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Market driven authentic non-timber forest products from the Baltic Sea region

Report

The determination of the authenticity of frozen and lyophilized bilberries and lingonberries of different regions in Lithuania, Latvia, Finland and Norway by Surface enhanced Raman spectroscopy (SERS)

DEVELOPMENT OF THE METHODOLOGY FOR RAMAN AND SERS MEASUREMENTS OF THE BERRIES SAMPLES

Raman vs SERS spectroscopy. Raman spectroscopy is a non-sensitive technique due to a weak scattering related to that only one of a million photons are scattered in-elastically. Therefore, low-intensity Raman bands are observed in the spectrum, what makes an analysis of a test object to be complicated. Especially in the cases, when the concentrations of the particular molecules of interest are extremely low. Moreover, when analyzing materials containing pigments, there is a probability that the fluorescent background will be observed in the spectrum, which interferes with the qualitative, as well as quantitative analysis of the test object. Since the bilberries and lingonberries are rich in anthocyanin, the main peaks of the bilberries expressed in the spectra are related to the vibrations of these organic compounds. Anthocyanin are pigments that give fruits, berries and vegetables a color varying from blue to red. However, the presence of anthocyanin usually determine a strong fluorescent background in the Raman spectra of bilberries as it is shown in Fig. 1 (A). As seen, the fluorescence is so intense that it suppress the weak Raman signal emitted by the pigment and makes it impossible to distinguish the peaks. Therefore, in order to obtain a spectrum of a good quality, surface enhanced Raman spectroscopy (SERS) was applied. 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, it is expected that the fluorescence quenching will occur after the adsorbance of the analyte will take place on the surface of the silver nanoparticles. The energy transfer occurring between the nanostructured metal and the fluorophore causes such phenomenon. Thus, in order to perform the SERS measurements of the bilberries/lingonberries, silver SERS substrates were prepared and analyzed. As it is shown in Fig. 1 (B), the morphological analysis of the SERS substrate revealed that the nanoparticles were homogeneously deposited on the silicon wafer. In addition, the EDX analysis of the SERS substrate was carried out that confirmed the presence of the silver (Fig. 1, C).

