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Surface wax composition of wild and cultivated Northern berries

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Abstract. Surface wax of plants is the outer layer, which protects the plant from dehydration, extreme temperatures, UV radiation and changes in the environment, as well as attacks from moulds and bacteria. Studies of berry surface wax are of importance to understand metabolism character (factors affecting wax layer composition in different berry species) as well as to increase the shelf life of berries and increase the microbial resistance. The aim of this study was analysis of surface wax composition of commercially grown 8 blueberry (*Vaccinium corymbosum*) varieties, wild bilberry (*Vaccinium myrtillus* L.) and bog bilberry (*Vaccinium uliginosum* L.). More than 80 different compounds were identified and quantified belonging to 9 groups of compounds, namely, alkanes, phytosterols, alcohols, fatty acids, phenolic acids, ketones, aldehydes, esters and tocopherols. Significant differences were found between blueberry (*Vaccinium corymbosum*) and bog bilberry (*Vaccinium uliginosum* L.) surface wax composition. Amongst studied berries differences were found in concentrations of triterpenes (up to 62% in blueberries), and fatty acids (up to 26% in bilberries) identifying species related differences influencing associated functional properties of berry wax (antimicrobial activity, stress caused by environmental changes). Blueberry variety 'Polaris' had the highest amount of ursolic acid (9.30 g 100 g^{-1}), alpha-amyrin (11.07 g 100 g^{-1}) and lupeol (10.2 g 100 g^{-1}). Research on berry surface wax composition could help reduce loss of commercially produced berries due to environmental impacts or microbial attacks, prolonging shelf life and overall quality of fruits and vegetables post-harvest.

Key words: blueberry, bilberry, bog bilberry, cuticular, cuticle, surface wax, chemical composition, lipids.

INTRODUCTION

Blueberries (*Vaccinium corymbosum* L.) are rich source of polyphenolic substances, carbohydrates, vitamins as well as lipids (Kim et al., 2013) and they are one of the commercially important berry species. Blueberries have large fruits, long shelf life, excellent taste properties and they are suggested as part of a healthy diet (Nile & Park, 2014). Blueberry cultivation takes place worldwide and the demand is steadily increasing (Venskutonis et al., 2016). Blueberries can be considered as functional food and many cultivars (varieties) are developed to differ in taste, size and colour of berries, improving productivity of blueberry bushes and prolonging growing season (Burdulis et al., 2007). Blueberries contain many phenolics responsible for their colour

(anthocyanins) and high radical scavenging capacity, which is considered beneficial for human consumption (Cho et al., 2004). Another important group of substances in the composition of *Vaccinium corymbosum* berries are their lipids and waxes. Berry lipids are berry skin waxes, seed lipids and cytoplasm lipids. By the chemical composition *Vaccinium* berry lipids include triglycerides, fatty acids, alcohols, alkanes as well as sterols, terpenes and other groups of substances with low polarity (Dulf et al., 2012).

An important group of berry lipids are their cuticular wax, which have glossy or glaucous appearance; it acts as an interface between plant and the environment (Yeats & Rose, 2013). The outermost layer of plant organs- the cuticle- protect the plant from abiotic stresses such as dehydration, extreme temperatures (frost, heat) and other factors presented by the environmental changes in the growth area. Cuticular wax is a complex mixture of various aliphatic and aromatic compounds. Plant waxes consist of low- to intermediate-polarity compounds, they are hydrophobic, non reactive, long-chain (chain length from C_{12} up to C_{70}) chemical compounds (Jetter et al., 2006). The main compound classes found in the wax are n-alkanes, fatty acids, primary alcohols, aldehydes, secondary alcohols, ketones, phytosterols and esters. To obtain waxes care should be taken to avoid co-extraction of cytoplasmic and membrane lipids and it is suggested to do it by treatment of fresh berries with hydrophobic solvents such as chloroform, hexane or petroleum ether (Sharma et al., 2018).

The importance of cuticular wax studies of blueberries is related to the need to understand the natural protection mechanisms of berries as well as to consider possibilities to increase the quality of produce by increasing the shelf-life during berry storage, fruit quality and reduce possible microbial infections (Lara et al., 2014). Functional foods, nutraceuticals and healthcare products containing plant lipids (waxes) are being developed as innovative, consumer friendly products (Weingartner et al., 2014). The hydrophobic properties of plant cuticular waxes are being investigated for implementation as part of antimicrobial paints, windshield coatings, stain resistant textiles and biodegradable plastics (Li et al., 2007; Yadav et al., 2014).

