ORIGINAL PAPER

Surface-enhanced Raman spectroscopy for the characterization of *Vaccinium myrtillus* L. bilberries of the Baltic–Nordic regions

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Abstract

In this study, an investigation of the wild bilberries (*Vaccinium myrtillus* L.) of the different Baltic–Nordic regions using surface-enhanced Raman spectroscopy (SERS) combined with principal component analysis (PCA) is presented. The bilberries were collected in Lithuania, Latvia, Finland and Norway. The set of the SERS spectra of the berry extracts (pH ~ 4) were recorded on the silver nanoparticles based SERS substrates. The SERS spectra of the extracts were acquired using 532 nm laser as an excitation source. The morphology of the SERS substrates was evaluated by scanning electron microscopy (SEM) and the presence of the silver nanoparticles was confirmed by the energy-dispersive X-ray spectroscopy (EDX). The enhancement factor (EF) of the silver SERS substrates was found to be 10^5. It has been shown that a strong fluorescence background, associated with the phenolic compounds found in bilberries, can be subtracted due to the fluorescence-quenching properties of the silver nanoparticles. Therefore, an application of the SERS technique allowed to observe the characteristic peaks of the bilberries and the PCA tool enabled to evaluate the spectral variation across the entire SERS data set. The results presented in this paper show that the SERS technique coupled with PCA chemometric analysis might serve as a complementary method that allows to identify the country of origin of the bilberries based on the spectral differences.

Keywords SERS · Raman spectroscopy · Bilberries · Anthocyanin · *Vaccinium myrtillus*

Introduction

Over the past decade, Raman spectroscopy and SERS began to compete intensively with traditional food research methods such as high-performance liquid chromatography (HPLC), gas chromatography (GS) and enzyme-linked immunosorbent assay (ELISA) [1, 2]. One of the main reasons for such competition is the potential of the SERS to provide the diverse information about the chemical composition of the materials with high sensitivity and specificity. Such capabilities make SERS technique attractive for the detection of molecular traces in various food matrixes leading to the next level control of food quality and safety. SERS has already been applied for the detection of pesticides [2, 3], antibiotics [4, 5], heavy metals [2, 6] and foodborne pathogens [7, 8] in various foods, indicating the versatility of the method. Among the mentioned merits, SERS technique also

holds an advantage in the research of epigenetic events such as DNA methylation occurring due to the alkylation of various bases [9]. Therefore, SERS-based analytical technology expands the possibilities in the research regarding bioactive food components such as probiotics [10]. This paper discusses an application of the SERS for the investigation of the wild bilberries. Due to their beneficial properties for human health, the wild fruits (blueberries, lingonberries, cherries, etc.) and their products are used for food and medical purposes. Therefore, more attention should be paid for the study of the wild fruits at the molecular level. Usually, different analytical methods are applied to test individual components of the berries [11, 12] and plant substances [13]. Meanwhile, SERS technique allows to obtain a complex spectra that consist of common molecular vibrations of all the sample's compounds at the same time. This is important for the initial qualitative assessment of raw materials and products, as the overall spectra also reflects the molecular fingerprints of toxic substances such as mycotoxins [14]. A chemically specific SERS tool also provides an opportunity to observe the delicate changes of the molecular structure of flavonoids (i.e., anthocyanin) [15–17].

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As an inexpensive technique, SERS demonstrates a significant breakthrough in the field of food authenticity and adulteration [18]. The ability to authenticate various food products is a major concern to the food industry as well as to the consumers. Therefore, the identification of ingredients and the detection of adulterants (authentication), as well as the determination of raw material origin (traceability) is an important issue. For this reason, further studies should involve a wider range of different species of the bilberries, as well as the development of the reference databases with defined parameters which may be considered as useful geographic origin markers. Reference values are required for the confirmation of a specific origin and the identification of an unknown origin [19, 20]. Today, innovative and sensitive spectroscopic methods show a potential to assess the authenticity of the products and to ensure the prevention of incorrect labeling.