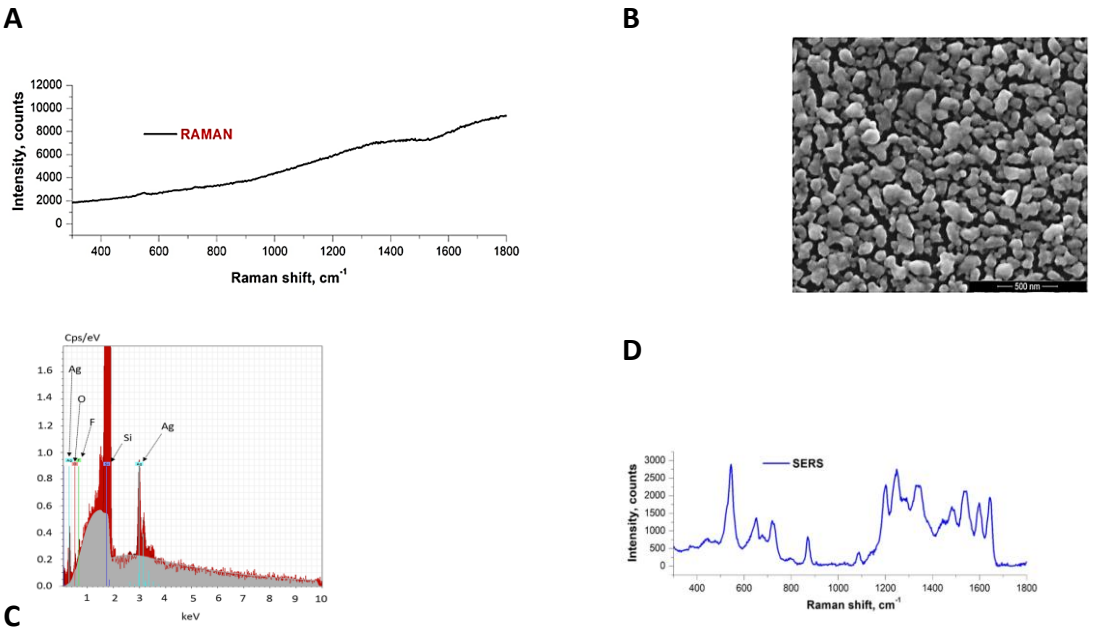


Fig. 1. (A) Raman spectra of bilberries obtained on the blank silicon wafer; (B) SEM image of the silver SERS substrate (magnification: 100 000×; high vacuum mode 30.000 kV); (C) EDX spectra of the silver SERS substrate; (D) SERS spectra of the bilberries obtained on the nanostructured silver surface

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. Hence, 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 Raman peaks. As seen in Fig. 1 (D), the peaks of the bilberries are well expressed in the full range of the spectra while performing the measurements on the silver SERS substrate.

CONCLUSIONS. Various parameters (Raman spectrometer equipped with lasers of 785 nm and 532 nm wavelength, acquisition time from 1 to 60 seconds, laser power and type of the sample) were experimentally tested in order to optimize the measurements' conditions. It was found that the seed spectrum of the berries is not informative enough while the solid berries show an extremely high fluorescence background due to the skin. Therefore, the optimal samples – the juice of the berries diluted with water were used for the experiments. Based on the number of experimental tests, the **final** methodology was built and applied for the SERS measurements of both – frozen and lyophilized bilberries and lingonberries.

METHODOLOGY FOR THE SERS MEASUREMENTS OF FROZEN AND LYOPHILIZED BILBERRIES AND LINGONBERRIES

Chemicals used for the analysis

Water, silver nitrate (≥ 99.0%) and hydrofluoric acid (48 wt. % in H_2O, ≥ 99.99%) were purchased from Sigma Aldrich. All the reagents for the SERS experiments were of analytical grade and used without further purification.

Materials and 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 microscopy (SEM, Hitachi S-3400 N).

Methodology for the preparation of the berries samples

The bilberries were homogenized in the blender. 5 g of the mashed berries was transferred into the flask and diluted with distilled water to 50 ml. The solution was centrifuged for 2 min. at 4000 rpm in order to precipitate the insoluble parts of the berries (skin and seeds). 5 ml of the centrifugate was transferred to the flask of 25 ml and diluted with distilled water. 25 µl of such prepared solution (pH 4.06) was dropped onto the silver SERS substrate for SERS measurements and on the blank silicon wafer for normal Raman measurements. The SERS spectra of the bilberries and lingonberries were recorded using Raman spectrometer (NTEGRA Spectra, NT-MDT Inc.). The objective magnification was 100 ×, numerical aperture NA = 1.49 and a spot diameter of optical beam focus – 500 nm. The excitation source was a laser with λ = 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 10 s. 20 SERS spectra of the bilberries and lingonberries were recorded at the different points of the sample. All the spectra were normalized and averaged using Origin Pro 9.0 software.

EVALUATION OF THE AUTHENTICITY OF THE BILBERRIES AND LINGONBERIES

Principal component analysis (PCA) was used to establish a novel approach for the detection of the authenticity of bilberries/lingonberries. 20 SERS spectra of each kind of the bilberries were recorded, normalized and analyzed by PCA. In order to demonstrate the developed methodology, an example of the SERS measurements and authenticity analysis by PCA is presented below. As seen in table 1, target objects for the SERS measurements were frozen bilberries obtained from three different regions in Lithuania and Norway. Fig. 2 shows the normalized spectra of the bilberries recorded at 20 different points of each sample.

Table 1. Frozen bilberries selected for the SERS measurements and evaluation of authenticity by PCA analysis.