The composition of berry waxes depend on the species, their growth location and thus it is important to study berries composition in each specific site. Lipids of *Vaccinium corymbosum* have been studied by other authors, however, no information is present for the wax composition of Northern blueberry varieties. The aim of the present article is to study and to compare the composition of waxes of 8 bilberry *Vaccinium corymbosum* cultivars as well as comparison with related species bilberry *Vaccinium myrtillus* L. and bog bilberry *Vaccinium uliginosum* L. cuticular wax.

MATERIALS AND METHODS

Plant material

In this study, three berry species were examined for their cuticular wax composition. Examined berries were- bog bilberry (*Vaccinium uliginosum* L.), bilberry (*Vaccinium myrtillus* L.) and eight varieties of blueberry (*Vaccinium corymbosum* L.), namely, 'Blue crop', 'Blue gold', 'Chandler', 'Chippewa', 'Duke', 'North blue', 'Patriot', 'Polaris'. The different blueberry varieties were harvested at a commercial blueberry farm Z/S 'Strelnieki' located on the outskirts of town Jurmala, Latvia. Bog bilberries and bilberries were harvested from the forests belonging to Kemeru National Park. To avoid contamination and possible damage to the outer layer of berries they were

harvested into glass containers previously washed with chloroform (≥ 99%, Sigma Aldrich) using metal forceps. In total approximately 700 berries were harvested for each sample, all berries were harvested in the summer/autumn of 2018. After the harvest berries were placed into a refrigerated sample box and delivered to the laboratory for immediate extraction of waxes.

Extraction of cuticular wax

Extraction of cuticular wax was done using two extraction solvents, chloroform and a mixture of hexane/ethyl acetate (1:1) (≥ 99%, Sigma Aldrich). Each extraction solvent was used three times for the extraction of respective berry species. For extraction, 150 mL of extraction solvent was poured into three separate glass beakers that were previously cleaned with the same solvent. A hundred berries were picked from the harvested sample and sequentially dipped one by one into the extraction solvent for 30 seconds in each of the three beakers containing the solvent. Clean metal forceps were used for the berry dipping. After the berry dipping all of the contents of the three used beakers were filtered and combined into an evaporation flask. Each beaker was further washed twice with extraction solvent and added to the combined extract. Samples were evaporated under reduced pressure using Hi-Vap Advantage (Heidolph, Switzerland) evaporator. Samples were evaporated to approximately 5 mL and transferred to clean glass tubes. The remaining solvent was evaporated in a water bath (40 °C) (Cole Parmer) under a gentle stream of nitrogen until dry. The dried berry cuticular wax samples were stored into a freezer (-20 °C) until analysis.

Analysis using Gas Chromatography-Mass Spectrometry (GC-MS)

The cuticular berry wax chloroform extracts were evaporated under a flow of nitrogen. Silylation was done using N,O-bis (trimethylsilyl) trifluoroacetamide, BSTFA (200 µl, Sigma-Aldrich) in pyridine (1,300 µl, Sigma-Aldrich), for 1 hour at 60 °C. Quantification was performed using three external standard curves: ergosterol (Sigma-Aldrich), stearic acid (Sigma-Aldrich) and tetracosane. GC- MS analysis was performed using GC-2010 plus coupled with GC/MS QP-2010 Ultra mass detector (Shimadzu, Japan). The column used was Restek Rxi®-5MS (30 m × 0.25 mm × 0.25 µm; Crossbond ® 5% diphenyl + 95% dimethyl polysiloxane) with working temperature range 40 to 350 °C. He (Helium) was used as carrier gas with a total flow rate of 10.8 mL min^{-1} and column flow rate of 0.71 mL min^{-1} flow rate. The split ratio was 1:10 and injection temperature 290 °C. The temperature programme used was: oven temperature 200 °C (2 min) increased 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 analyser was used with electron impact (EI) ionisation, ionization voltage of 70eV. The ion source temperature was 230 °C and interface temperature 290 °C. Identification of the compounds separated in the GC was performed using Shimadzu LabSolutions 4.30 software, coupled with NIST'14 spectral library.