One of the main challenges that the manufacturers face today is the synthetic dyes, such as amaranth, added to the berry extracts [21, 22]. Such adulteration aims to artificially intensify the color of the berries leading to the false evaluation of the total anthocyanin content by UV–Vis method. Incorrect results are observed because the absorption of the dye occurs at the same wavelength as anthocyanin. Therefore, it becomes impossible to distinguish whether the absorption peak belongs to anthocyanin or adulterant substance. In contrast to UV–Vis, SERS allows to perform the qualitative and quantitative detection of amaranth at the trace level with high specificity, which is determined by the unique molecular vibrations of the dye in the spectral fingerprint region [23].

In this work, we focus on the application of the SERS technique for the investigation of the wild bilberries naturally growing in the different Baltic–Nordic regions. Previous research demonstrates that the composition of anthocyanin as well as other bioactive compounds present in the wild bilberries depend not only on the internal (genotype) but also on external (environmental) factors [24]. Based on these findings, we hypothesized that the SERS approach will allow to distinguish the wild bilberries growing at the different locations by analyzing their spectral data. To achieve the aim, we applied SERS technique for the generation of the berries' spectra and PCA tool for an evaluation of the spectral variation across the entire SERS data set. To the best of our knowledge, the comparison of the wild bilberries of the different Baltic–Nordic regions was not reported before.

Materials and methods

Materials

Water, silver nitrate ($\geq 99.0\%$), hydrofluoric acid (48 wt. % in H_2O, $\geq 99.99\%$), pentacene (99.0%), methanol (HPLC, $\geq 99.0\%$), HCl (37.0%), D-fructose ($\geq 99.0\%$), D-glucose (99.5%), and sucrose ($\geq 99.5\%$) were purchased from Sigma Aldrich. $ZnSO_4 \times 2H_2O$ and $K_4[Fe(CN)_6] \times 3H_2O$ were purchased from Eurochemicals. All the reagents used for the experiments were of analytical grade and used without further purification. All the bilberries (*Vaccinium myrtillus*) were harvested at the same ripening stage and frozen at $-20\,°C$ without further processing.

Methods

Preparation of the silver SERS substrates

The solution of silver precursor was prepared by dissolving $AgNO_3$ in water to the final concentration of 0.1 M. The prepared HF (24%) and $AgNO_3$ (0.1 M) solutions were mixed in a ratio of 1:1 (v:v). The silicon wafers were immersed into the reaction solution for 5 s, immediately transferred to the distilled water and finally dried under the nitrogen flow. Morphological characterization of the synthesized silver SERS substrates was carried out using SEM microscope (SEM, Hitachi S-3400 N).

For the determination of the EF of the silver SERS substrates, the monolayer of the pentacene was deposited on the nanostructured SERS surface by vacuum evaporation. The thickness of the monolayer was 1–1.5 nm. The conditions applied for the acquirance of the spectra are provided in the next section.

Preparation of the bilberry extracts for SERS measurements

1000 g of the bilberries was collected in each country. The berries were divided into 3 containers (each container contained 330 g of the bilberries) and homogenized using a blender. 5 g of the mashed berries from each container was transferred to the separate flasks and diluted with distilled water to 50 ml. The solutions were centrifuged for 2 min at 4000 rpm to precipitate the insoluble parts of the berries (skin and seeds). 5 ml of each centrifugate was transferred to the flasks and diluted with distilled water to 10 ml. 25 µl of the prepared solutions (pH 4.06) was dropped on the separate silver SERS substrates. Therefore, three samples of the bilberries were prepared for each country and 20 SERS spectra of the bilberries were recorded at the different points of each sample. The graphical illustration of the preparation of the bilberries samples is presented in Fig. S1 (see supplementary material).

The SERS spectra of the bilberries were recorded using Raman spectrometer (NTEGRA Spectra, NT-MDT Inc.). The objective magnification was $100\times$, numerical aperture $NA = 1.49$ and a spot diameter of the optical beam focus—500 nm. The excitation source was a laser with $\lambda = 532$ nm and optical beam power of 25 mW. The laser power on the

samples was 0.20 mW. An integration time for the collection of the spectra was set to be 1 s. All the spectra were normalized and averaged using Origin Pro 9.0 software.