No.	TARGET OBJECT	LOCATION
1	FROZEN BILBERRIES	LT 1 (LITHUANIA)
2	FROZEN BILBERRIES	LT 2 (LITHUANIA)
3	FROZEN BILBERRIES	LT 3 (LITHUANIA)
4	FROZEN BILBERRIES	NOR 1 (NORWAY)
5	FROZEN BILBERRIES	NOR 2 (NORWAY)
6	FROZEN BILBERRIES	NOR 3 (NORWAY)

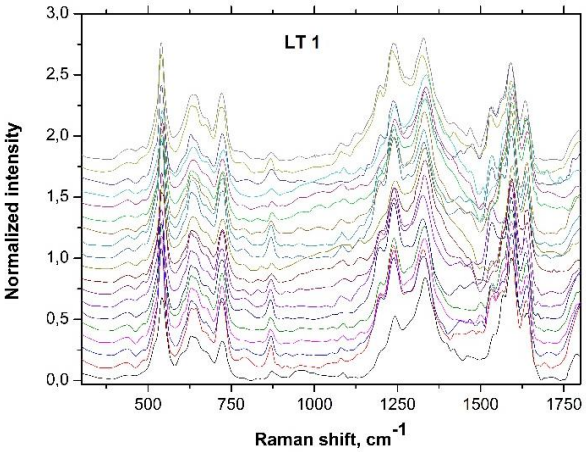


Fig. A. SERS spectra of frozen bilberries recorded at 20 different point of the sample. Bilberries were collected in Lithuania, location 1.

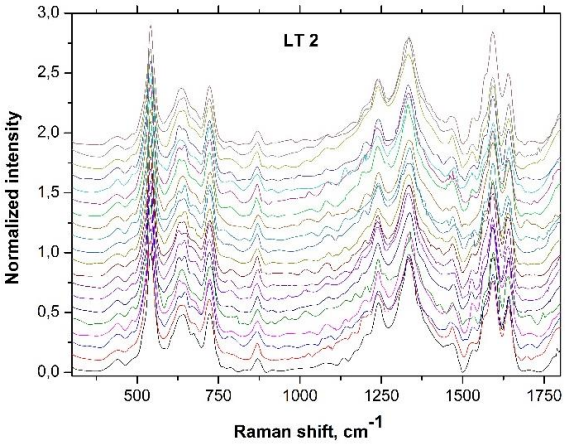


Fig. B. SERS spectra of frozen bilberries recorded at 20 different point of the sample. Bilberries were collected in Lithuania, location 2.

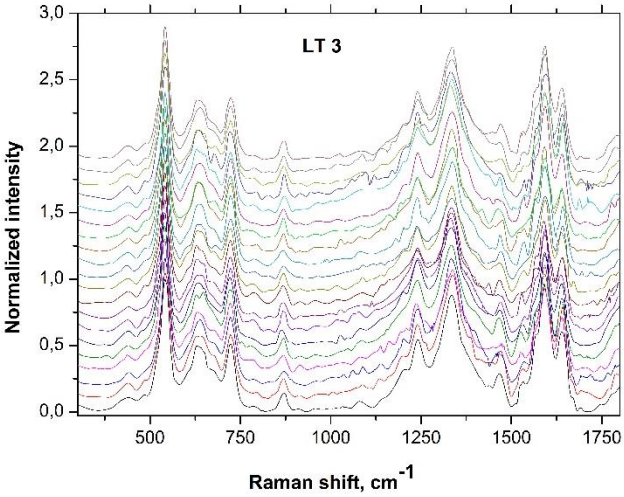


Fig. C. SERS spectra of frozen bilberries recorded at 20 different point of the sample. Bilberries were collected in Lithuania, location 3.

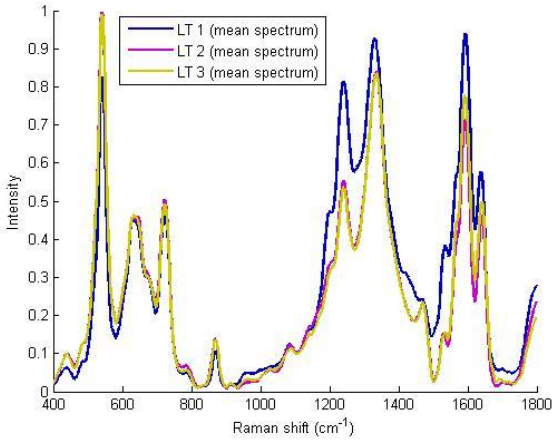


Fig. D. Mean SERS spectra of frozen bilberries obtained from 3 locations in Lithuania.