Data analysis

Quantitative data of cuticular wax composition was subjected to two-way analysis of variance (ANOVA) to evaluate the differences between the analysed berries. Principal component analysis (PCA) on correlation matrix and hierarchical cluster analysis using

Ward's method with standardized data was performed to evaluate relationship among various tested berries. Statistical analysis and data visualisation was done using SAS JMP®, Version 13 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Composition of berry cuticular wax was investigated for three species, both grown commercially and found in the wild forests and bogs of Latvia. Studied berries were chosen in order to cover *Vaccinium* genus berries and compare wild and cultivated berries (Table 1). Substances found as part of the cuticular wax were identified and quantified to evaluate variations of composition and contents among berries.

The amount of extracted cuticular wax ranged from 0.65 to 0.90 mg berry^{-1} for the investigated blueberry varieties (Table 1). The amount of extracted wax is similar in the blueberry varieties, bilberries and bog bilberries, these berries have white, textured cuticular wax layer (crystal forming) (Jeffree, 2006). The glaucous appearance in analysed berries could be attributed to the presence of high triterpenoid contents (Fig. 1, B), however, in this study the morphology of cuticular wax layer was not investigated. In terms of morphology, wax layer of blueberries is considered to belong to β-diketone tubes (tubule shaped wax crystals (Barthlott et al., 1998)), where diketones and triterpenoids were found to be the major compounds (Chu et al., 2017).The results reported by Chu et al. (2017) are in agreement with our findings - blueberry cultivars have high triterpenoid contents and, among other studied berries, contained high amounts of hentriacontane-10,12-dione (up to 6.0 g 100 g^{-1} extract in 'North Blue').

Table 1. Studied berries and the extracted cuticular wax amount per berry

Studied berry	Variety	Wax, mg berry^{-1}
Bog bilberry		$0.95^{a} \pm 0.09$
Bilberry		$0.63^{b} \pm 0.05$
Blueberry	Blue crop	$0.74^{c} \pm 0.04$
	Blue gold	$0.67^{b} \pm 0.03$
	Chandler	$0.83^{c} \pm 0.05$
	Chippewa	$0.90^{c} \pm 0.07$
	Duke	$0.57^{c} \pm 0.02$
	North blue	$0.65^{b} \pm 0.02$
	Patriot	$0.84^{c} \pm 0.03$
	Polaris	$0.87^{c} \pm 0.03$

^{a,b,c} – represent significant differences ($\alpha = 0.05$) between different groups (ANOVA, Tukeys HSD).

As part of the berry waxes 9 groups of compounds were found, namely, alkanes, phytosterols (triterpenoids), alcohols, fatty acids, phenolic acids, ketones, tocopherols and aldehydes (Fig. 1, B). Obtained cuticular wax extracts from different berries show a similar pattern of plant wax constituents, where triterpenes (up to 62% of total wax content in 'Blue Gold' and 'Blue Crop') and alcohols (up to 28% of total wax content in 'North Blue') are the major groups of cuticular wax components (Fig. 1). Aldehydes were found in all of the berries, however, their concentrations were low (Fig. 1, A). As minor groups of compounds found in the berries, phenolic acids and tocopherols were identified, despite the low concentration of these substances, they have a vital role in the plant - pathogen interaction (Fig. 1, B) (Kolattukudy et al., 1995). Phenolic acids and tocopherols have been reported to have protective abilities against UV radiation and antimicrobial activity, respectively (Kolb et al., 2003; Ahmed et al., 2014; Andrade et al., 2014; Ghimire et al., 2017).

Triterpenes were the most abundant components in the cuticular wax of the studied 3 species of berries, varying from 32% ('Chippewa') to 62% ('Blue Gold') of total wax contents (Fig. 1). Eleven different triterpenes were identified as part of the cuticular wax. The triterpene acid ursolic acid was found in all of the studied berries in varying amounts. In the blueberry variety 'Chippewa' 0.46 g 100 g^{-1} ursolic acid was found. The analysed blueberry varieties show different triterpene composition patterns. Variety 'Polaris' has the highest amount of ursolic acid (9.30 g 100 g^{-1}), alpha-amyrin (11.07 g 100 g^{-1}) and lupeol (10.2 g 100 g^{-1}) among all of the studied berries. Alpha – amyrin, beta – amyrin and lupeol are triterpene alcohols that are dominant in both, the cultivated and wild *Vaccinium* berries. Lanosterol was found only in, blueberry variety 'Chandler' (0.34 g 100 g^{-1}).