Preparation of the bilberries samples for UV–Vis measurements

The preparation of the berries samples for the analysis of total anthocyanin content was based on the European pharmacopeia protocol [25]. Briefly, 5 g of the homogenized berries was added to the flask and diluted with 95 ml of methanol. The flask was placed into the ultrasonic bath for 20 min. Then, the extract was filtered and diluted with methanol to 100 ml. 1 ml of the prepared extract solution was transferred to the flask and diluted with acidified methanol (0.1% HCl) to 50 ml. The prepared solution was analyzed at 528 nm wavelength using UV–VIS spectrophotometer (Agilent, Cary 60). The total content of anthocyanin was calculated according to the following equation:

$$\text{Total anthocyanin content} \ (\%w/w) = \frac{A \times 5000}{718 \times m}, \quad (1)$$

where 718 is the absorption of cyaniding-3-glucoside at 528 nm; A is the absorbance of the test sample at 528 nm; m is the sample weight, mg.

High-performance liquid chromatography analysis of saccharides

Preparation of the standard solutions 200 mg of fructose, glucose and sucrose were added to the separate flasks and diluted with water to 100 ml. The final concentrations of the saccharides in the solutions were 2 mg/ml.

Preparation of the test solutions 3 g of the homogenized berries was transferred to the flask and diluted with water to 50 ml. The prepared solution was placed into the water bath for 15 min at 60 °C. Then, the solution was cooled to room temperature and treated with Carrez I (7.20 g of $ZnSO_4 \times 2H_2O$ diluted with water to 100 ml) and Carrez II (3.60 g of $K_4[Fe(CN)_6] \times 3H_2O$ diluted with water to 100 ml) solutions. The final transparent solutions were filtered through membrane filters (size of the pores—0.45 μm) and analyzed by HPLC system (Shimadzu). The content of the saccharides was calculated according to the following equation:

$$\text{Saccharides content} \ (\%w/w) = \frac{S_s \times C_{st} \times V \times 100}{S_{st} \times 1000 \times m}, \quad (2)$$

where S_s is the sample peak area; C_{st} is the saccharide standard concentration mg/ml; V is the volume in which the sample was prepared, ml; S_{st} is the saccharide standard peak area; m is the sample weight, g.

HPLC operating conditions Mobile phase flow rate 1.2 ml/min; injection volume of the sample—20 μl; column temperature—28 ℃; an evaporative light scattering detector, isocratic elution, a mixture of acetonitrile and water (75:25, v: v) was used as the mobile phase.
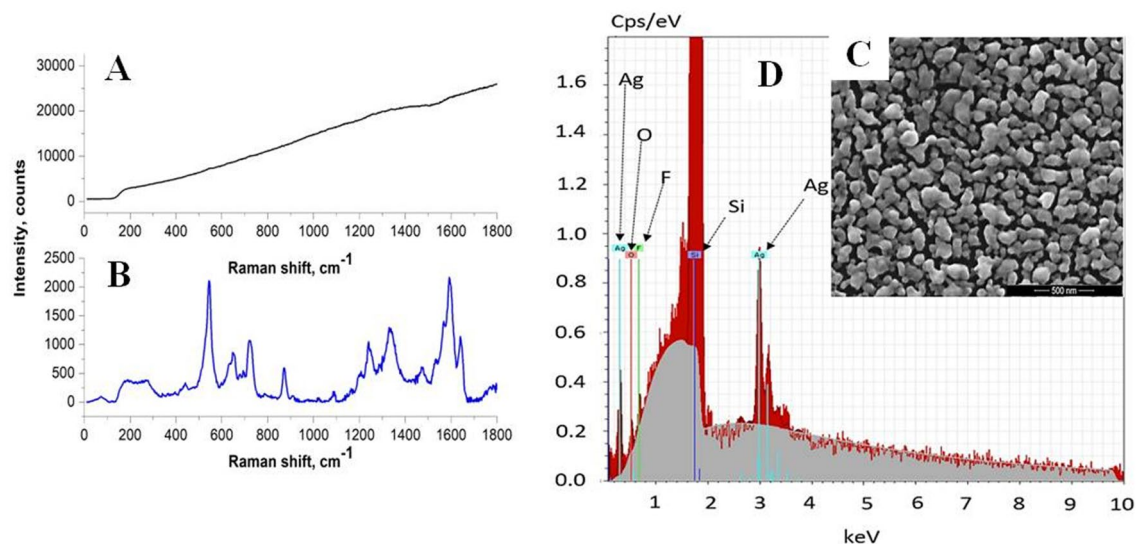
Principal component analysis

The PCA was used for the spectral data clustering to evaluate the differences among the SERS spectra of the bilberries. PCA analysis in a spectral range from 250 to 1800 cm^{-1} was carried out in the Matlab platform using Raman preprocessing software. The normalized SERS spectra of the bilberries of each country were grouped and the PCA scores were plotted. PCA score distributions were used to distinguish the spectral properties contributing to the spectral variance.