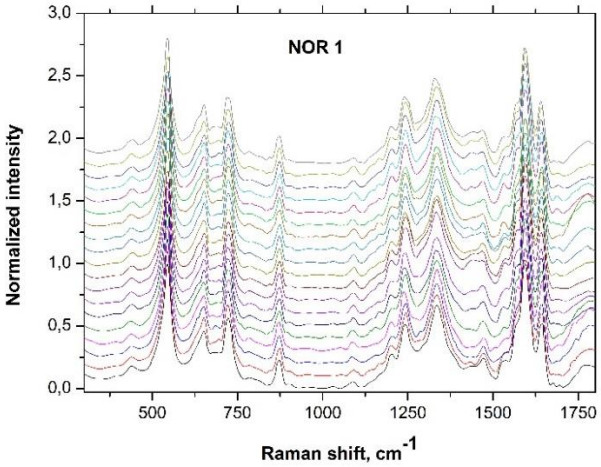


Fig. E. SERS spectra of frozen bilberries recorded at 20 different point of the sample. Bilberries were collected in Norway, location 1.

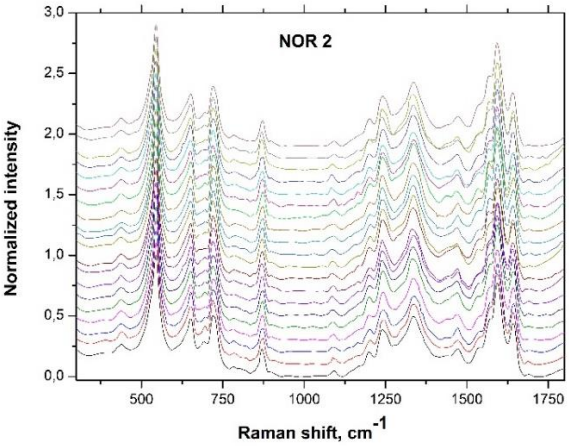


Fig. F. SERS spectra of frozen bilberries recorded at 20 different point of the sample. Bilberries were collected in Norway, location 2.

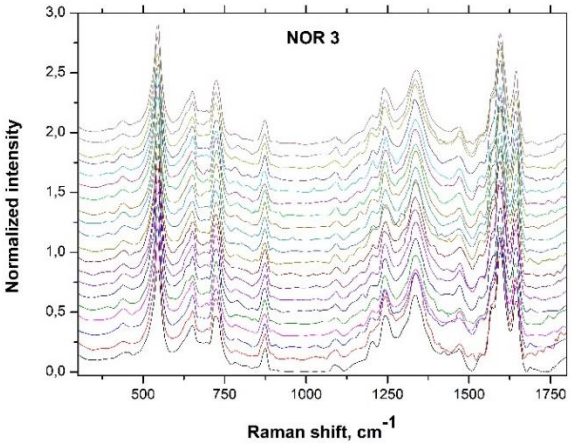


Fig. G. SERS spectra of frozen bilberries recorded at 20 different point of the sample. Bilberries were collected in Norway, location 3.

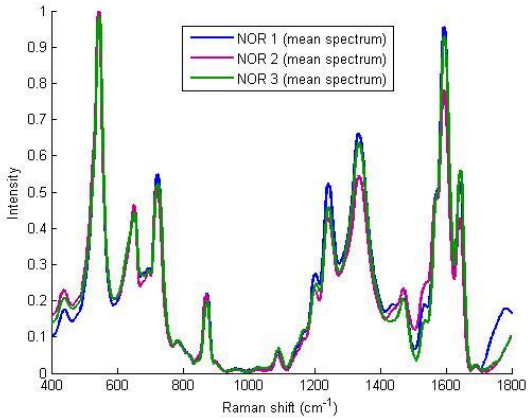


Fig. H. Mean SERS spectra of frozen bilberries obtained from 3 locations in Norway.

Fig. 2. Normalized SERS spectra frozen bilberries obtained from three different regions in Lithuania and Norway.