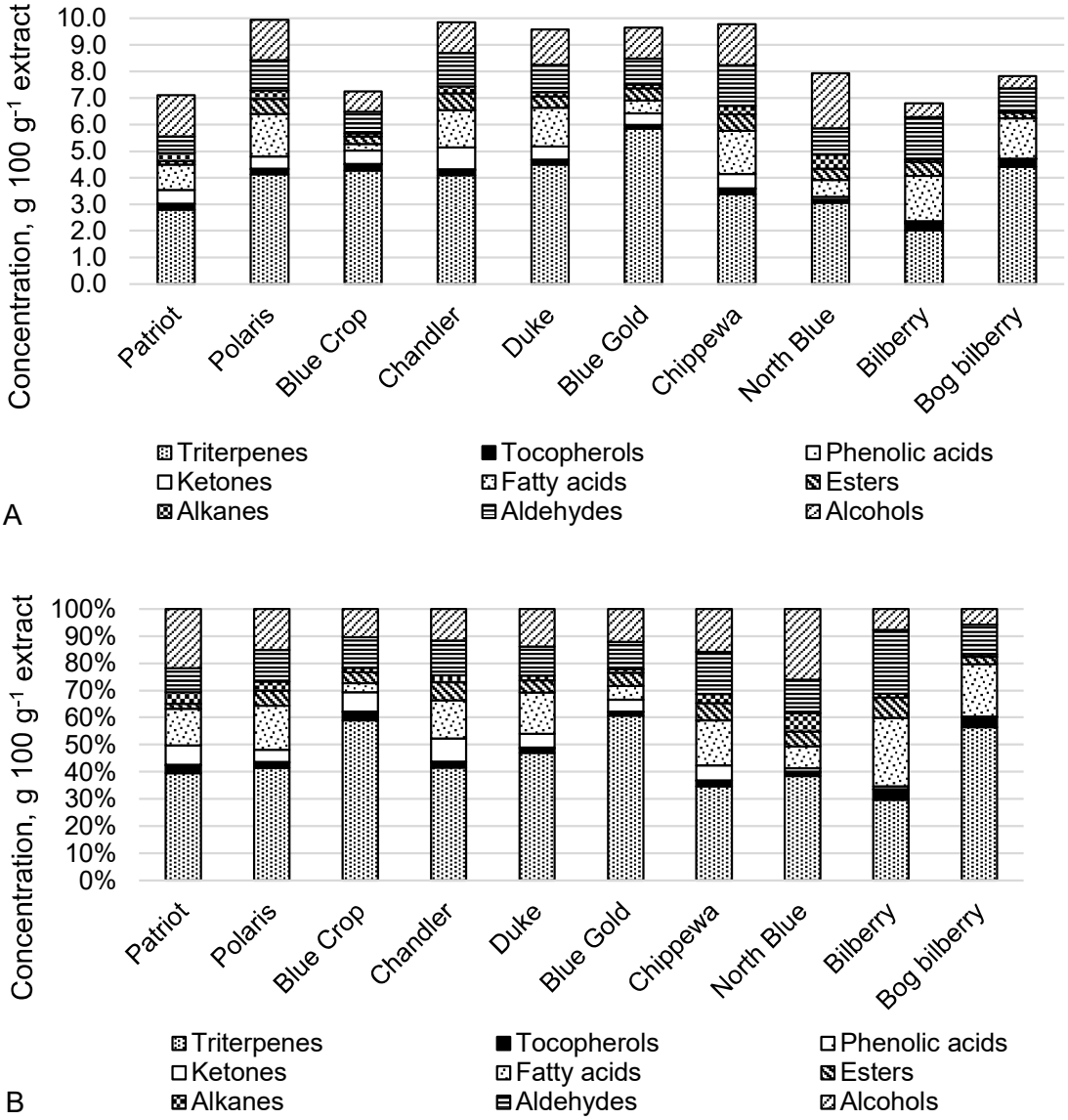

Figure 1. Absolute (A) and relative (B) amounts of identified compound groups in studied berries.