Results and discussion

Raman spectroscopy is a non-sensitive technique related to a weak scattering because only one of a million photons are scattered in-elastically. As a result, a low-intensity Raman bands are observed in the spectrum leading to the complicated analysis of the test object. Especially in the cases, when the concentrations of the particular molecules are low. Moreover, during the analysis of the materials that contain pigments, there is a probability that the fluorescent background will be observed in the spectra that will affect the qualitative as well as quantitative analysis of the sample. Given that the bilberries are rich in anthocyanin, the main peaks expressed in the spectra belong to the molecular vibrations of these phenolic compounds. Anthocyanins are pigments that give fruits, berries and vegetables a beautiful color varying from blue to red. However, the presence of anthocyanin usually determines a strong fluorescent background in Raman spectra as it is shown in Fig. 1A. As seen, the fluorescence is so intense that it suppresses a weak Raman signal emitted by the pigment. For this reason, it becomes impossible to distinguish the peaks. To avoid this issue, SERS approach was applied for the analysis of the bilberry extracts.

The principle of the SERS technique can be described as a phenomenon when the analyte adsorbs on the nanostructured metal surface leading to the enhanced intensity of Raman signal. Moreover, the fluorescence quenching occurs after the adsorbance of the analyte takes place on the surface of the nanoparticles [26]. This phenomenon is caused by the energy transfer occurring between the nanostructured metal and the fluorophore. Therefore, to perform the SERS measurements of the bilberry extracts, silver SERS substrates were prepared and analyzed. As seen in Fig. 1C, the morphological analysis of the SERS substrate showed

that the nanoparticles were successfully deposited on the silicon wafers. In addition, the EDX analysis of the SERS substrate was carried out that confirmed the presence of the silver (Fig. 1D). Random arrangement and distribution of the metal nanoparticles ensure a high density of the plasmonic hot spots—the nanometric gaps (1–2 nm) between the nanoparticles. Therefore, when the analyte is "entrapped" into the hot spot and irradiated with a laser beam, a strong electromagnetic field is generated inside the nano-gap resulting in the increased intensity of the peaks. The quantitative parameter describing the ability of the SERS substrate to enhance the Raman signal of the analyte is the enhancement factor. In this work, the EF was determined by evaporating pentacene monolayer on the nanostructured silver surface. As seen in Fig. S2 (see Supplementary Material), the pentacene was almost undetectable on the blank silicon wafer. Meanwhile, silver SERS substrate allowed to obtain the intense Raman signals. Based on the results of pentacene measurements, the EF of the synthesized silver SERS substrates was found to be 10^5. The sensitive SERS substrates were further applied for the measurements of the bilberry extracts. As seen in Fig. 1B, the bands of the bilberries are well expressed in the full range of the spectra.

After the evaluation of the morphological and sensitivity properties of the nanostructured silver substrates, SERS measurements were carried out for the wild bilberries growing in the particular regions in Lithuania (LTU), Latvia (LVA), Norway (NOR) and Finland (FNL). The results are presented in Fig. 2A.

