% of total cuticular wax content, respectively, while in bog bilberry
214 and crowberry, triterpenoids accounted for only 3.2% and 3.4% of the total cuticular wax,
215 respectively (Fig. 2b). Both in bilberry and lingonberry wax, triterpene alcohols ($\beta$-amyrin, $\alpha$-amyrin,
216 lupeol) and triterpene acids (oleanolic acid, ursolic acid) were identified, while only lingonberry wax
217 contained adriaticol and uvaol (Table 1). In bog bilberry, oleanolic acid (3.1%) and ursolic acid
218 (1.8%) were identified (Table 1).
219 In bilberry, $\beta$-amyrin was found to be the most abundant triterpenoid (20.2% of total wax)
220 followed by oleanolic acid (8.9%) and $\alpha$-amyrin (7.1%). Among *Vaccinium* species, reports of
221 triterpenoid profiles in bilberry and blueberry cuticular waxes have concluded triterpene acids,

namely oleanolic acid and ursolic acid, as the dominant compounds (Szakiel et al., 2012a; Chu et al., 2017). This result is different from our study that indicated *β*-amyrin as the most abundant triterpenoid followed by oleanolic acid in bilberry cuticular wax. The variability in bilberry triterpenoid profiles between the two studies can be due to the difference in geographical origin of the sample or timing of sample collection.

In lingonberry cuticular wax, adriaticol (14.2%) followed by *α*-amyrin (13%) and *β*-amyrin (12.8%) were the dominant triterpenoids. Adriaticol has a structure similar to isoarborinol, a C3-oxygenated pentacyclic triterpenol. Isoarborinol derivatives, used as plant biomarkers are rarely reported in cuticular wax of plants. Isoarborinol derivatives have been reported earlier in leaf epicuticular wax of *Euphorbia lathyris* and plants of angiosperm families such as *Gramineae* (Van Bree et al., 2018). The finding of adriaticol in lingonberry wax gives possibility to use the compound as a biomarker for the identification of lingonberries. However, for that purpose, further studies of the wax profile of different berry species are still required.

The detection of platelets on the surface of bilberry and lingonberry in our study may be due to the dominance of triterpenoids in the cuticular wax. Triterpenoid rich platelets have been reported in *Sedum rupestre* leaf wax (Stevens, 1995). Also, triterpenoid rich wax of olive fruits (*Olea europaea)* and leaves of southern mahogany (*Eucalyptus botryoides)* recrystallized as platelets (Baker, 1982). In cases of syntopism, mostly platelets are found in combination with other crystalloid structures, which is consistent with our SEM analysis of bilberry and lingonberry surface. In the light of current knowledge, the chemical basis behind the presence of other crystalloid forms (rodlets in bilberry, plates in lingonberry) along with platelets cannot be determined but might either be due to the presence of other chemical compounds in cuticular wax, or due to specific genetic regulation or environmental conditions during crystallization of cuticular wax.

*3.2.2. Fatty acids*

Fatty acids were the major components of cuticular wax in bog bilberry accounting for 54.8% of the total wax (Fig. 2b). In bilberry, lingonberry and crowberry, fatty acids accounted for 31.7%, 15.9% and 14.4% of the total wax content, respectively (Fig. 2b). Fatty acids have not earlier been reported as the dominant component in fruit cuticular waxes although some fruits contain high amounts of fatty acids in their cuticles such as Asian pear (Yin et al., 2011) and cucumber (Wang et al., 2015) In bilberry cuticular wax, a vast variety of fatty acids was detected of which montanic acid ($C_{28:0}$) and cerotic acid ($C_{26:0}$) were the predominant ones (Table 1). Bog bilberry wax also had a wide variety of fatty acids with arachidic acid ($C_{20:0}$) being the dominant (Table 1). Crowberry fruit contained oleic acid ($C_{18:\ 1n-9}$) as the most abundant cuticular fatty acid while lingonberry wax contained mainly lignoceric acid ($C_{24:0}$). In blueberry, $C_{30}$ was dominant fatty acid (Chu et al., 2017), $C_{26}$, $C_{28}$ in newhall orange and satsuma mandarin respectively (Wang et al., 2014), while in pear $C_{16}$ and $C_{18}$ were predominant fatty acids (Wu et al., 2018). Bog bilberry shows unique composition with high arachidic acid content.