Triterpenoids as part of cuticular wax have been reported previously in a wide variety of plants, for example, grapes, apples cherries, tomatoes, blueberries and plums (Szakiel et al., 2012; Lara et al., 2014; Pensec et al., 2014; Chu et al., 2017). Considering the knowledge about the function of cuticular wax, where, for example, alkanes are believed to be responsible for the prevention of water loss, triterpenoids, on the other hand, might play a role in plant-pathogen interaction/recognition. Various biological activities and health promoting effects are attributed to triterpenoid molecules as part of human diet. Triterpenoids were reported to be the main group of compounds in blueberry cuticular wax (Chu et al., 2017), it was found that triterpenoids composed 64.2% of total wax, which is in agreement with the reported total wax amounts of specific varieties of blueberries analysed in this study (up to 62%, Fig. 1), also, the reported dominant triterpenoids are the same in both studies. During the GC/MS analysis, compounds in minor concentrations with unidentified MS spectra were recorded, possibly belonging to ursane- or oleanane type triterpenes. The unidentified compounds could contribute to species specificity and cuticular wax protective properties.

Alkanes with chain length from C20 to C33 were found in all of the studied berries. Blueberry varieties and the rest of the studied berries contained from 1.5–7% alkanes of total wax contents (Fig. 1). Differences among the relative alkane distribution in berries could be explained by the morphology of the cuticular wax- alkanes are possibly related to the glossiness of the berry, as glaucous berries have lower alkane concentration (Barthlott et al., 1998). The main alkanes found in the cuticular wax of studied berries were the C29 (nonacosane) and C31 (hentriacontane) alkanes. The dominant alkanes found on the surface of the berries are odd-numbered chain length.

Analysed blueberry varieties contained from 1.5 to 7% alkanes of total wax, which is higher than that reported by Chu et al., 2017, however, the previously reported alkane composition seems to be similar among the tested varieties, where the odd-numbered alkanes, specifically, C29 and C31were the dominant alkanes (Chu et al., 2017). Alkanes have been found to be part of outermost layer (cuticle) of many fruits, the dominant C29 alkane has been found on the surfaces of plums, apples and cherries (Lara et al., 2014). Main function of alkanes as part of the cuticular wax is to control transpirational water loss of the plant (Parsons et al., 2012).

Saturated fatty acids contribute to 26% in bilberry and 20% of total wax in bog bilberry. Blueberry varieties ‘Chippewa’ and ‘Chandler’ contained 24% and 20% fatty acids of total wax content (Fig. 1). Overall, the fatty acid distribution in studied berries was higher in the blueberries and bilberries. Also, the total amount fatty acids was higher in bluberries with up to 15.6 g 100 g^{-1} in ‘Chandler’. As the most abundant fatty acid in the blueberry varieties triacontanoic acid (C30:0) was found, however, this fatty acid was not found in variety ‘Chippewa’, while variety ‘North Blue’ contained 9.6 g 100 g^{-1} of this fatty acid. Bilberry and bog bilberry present hexacosanoic acid (C26:0) as the major fatty acid with 7.6 and 5.2 g 100 g^{-1} extract, respectively. Identified fatty acids ranged from C16:0 to C30:0 in different berry species. In blueberry varieties grown in China the same chain length fatty acids were identified as part of the cuticular wax with C30:0 beeing the most abundant, followed by C28:0 (Chu et al., 2017).