Figure 2B shows the set of the bilberries SERS spectra of each country (first replicate). At the glance, the spectra are very similar due to the multiple overlapping bands that belong to a number of molecules in the sample. Therefore, to perform a precise analysis of the spectra, the deconvolution algorithm (Gaussian) was applied to fit the peaks. As a model for fitting, the averaged SERS spectrum of the Lithuanian bilberries was selected. The fitted bands were numbered and the vibrational assignments were provided in Table 1. As seen in the deconvoluted SERS spectra (Fig. 3), the most intensive bands arise from the vibrational modes of the most abundant anthocyanin found in bilberries—delphinidins, cyanidins, petunidins, malvidins, pelargonidins and peonidins [15, 16, 27, 28]. The intensive peaks located at ~ 1530 cm^{-1}, ~ 1590 cm^{-1}, and ~ 1640 cm^{-1} are related to the ring stretching vibrations and a band observed at ~ 1243 cm^{-1} belongs to the stretching mode of C–OH bond in anthocyanin [16]. At low pH value (~ 4.0), the spectral pattern of the bilberry extracts is determined by the vibrations of the glycosylated anthocyanin.

The vitamin C related vibrations are observed at ~ 467 cm^{-1} and ~ 1758 cm^{-1} [29]. Meanwhile, the peaks located at ~ 1590 cm^{-1} and ~ 794, ~ 1162 cm^{-1} belong to retinol and phenolic acids, respectively [27, 30]. Bilberries contain a significant amount of polyunsaturated essential fatty acids (i.e., linoleic and linolenic acids) [31] and exhibit a C=O stretching vibrations at ~ 1744 cm^{-1} and ~ 1771 cm^{-1} [32]. Given that glutamic acid and valine are the dominant amino acids in bilberries [33], they also generate SERS signals at ~ 902 cm^{-1}, ~ 1275 cm^{-1}, and ~ 1562 cm^{-1} [34]. Other important compounds present in bilberries are carbohydrates that include monosaccharides (fructose, glucose) and polysaccharides (fibers). Therefore, the peaks
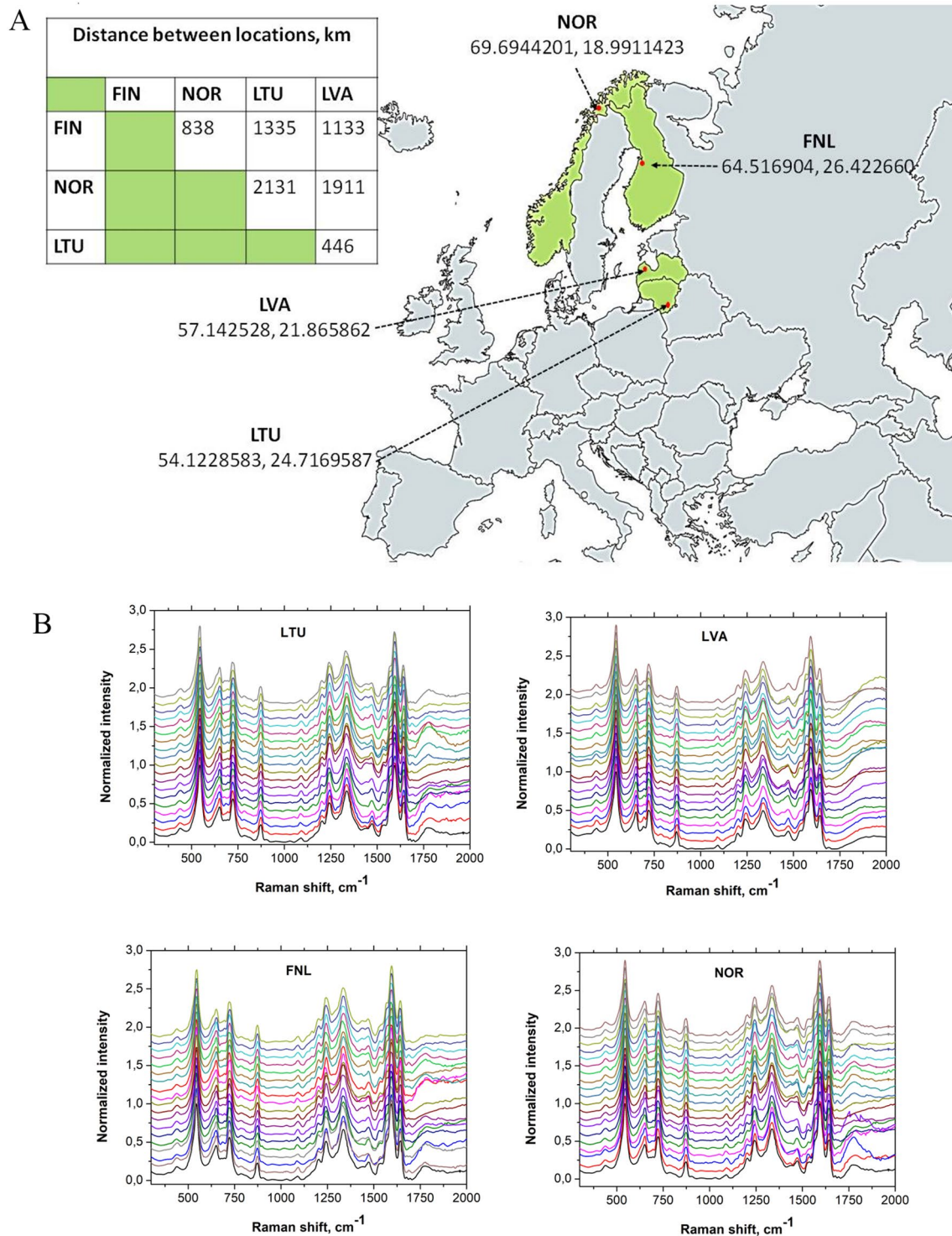
Fig. 2 A The geographical coordinates of the regions in Lithuania, Latvia, Norway and Finland where the wild bilberries were harvested; **B** the set of the bilberries SERS spectra of each country recorded at 20 different points of the sample