*3.2.3 Alkanes*

Alkanes were the predominant compounds in cuticular wax of crowberry fruits, constituting 70.6% of the total wax (Fig. 2b). Especially the amount of nonacosane was high followed by hentriacontane in crowberry (Table 1). Both nonacosane and hentriacontane are common compounds found in fruit epicuticular waxes (Trivedi et al., 2019). Nonacosane has been reported as the dominant compound in cuticular waxes of many fruits such as apple (Belding, Sutton, Blankenship, 2000), Asian pear (Yin et al., 2011) and cucumber (Wang et al., 2015). In lingonberry cuticular wax, alkanes represented 11.5% of total wax (Fig. 2b) with nonacosane as the predominant alkane (Table 1). Alkanes were a minor fraction in cuticular wax of bilberry and bog bilberry fruits constituting only 2.4% and 1.4% of total wax, respectively (Fig. 2b). Our morphological analyses with crowberry support the previous suggestions that waxes containing high amounts of alkanes, nonacosane and hentriacontane often have plate-like morphology (Jeffree, Riederer & Müller, 2006).

### *3.2.5 Other very-long-chain aliphatic compounds*

Aldehydes were found in cuticular wax of fruits of all studied berry species: 10.3%, 7.2%, 1.8% and 0.9% in bilberry, bog bilberry, crowberry and lingonberry, respectively (Fig. 2b). Octacosanal was the predominant aldehyde in bilberry and bog bilberry fruits while in crowberry and lingonberry, tetracosanal was the predominant one (Table 1). Aldehydes are rarely found abundantly in fruit cuticles with the exception in cucumber, cranberry (*Vaccinium macrocarpon*), and Citrus fruits (Trivedi et al., 2019). The detected aldehyde amount in bilberry cuticular wax in this study is close to that reported in cranberry earlier (Croteau and Fagerson, 1971).

Ketones accounted for the second largest fraction in bog bilberry wax (22.5%) with 2-heneicosanone as the most prominent ketone (Table 1). Ketones were also present in small quantities in bilberry (3.6%) and crowberry (0.03%) fruit cuticular wax (Fig. 2b).

Primary alcohols were present in berry waxes only in small quantities accounting for 1.3%, 2.3%, 0.6% and 2.6% in cuticular wax of bilberry, bog bilberry, crowberry and lingonberry fruits, respectively (Fig. 2b). Cinnamic acid in small quantities was found in bog bilberry cuticular wax. Minor quantity of p-coumaric acid was found in lingonberry cuticular wax (Table 1).

### *3.3 SFE extraction and chemical composition of wax*

By SFE extraction, green semisolid wax was obtained from bilberry press cake while the wax from lingonberry press cake was more yellow and greasy (Fig. 3a). The yield of lingonberry fruit wax was 1.02 % (10.2 mg/g DW) while the yield for bilberry was 0.45% (4.5 mg/g DW).

Compositional analysis by GC-MS showed that fatty acids were the most abundant constituents in the SFE extracts accounting for 83.4% and 76.9% in bilberry and lingonberry wax, respectively (Fig. 3b). The wax components also included alkanes, triterpenoids, phytosterols, vitamin E, and small amounts of aldehydes and cinnamic acid (Fig. 3b, Supplementary Table 2). In our study, linoleic acid and γ-linolenic acid were the predominant compounds constituting a total of 53.9% and 54.8%

of total wax for bilberry and lingonberry, respectively (Supplementary Table 2), that is similar as reported from SFE extraction of bilberry earlier (Jummah et al. 2015). Triterpenoids accounted for 3.0% and 14.3% for bilberry and lingonberry wax, respectively, with *β*-amyrin and lupeol as predominant compounds. Alkanes formed a minor fraction (9.9% for bilberry and 4.8% for lingonberry wax) with nonacosane as the dominant alkane in both bilberry and lingonberry press cakes. *β*-sitosterol was found in bilberry and lingonberry wax constituting 3% and 4.7% of total wax composition, respectively. Small quantities of cinnamic acid was also found in bilberry and lingonberry wax.

