Near-infrared spectroscopic characterization of steviol glycosides extracted from *Stevia rebaudiana* Bertoni using high-power ultrasound and gas-phase plasma

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Summary

The purpose of this study was to examine the possibility of extraction of steviol glycosides from dried leaves of *Stevia rebaudiana* by application of high-intensity ultrasound and gas atmospheric plasma, in which water is used as a solvent. In contrast to Soxhlet extraction (by water), an increase in the yield of average value of steviol glycosides was achieved by ultrasound (59 %) and gas plasma (43 %). Highest effect was at treatment for 30 min, the amplitude of 90 μm, and a temperature of 45 °C. After gas plasma treatments, the average yield of steviol glycosides (101.34 g·kg^{-1}) was slightly lower than that after the application of ultrasound. This study also established a near-infrared spectroscopy (NIR) model for direct and rapid analysis of the expected content of steviosides. NIR spectroscopy showed the capacity that, combined with multivariate analysis tools (principal component analysis, partial least squares regression), was successful in grouping data.

Keywords

steviol glycosides; near-infrared spectroscopy; gas-phase plasma; high-intensity ultrasound; principal component analysis; partial least squares regression

One of the major problems in the diet of consumers represents the use of sweeteners or sugar, which is attributed to various health problems such as obesity, diabetes, tooth decay, hypertension and pancreatitis [1, 2]. Therefore, intensified studies have recently dealt with the use of *Stevia rebaudiana* Bertoni in human diet. Dried leaves of stevia are used, in some countries, as a natural sweetener for a long time [3]. Sweetening components in stevia leaves are sweet steviol glycosides, the most important being stevioside and rebaudioside A, which represent about 90 % (w/w) of total steviol [4].

Extraction of valuable compounds from plant material is generally conducted by the following procedures: macroscopic pre-treatment, the separation of macro- and micromolecules, extraction, purification and forming of products [5]. Among mentioned procedures, extraction is the most important step of recovering highly valuable compounds from natural materials. Conventional extraction processes, namely, distillation, solvent extraction and cold-pressing, have numerous disadvantages [6, 7]. Therefore, in recent years, the food and pharmaceutical industry express growing interest in the development and application of new methods, which will contribute to the reduction and/or elimination of the disadvantages of conventional extraction techniques. Numerous studies aim at possibilities to use thermal extraction method (assisted) such as microwaves [8, 9], and non-thermal methods, such as ultrasound [10], gas and liquid plasma [11], sub- and supercritical fluid extraction [12], pulsed electric field [13, 14] or enzyme preparations [15]. The advantages of these methods are shorter time of extraction,

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without the use of organic solvents and elevated temperatures that affect the quality of the final product, lower consumption of energy, increased yield of extracted components, which in the end improves environmental protection [16, 17]. Even slight changes of the extraction conditions can result in significant changes in the nature of the compounds to be extracted. Therefore, at selecting the extraction method, researchers should perform the procedures of optimization, which test the impact of various factors. Selection of extraction conditions is aimed at better yield and preservation of the extracted compounds [8, 18–21].

In the focus of some studies was to establish a rapid and accurate method to determine the content of glycosides [1, 3, 15, 22, 23]. A contemporary technology of near-infrared spectroscopy, which is widely applied in agricultural and food technology [24–27] in rapid determination of the sample [28, 29], can be used for calibration and prediction of characteristics of extracts and chemical constituents [15]. As a rapid, non-destructive and efficient technology for food analysis, it could also overcome the disadvantage of chemical measurement of steviol glycosides in differently treated extracts, besides the recently introduced methodology of rebaudioside A and stevioside determination based on capillary electrophoresis with contactless conductivity detection [30].

The purpose of this study was to examine the possibility of extraction of steviol glycosides from dried leaves of *Stevia rebaudiana* by application of innovative methods of extraction, namely, high-intensity ultrasound and gas atmospheric plasma, in which water is used as a solvent. This study was also aimed to establish a near-infrared spectroscopy model for direct and rapid analysis of the expected content of steviol glycosides in differently treated extracts.