located at ∼ 423 cm^{-1}, ∼ 730 cm^{-1}, and ∼ 1204 cm^{-1} and at ∼ 1142 cm^{-1} and ∼ 1471 cm^{-1} belong to D-glucose and D-fructose, respectively. Based on the analysis of the deconvoluted spectrum, the comparison of the series of the bilberries SERS spectra was carried out (Fig. 2B). Despite the visual similarity of the SERS spectral pattern, the spectral clusters of each country were observed in PCA plot. As seen in Fig. 4A, the highest spectral similarity was common

Table 1 Assignment of the observed SERS bands

Peak number	Peak position, cm^{-1}	Assignment	Source
1	423	D-Glucose	[35]
2	467	Vitamin C, CO in plane deformation	[29]
3	539	Cyanidin, CC, in plane bending	[15]
4	542	Malvidin, CC in plane bending	
5	645	Malvidin	
6	651	Peonidin	
7	730	D-Glucose	[35]
8	794	Chlorogenic acid	[30]
9	875	Pelargonidin, CH, cyanidin, CH, out of plane bending	[15]
10	902	Amino acids	[34]
11	1082	Petunidin, delphinidin, aromatic CH bending and rocking, C–OH bending	[15]
12	1142	D-Fructose	[35]
13	1162	Phenolic acids	[30]
14	1204	D-Glucose	[35]
15	1243	C–OH, malvidins	[15, 16]
16	1275	Amino acids	[34]
17	1341	Delphinidin, CC stretching (interring), CH in plane bending	[15]
18	1439	D-Fructose	[35]
19	1471	D-Fructose	
20	1530	Petunidins, ring stretching	[15, 16]
21	1562	Amino acids	[34]
22	1590	Petunidins, peonidins, malvidins, ring stretching, retinol, C=C	[15, 16, 27]
23	1643	Pelargonidins, peonidins, ring stretching	[15, 16]
24	1744	Fatty acids, C=O stretching, pectins	[32]
25	1758	Vitamin C, C=O stretching	[29]
26	1771	Linoleic acid, C=O	[32]
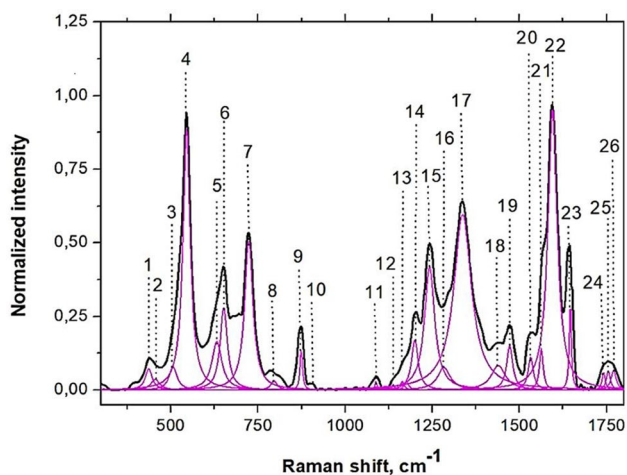
Fig. 3 The deconvoluted average SERS spectrum of Lithuanian bilberries

for the Norwegian and Finnish bilberries. Similar results were common for the second and the third experimental replicates (see Figs. S3 and S4, supplementary material).