The wax derived from bilberry and lingonberry press cakes, in the present study, had different chemical composition compared to berry cuticular waxes. Phytosterols and vitamin E were detected in press cakes, but not in cuticular wax of berries. The difference in composition is most likely due to the presence of seeds in the berry press cakes. Bilberry and lingonberry seed oil have been reported to contain vitamin E and other bioactive compounds (Gustinelli et al., 2018; Yang, Ahotupa, Määttä & Kallio, 2011). Since SFE is a clean, sustainable method to extract valuable waxes from various agricultural residues (Attard et al., 2018), it suites well for the extraction of waxes rich in bioactive compounds for commercial applications, such as purposes for food and cosmetic industry. Dietary as well as topical application of γ-linolenic acid has been reported to have protective effect on structure and physiology of skin (Andreassi et al., 1997, Kawamura et al., 2011). Minor quantities of benzoic acid were also detected in bilberry and lingonberry wax in our study (Supplementary Table 2), and can contribute to the shelf-life of wax (Brul & Coote, 1999). Therefore, berry waxes can also have potential applications in food packaging and preservation. Our study signifies that residues of berry juice industry can be used to extract wax using sustainable extraction process (SFE). This wax has potential as suitable and effective additive for applications in food formulations as well as cosmetic industry.

*3.4 SPF of berry wax*

SPF is a universal term used to assess the UV absorption/blocking potential of compounds. There are studies on *in vitro* SPFs of various plant extracts (Maske, Lokapure, Nimbalkar, Malavi & D'souza, 2015; Kumar, Datta & Dutta Gupta, 2009). However, to our knowledge the UV blocking potential of berry waxes has not been studied yet. All extracted berry waxes were tested for SPF in our study. The results show high UV-B absorption properties as revealed by their SPFs as well as demonstrate dose dependent increase of SPF with the wax concentration (Table 2). Cuticular wax of bog bilberry fruit showed the highest SPF. From SFE extracted waxes of berry press cakes, bilberry wax showed higher SPF than lingonberry wax (Table 2). The high SPF could be attributed to the presence of higher amount of cinnamic acid and vitamin E in bilberry wax compared to lingonberry wax (SFE extractions), and high cinnamic acid amount in bog bilberry cuticular wax. Cinnamic acid has been shown to absorb broad range of UV-light, and it is used artificially as a UV-absorber in cosmetic products (Li et al., 2017). Vitamin E has photoprotective effect against UV radiation (Podhaisky & Wohlrab, 2002).

One of the most important physiological functions of cuticular wax in plants is the protection from UV-B and, thus, it is not surprising that the extracted cuticular waxes showed high UV-B absorption. In our study, the wild berry waxes show good SPFs that is comparable to SPFs of common commercial sunscreen products (Dutra, Oliveira, Kedor & Santoro 2004). Secondary metabolites such as triterpenoids (Hashim, Sidek, Helan, Sabery, Palanisamy & Ilham, 2011), phenolic acids (Kumar, Datta & Dutta Gupta, 2009) have been attributed for the potential UV-B absorption activities of plant extracts. The presence of triterpenoids and phenolic acids in berry wax could be responsible for UV-B absorbing properties of the studied berry waxes.