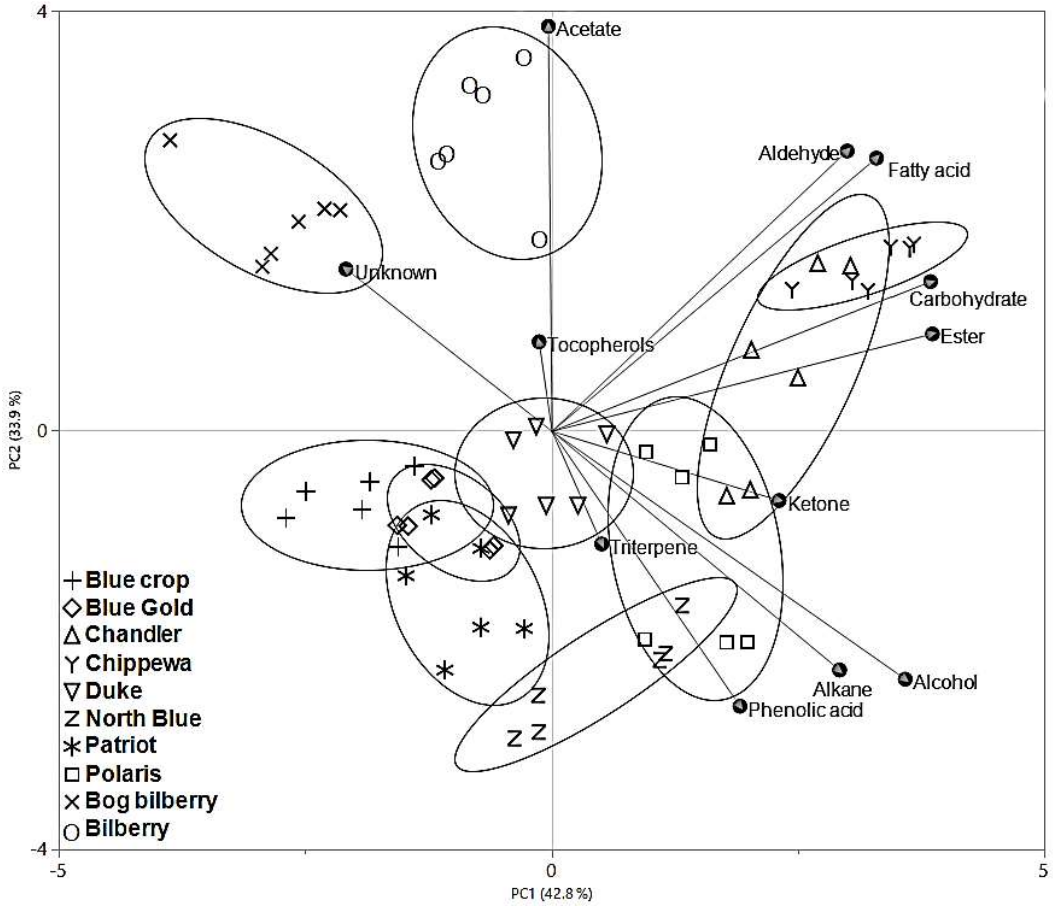

Figure 2. Principal components analysis of different berry species and blueberry varieties based on the identified compound classes of berry waxes.

Principal component analysis of the studied berry species and blueberry varieties based on the identified compound classes (Fig. 2) demonstrates significant differences amongst bluberry varieties on one hand and bilberies and bog bilberries on the other hand, thus supporting relationship between these berries based on chemical composition. However, also among varieties of blueberries, which belong to the same species, significant differences can be found. Also, main classes of compounds forming waxes can be grouped in the PCA plot, indicating similarities in their metabolic functions: long chain aldehydes, fatty acids, esters in one gorup, ketones, fatty alcohols, alkanes in another group (Fig. 2). Acyl- coenzymeA is directly responsible for the synthesis of esters, fatty acids and primary alchols which are later transformed to alkanes, seccondary alcohols and ketones by different oxidases (Goodwin et al., 2005). Grouped separately are tocopherols and terpenes indicating their different biological functions probably related to berry protective functions against microrganisms.

CONCLUSIONS

Study of berry waxes are of importance for commercially cultivated species as waxes influence berry appearance and storage quality. By means of gas chromatography-mass spectrometry more than 80 different compounds were identified and quantified in

8 varieties of commercially cultivated blueberry (*Vaccinium corymbosum*), bilberry (*Vaccinium myrtillus* L.) and bog bilberry (*Vaccinium uliginosum* L.). Identified berry wax components belong to 9 groups of compounds, namely, alkanes, triterpenes, alcohols, fatty acids, phenolic acids, ketones, aldehydes, esters and tocopherols. Significant differences in the wax composition was found amongst berries from different species, as well as differences among varieties within the same species. Presented results suggest that analysis of cuticular wax can be used to distinguish between berries within the same species and among different species, implicating use in berry authenticity testing, however, more testing should be done to avoid the interference of varying environmental factors.

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