Such similarity is determined by the similar geographical situation—both countries are located in the Northern Europe. According to the previous research, a significantly higher content of total anthocyanin is abundant in bilberries of Northern regions [36]. It is related to the lower temperatures at which the accumulation of anthocyanin increases [24]. In order to simplify the analysis and to determine the main spectral differences leading to the separation of the spectral groups in PCA plot, all the SERS spectra of each country were averaged and plotted on the same graph (Fig. 4B). It was noticed that the different qualitative/quantitative content of anthocyanin reflects in the SERS spectra. As seen in Fig. 4B, the vibrations at ~645 cm^{-1} and ~651 cm^{-1} correspond to anthocyanin and are a slightly shifted in the case of Finnish and Norwegian bilberries. Moreover, a band located at ~1341 cm^{-1} also belongs to anthocyanin and is of higher intensity in the case of Northern bilberries. These observations suggest that the Finnish and Norwegian bilberries contain higher amount of anthocyanin compared to the bilberries growing on the Eastern shore of the Baltic Sea. Another band which significantly differs in shape and intensity is located at ~1471 cm^{-1} and corresponds to D-fructose.
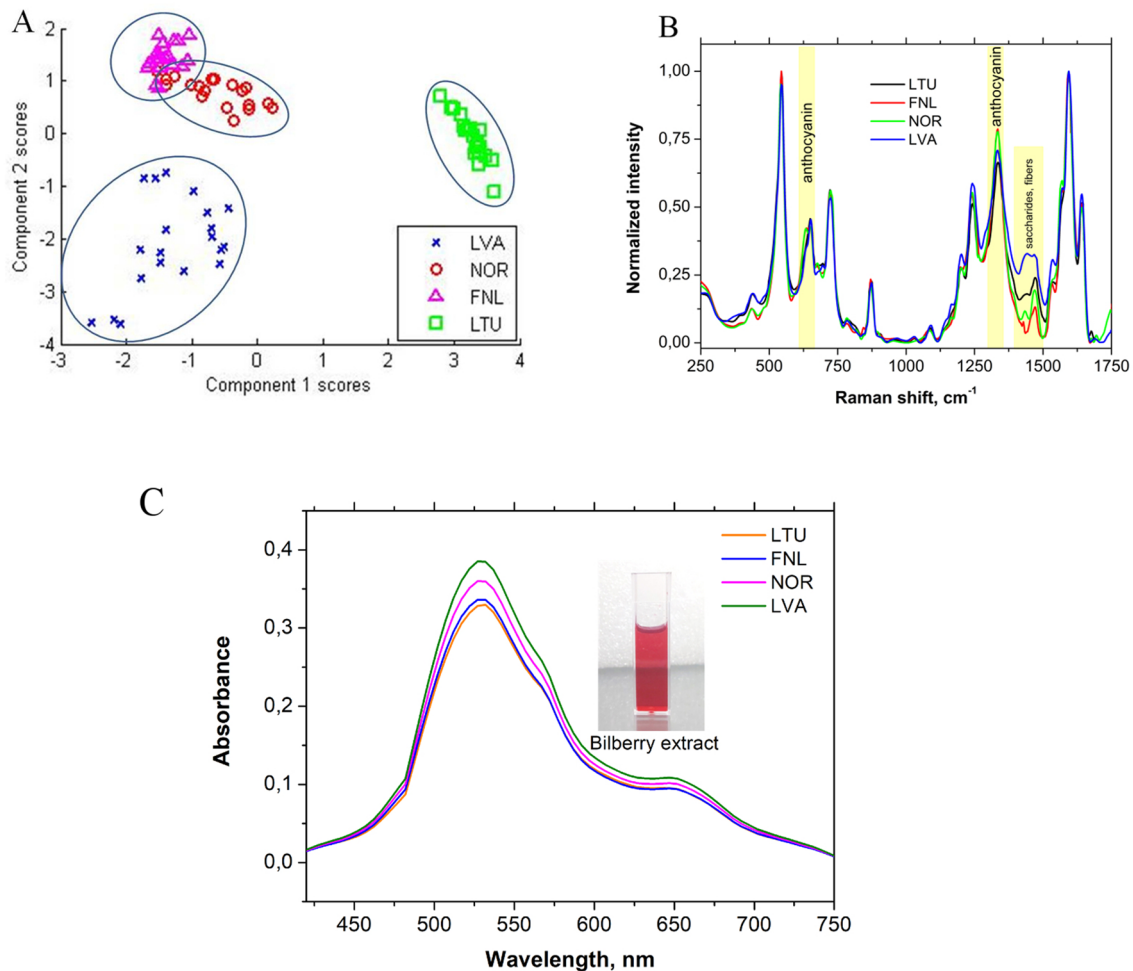
Fig. 4 **A** PCA score plot that shows the spectral discrimination between groups; **B** the averaged SERS spectra of bilberries of each country; **C** UV–Vis spectra of the bilberry extracts