## 4. Conclusion

We have reported the chemical composition, morphology and SPF of cuticular wax of fruits of four important northern wild berry species. The variation in amount, morphology and chemical composition as well as high SPF of cuticular wax in different berry species was detected. Our results

may contribute the exploration of various applications of the berry waxes in food engineering, food packaging/preservation and cosmetic industry. Berry wax might be suitable for applications in food grade films/coatings to improve water barrier, optical and mechanical properties of films. There is an increasing demand for natural plant based waxes due to irregular supply of petroleum based waxes as well as consumer inclination for natural products. It is therefore, imperative to explore more plant based waxes for a sustainable greener economy. Therefore, we utilized SFE of industrial residual berry press cakes from berry juice industry to present a potential source of natural berry waxes.

## 5. Acknowledgements

We acknowledge the research grant from InterregNord (Natural Wax of Arctic Berries as Our Treasure – WAX project (number 20201089 to University of Oulu and grant IR16-020 and grant RMF16-026 to Troms Fylkeskommune and NIBIO). Work of PT was financially supported by I4future doctoral program funded by EU Horizon 2020 research and innovation program under Marie Skoldowska Curie grant agreement number 713606. Work of JK was financially supported by the ERDF project No. 1.1.1.1/16/A/047 “Genus Vaccinium berry processing using "green" technologies and innovative, pharmacologically characterized biopharmaceutical products” and work of LK was financially supported by the patron “Mikrotīkls” Ltd. administered by the Foundation of University of Latvia.

## Conflicts of interest

The authors declare no conflict of interest.

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513 Legends of figures:

514

515 Figure 1. Morphology of epicuticular wax on the surface of the wild berries: bilberry (a), bog
516 bilberry (b), lingonberry (c), and crowberry (d). Red arrows in figures indicate platelets on bilberry
517 surface and plates on lingonberry surface.

518

519 Figure 2. Amount of cuticular wax (μg/cm$^2$) in wild berry species (a). Cuticular wax profile in wild
520 berry species (b). Principal component analysis of cuticular berry wax composition (c). Bars
521 represent means ± SD of three replicates.