The set of the normalized SERS spectra of the different regions from one country are transferred to the statistical software with the purpose to perform principal component analysis. As seen in Fig. 3, PCA analysis allow the different groups of the bilberries to be identified according to the series of SERS spectra.

A **B**

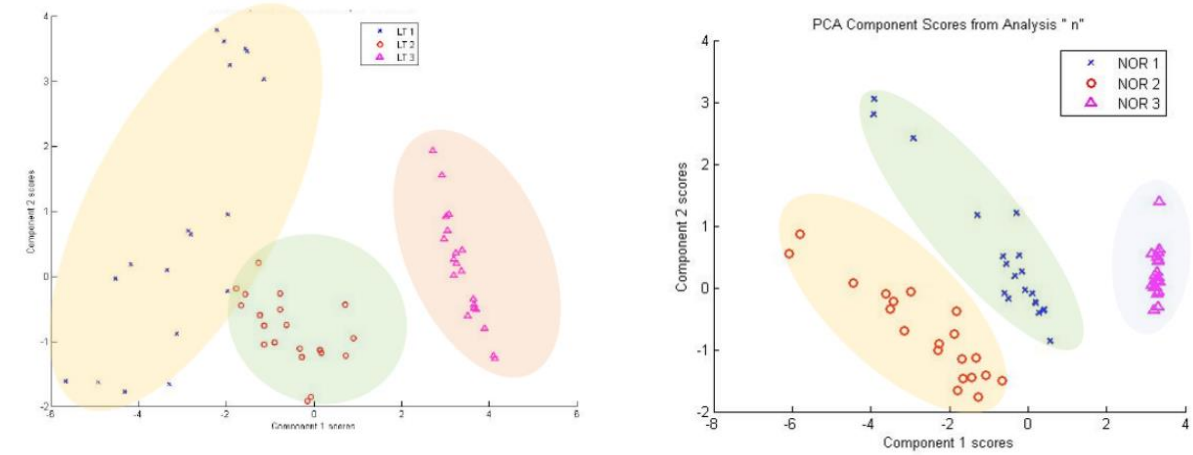
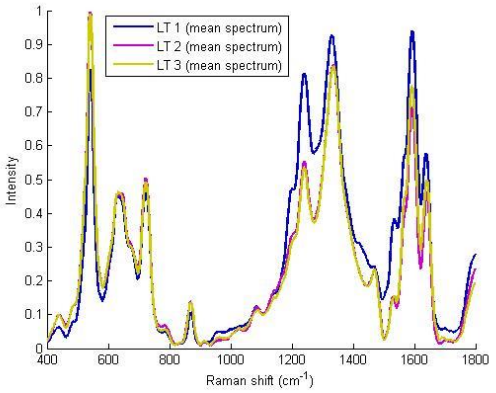


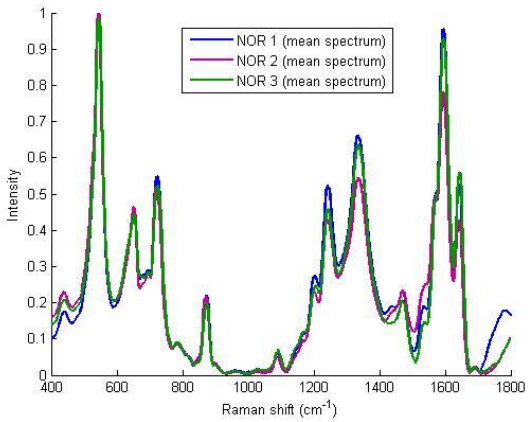
Fig. 3. PCA analysis of the frozen bilberries obtained from 3 different locations in (A) Lithuania and (B) Norway

As the indicators that determine the spectral differences leading to the statistical clusters formation in the PCA graph, flavonoids (anthocyanidins, quercetine, myricetin, etc.), vitamins and chlorogenic acids were selected. In order to develop a business-oriented methodology for the authentication of the berries, it is recommended to record the full-ranged spectra. Fig. 4. (A) and (B) demonstrates the averaged SERS spectra of the frozen bilberries collected from 3 different regions in Lithuania and Norway. The main peaks are marked by numbers and associated to the specific bond vibrations of the particular molecules. These peaks are identified and listed in the table (Fig. 4., C).