The predominant saccharides in bilberries are fructose and glucose, while sucrose is found only at trace amounts due to the enzymatic hydrolysis of disaccharide [37]. The broadened peak arising from the vibrations of D-fructose leads to the hypothesis that Lithuanian and Latvian bilberries contain higher amount of fructose compared to those growing in Finland and Norway.

To confirm the discussed hypotheses, the determination of the total anthocyanin content in bilberries was carried out by UV–Vis spectroscopy. As seen in Fig. 4C, the highest absorbance was common for the Finnish and Norwegian bilberries. The calculated quantities of anthocyanin were found to be 445, 459, 508 and 525 mg/100 g for Lithuanian, Latvian, Norwegian and Finnish berries, respectively (see Table 2).

HPLC analysis of the saccharides revealed that the highest amount of D-fructose (4.71%) is common for Lithuanian bilberries. A similar amount (4.25%) was found in Latvian berries. Meanwhile, Norwegian and Finnish bilberries

contained 3.83% and 3.72% of D-fructose, respectively. These findings are consistent with the SERS experimental results and confirm the applicability of the SERS-PCA method for the determination of the geographical origin of the bilberries.

Conclusions

The wild bilberries growing at the different geographical locations of the Baltic–Nordic regions were investigated by the SERS. Silver nanoparticles based SERS substrates allowed to quench the fluorescence background, to enhance the Raman signal and to distinguish the characteristic Raman signals of the compounds present in bilberries. To evaluate the differences between spectral data of the bilberries, the chemometric analysis was performed. It was found that SERS approach in combination with PCA enables to distinguish bilberries growing in the different countries. It was

Table 2 The amount of the saccharides and anthocyanin found in Lithuanian, Latvian, Norwegian and Finnish bilberries

Country	D-Glucose	D-Fructose	Sucrose	Total values of saccharides, % (HPLC)	Total content of anthocyanin, mg/100 g (UV–Vis)
LTU	3.50	4.71	0.00	8.21	445
LVA	3.11	4.25	0.01	7.36	459
NOR	3.15	3.83	0.00	6.98	508
FNL	2.93	3.72	0.00	6.65	525

determined that significant differences in the averaged and normalized SERS spectra were determined by the qualitative and quantitative compositions of anthocyanin and saccharides. However, it is possible that other components such as amino acids, vitamins, phenolic and fatty acids, also contributed to the clusterization of the groups in the PCA plot. The proposed SERS-PCA approach was found to be fast, sensitive and cost-effective for the analysis of the bilberries at the molecular level. Therefore, the systematic collection of the SERS spectral libraries opens up opportunities for the rapid evaluation of the bilberries' geographical origin.

Author contributions LT: conceptualization, methodology, investigation, analysis, and writing an original draft. VS: measurements, investigation, analysis, writing an original draft, review and editing.

Declarations

Competing interest The authors declare no competing interests.

Compliance with ethics requirements This article does not contain any studies with human participants or animals performed by any of the authors.

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