522

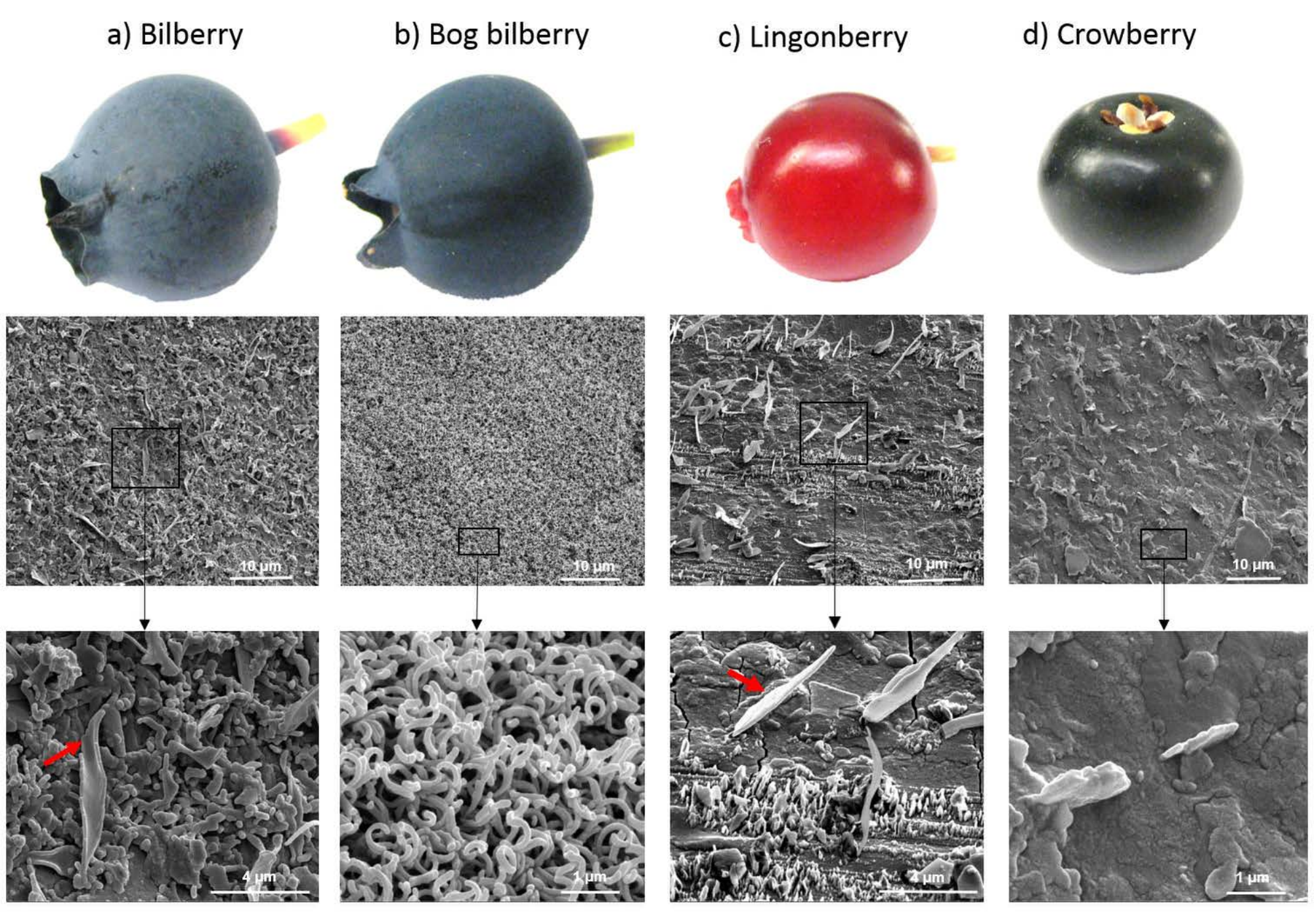
523 Figure 3. Wax obtained from bilberry and lingonberry press cakes by SFE (a). Wax profile in berry
524 press cakes (b).

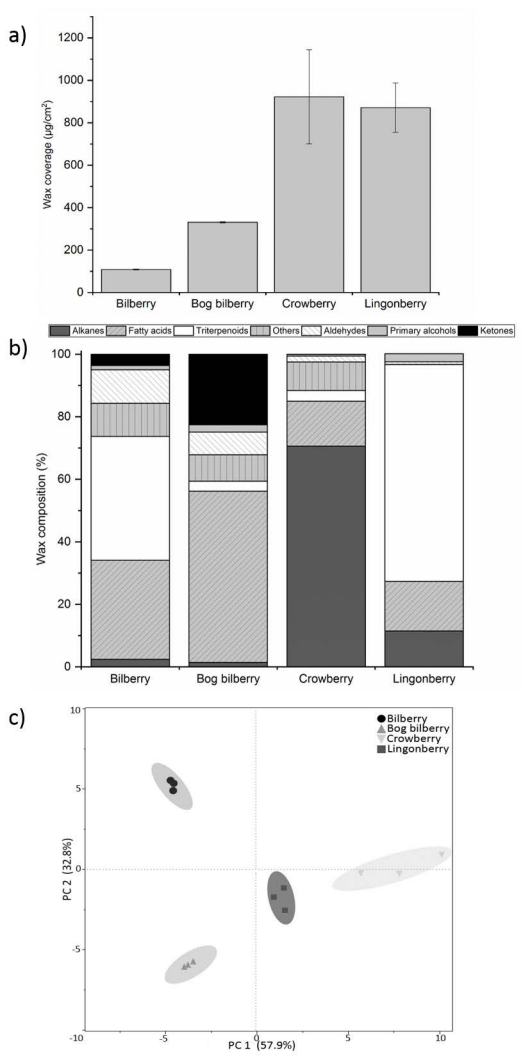
Table 1. Quantities (μg/cm$^2$) of cuticular wax compounds in the different wild berry species.