A **B**



Mean SERS spectra of bilberries obtained from 3 locations in Lithuania



Mean SERS spectra of bilberries obtained from 3 locations in Norway

C

Peak position, cm^{-1}	Assignment
423	D-glucose
467	Vitamin C, CO in plane deformation
539	Cyanidin, CC, in plane bending
542	Malvidin, CC in plane bending
630	Amino acids
651	Peonidin
730	D-glucose
794	Chlorogenic acid
875	Pelargonidin, CH, cyanidin, CH, out of plane bending
902	Amino acids
1082	Petunidin, delphinidin, aromatic CH bending and rocking, C–OH bending
1142	D-fructose
1162	Phenolic acids
1204	D-glucose
1243	Malvidin, CO, stretching
1275	Amino acids
1341	Delphinidin, CC stretching (inter-ring), CH in plane bending, phenolic acids (chlorogenic acids)
1439	Polysaccharides (fibers), flavonols (quercetine)
1471	D-Fructose
1528	Petunidin, CC stretching, flavonols backbone
1562	Amino acids
1590	Petunidin, peonidin, malvidin, CC stretching, retinol C=C
1643	Pelargonidin, peonidin, CC stretching
1744	Linoleic acid, C=O stretching
1758	Vitamin C, C=O stretching
1771	Linoleic acid, C=O

Fig. 4. Mean SERS spectra of the frozen bilberries of (A) Lithuania and (B) Norway; (C) Band assignments for the SERS spectra of bilberries

As seen from the table, most of the vibrations that belong to the different compounds overlap in the SERS spectra. For this reason, **the general** spectra consisting of the peaks exhibited by different bonds vibrations at the particular wavenumber is observed. These peaks may be deconvoluted and analyzed separately. However, PCA analysis allow the spectra of the bilberries from the different regions to be separated by avoiding deconvolution due to the shape and shifted peak positions. This phenomenon is related to the concentration of the specific compounds. For example, as seen from Fig. 5, the peaks at around 1590 cm^{-1} strongly differs in shape and are recorded at slightly shifted X-axis position due to the different concentration of retinol.

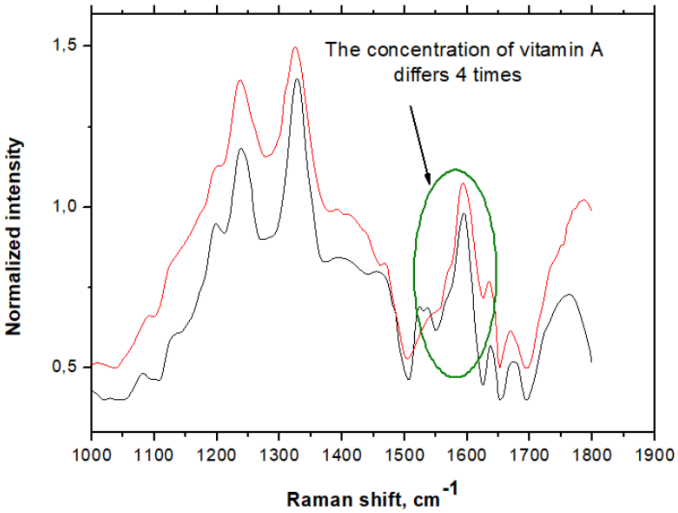


Fig. 5. Normalized SERS spectra of the bilberries of the different regions. The marked peak belongs to the retinol.

Therefore, it can be concluded that the developed methodology is appropriate for industry because of the following advantages:

- No specific preparation of the berries samples;
- No hazardous chemicals are used for the measurements;
- The time of the analysis is shortened because no deconvolution procedure is required;
- No sophisticated knowledge is required for the analysis of the authenticity results.

REFERENCE SERS SPECTRA OF THE BILBERRIES AND LINGONBERRIES (BOTH, FROZEN AND LYOPHILIZED) ARE AVAILABLE IN *.TXT FORMAT.