| Wax compounds | Quantity (μg/cm$^2$) | | | |
|---|---|---|---|---|
| | Bilberry | Bog bilberry | Crowberry | Lingonberry |
| *Triterpenoids* | | | | |
| β-Amyrin | 6.12 ± 0.51 | nd | nd | 49.48 ± 1.59 |
| α-Amyrin | 2.16 ± 0.08 | nd | nd | 50.37 ± 0.72 |
| Lupeol | 0.30 ± 0.29 | nd | nd | 37.83 ± 0.98 |
| Oleanolic acid | 2.73 ± 0.27 | 2.47 ± 1.24 | nd | 20.67 ± 00.92 |
| Ursolic acid | 0.61 ± 0.03 | 1.41 ± 0.71 | nd | 36.93 ± 0.31 |
| Adriaticol | nd | nd | nd | 55.05 ± 0.68 |
| Uvaol | nd | nd | nd | 17.17 ± 0.20 |
| Others | 0.10 ±0.10 | nd | 6.24 ± 0.36 | nd |
| *Fatty acids* | | | | |
| Linoleic acid | 0.01 ± 0.01 | nd | 2.01 ± 0.16 | 0.90 ± 0.01 |
| Oleic acid | 0.01 ± 0.01 | nd | 4.16 ± 0.22 | 0.88 ± 0.02 |
| Elaidic acid | 0.01 ± 0.01 | nd | nd | nd |
| Stearic acid | 0.43 ± 0.03 a | 2.46 ± 0.03 b | 1.76 ±0.08 c | 2.70 ± 0.05 c |
| 9,10-Dihydroxystearicacid | 0.29 ± 0.02 | nd | nd | 5.53 ± 0.26 |
| Nonadecanoic acid | 0.03 ± 0.02 | 0.45 ± 0.01 | nd | nd |
| 11-Eicosenoic acid | 0.03 ± 0.02 | 0.62 ± 0.05 | nd | nd |
| Arachidic acid | 1.21 ± 0.17 a | 27.21 ± 0.17 b | 1.01 ± 0.08 a | 2.93 ± 0.07 a |
| Heneicosanoic acid | 0.11 ± 0.01 | 0.45 ± 0.01 | 1.20 ± 0.05 | nd |
| Lignoceric acid | 0.44 ± 0.01 a | 3.54 ± 0.07 b | 1.55 ± 0.17 a | 30.69 ± 0.50 c |
| Hyenic acid | 0.07 ± 0.01 | 0.77 ± 0.01 | nd | nd |
| Cerotic acid | 2.10 ± 0.14 a | 2.75 ± 0.10 b | 0.59 ± 0.06 c | 4.54 ± 0.17 d |
| Carboceric acid | 0.18 ± 0.02 | 0.26 ± 0.01 | nd | nd |
| Montanic acid | 2.93 ± 0.18 a | 2.77 ± 0.14 a | 1.81 ± 0.21 b | 5.03 ± 0.15 c |
| Nonacosanoic acid | 0.18 ± 0.02 | nd | nd | nd |
| Melissic acid | 0.65 ± 0.05 a | 0.05 ± 0.05 b | 2.50 ± 0.34 c | 0.71 ± 0.02 a |
| Lacceric acid | 0.08 ± 0.03 | nd | nd | nd |
| *Alkanes* | | | | |
| Tricosane | 0.01 ± 0.01 | 0.34 ± 0.05 | 0.42 ± 0.02 | nd |
| Pentacosane | 0.10 ± 0.00 | 0.26 ± 0.02 | 0.17 ± 0.05 | nd |
| Hexacosane | 0.04 ± 0.00 | 0.16 ± 0.02 | nd | nd |
| Heptacosane | 0.23 ± 0.01 | 0.53 ± 0.02 | 4.67 ± 0.39 | 3.34 ± 0.07 |
| Octacosane | nd | nd | 1.41 ± 0.18 | 0.57 ± 0.04 a |
| Nonacosane | 0.09 ± 0.00 a | 0.05 ± 0.02 a | 97.71 ±11.40 b | 37.11 ± 0.63 |
| Triacontane | 0.21 ± 0.21 | nd | 1.67 ± 0.25 | 0.73 ± 0.00 a |
| Hentriacontane | 0.04 ± 0.01 a | 0.15 ± 0.05 a | 24.66 ± 3.22 b | 2.14 ± 0.03 |
| Dotriacontane | nd | nd | nd | nd |
| Tritriacontane | nd | nd | 0.76 ± 0.06 | nd |
| *Aldehydes* | | | | |
| Tetracosanal | 0.06 ± 0.00 a | 1.56 ±0.04 b | 1.39 ± 0.16 b | 1.02 ± 0.08 c |
| Hexacosanal | 0.96 ± 0.04 a | 3.02 ± 0.37 b | 0.48 ± 0.04 a | 0.83 ± 0.10 a |
| Heptacosanal | 0.02 ± 0.02 | nd | nd | nd |
| Octacosanal | 1.68 ± 0.10 a | 3.64 ± 0.13 b | 0.79 ± 0.07 c | 0.96 ± 0.10 c |

| | | | | |
|---|---|---|---|---|
| Triacontanal | 0.02 ± 0.00 | nd | nd | 0.54 ± 0.10 ab |
| Hentriacontanal | 0.52 ± 0.02 ab | 0.05 ± 0.03 b | 0.69 ± 0.31 c | nd |
| | | | | |
| *Ketones* | | | | |
| 2-Nonadecanone | 0.08 ± 0.00 | 1.65 ± 0.05 | nd | nd |
| 2-Heneicosanone | 2.33 ± 0.21 | 16.42 ± 1.18 | 0.04 ±0.04 | nd |
| | | | | |
| *Primary alcohols* | | | | |
| 1-Octadecanol | nd | 1.30 ± 0.80 | nd | 0.94 ± 0.08 |
| 1-Nonadecanol | 0.19 ± 0.03 | 0.50 ± 0.30 | nd | nd |
| 1-Eicosanol | nd | 0.12 ± 0.05 | nd | 2.13 ± 0.02 |
| 1-Docosanol | nd | nd | nd | 1.92 ± 0.08 |
| 1-Tetracosanol | nd | nd | nd | 2.48 ± 0.21 |
| 1-Hexacosanol | nd | nd | nd | 1.31 ± 0.09 |
| 1-Octacosanol | nd | nd | nd | 1.06 ± 0.06 |
| | | | | |
| Phytol | 0.06 ± 0.01 | 0.31 ± 0.01 | nd | nd |
| *Phenolic acids* | | | | |
| Cinnamic acid | nd | 0.39 ± 0.21 | nd | nd |
| p-coumaric acid | nd | nd | nd | 0.19 ± 0.01 |

Data represents means ± SE of three replicates
Different letters in chemical class in different berry species indicate significant differences ($P < 0.05$)
nd, not detected


a) Bilberry
b) Bog bilberry
c) Lingonberry
d) Crowberry
10 µm
10 µm
10 µm
10 µm
4 µm
1 µm
4 µm
1 µm


a)
Wax coverage (μg/cm²)
1200
1000
800
600
400
200
0
Bilberry
Bog bilberry
Crowberry
Lingonberry
b)
Alkanes
Fatty acids
Triterpenoids
Others
Aldehydes
Primary alcohols
Ketones
Wax composition (%)
100
80
60
40
20
0
Bilberry
Bog bilberry
Crowberry
Lingonberry
c)
Bilberry
Bog bilberry
Crowberry
Lingonberry
PC 2 (32.8%)
10
5
0
-5
-10
-10
-5
0
5
10
PC 1 (57.9%)

a)

Bilberry wax

Lingonberry wax

b)