MATERIALS AND METHODS

Materials

The plant *Stevia rebaudiana* Bertoni was grown in the experimental field of the Crops Department in Zagreb (Zagreb, Croatia). After harvesting, the leaves were dried in a shaded area, at ambient temperature (approximately 25 °C). The dry matter of the dried leaves of stevia was 3 % (w/w). Dried leaves were packed into plastic bags and stored until extraction in a dark place at laboratory temperature, without contact with air. Dry leaves were milled before extraction by an electric mill (Iskra, Kranj, Slovenia), for better contact with the solvent. All extractions were carried out with 5 g ± 0.0001 g of milled dry leaves, homogenized with 100 ml of distilled water. After the treatment, extracts were filtered through Whatman filter paper No. 40 (Whatman, Maidstone, United Kingdom) and stored in plastic vials at a temperature of + 4 °C until analysis.

Chemicals and standards

Potassium dihydrogen phosphate (ultrapure, 99%, for high-performance liquid chromatography, HPLC) and acetonitrile (ultra gradient HPLC grade) were obtained from J. T. Baker (Center Valley, Pennsylvania, USA). Phosphoric acid (p.a) was obtained from Kemika (Zagreb, Croatia). Steviol glycoside standards rebaudioside A and stevioside hydrate were obtained from Sigma (St. Louis, Missouri, USA). Steviol glycosides reference standard was obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Extraction methods

Soxhlet extraction

The principle of Soxhlet extraction is multiple-cycle continuous extraction. The main parts of the Soxhlet extractor are round bottom flask, extraction tube with reflux and bypass sidearms, condenser and heating element. Cellulose thimble with 5 g ± 0.0001 g of sample covered with a layer of clean cotton was inserted into extractor. Round bottom flask with 100 ml distilled water was attached to extractor and condenser, and the heating was started. Water vapour passed upwards, through the bypass sidearm of the extraction tube, into the condenser and condensed water dripped down into the thimble with the sample. Once reached the level of the bypass arm, the liquid was siphoned back into the flask. This operation continuously repeated until extraction was complete [31]. Extraction of steviol glycosides was carried out for 3 h (sample marked as S1) and 6 h (sample marked as S2), the experiments being performed in triplicate (Tab. 1).

Extraction with high-intensity ultrasound

Ultrasonic processor Misonix Sonicator S-4000 (Misonix, Farmingdale, New York, USA) was used for extraction at 600 W and 20 kHz. Samples were treated with an ultrasonic probe of a diameter of 12.7 mm, at amplitude 60 μm, 90 μm and 120 μm. Before extraction, 5 g ± 0.0001 g dry milled leaves of *Stevia rebaudiana* were transferred to a glass beaker (volume of 250 ml), to which 100 ml distilled water pre-heated to a temperature of extraction was poured. In the experiments, sonication time, amplitude and temperature of the solvent varied (Tab. 1). In order to prevent temperature

Tab. 1. Treatment parameters and extraction yield of rebaudioside A and stevioside in stevia extracts.

Sample	Time [min]	Amplitude [μm]	Temperature [°C]	Flow [$dm^3 \cdot min^{-1}$]	Measured content of steviosides [$g \cdot kg^{-1}$]		
					Rebaudioside A	Stevioside	Rebaudioside A + Stevioside
Ultrasound-treated samples							
U1	10	60	35	constant	25.3	89.0	114.3
U2	10	120	35	constant	24.6	84.4	108.9
U3	20	60	45	constant	25.1 e	90.6 e	115.7 e
U4	20	90	55	constant	26.4 dg	90.1 dg	116.4 dg
U5	30	90	45	constant	25.7 f	95.7 f	121.4 f
U6	30	120	55	constant	27.0	93.8	120.9
U7	10	120	55	constant	23.9	90.6	114.6
U8	10	60	55	constant	25.8	84.7	110.5
U9	30	120	35	constant	25.0	85.2	110.2
U10	20	120	45	constant	25.1 e	84.0 e	109.1 e
U11	20	90	35	constant	25.2 dg	91.3 dg	116.5 dg
U12	20	90	45	constant	26.9 dfg	87.1 dfg	114.0 dfg
U13	30	60	55	constant	24.5	89.9	114.4
U14	10	90	45	constant	23.3 f	82.6 f	105.9 f
U15	30	60	35	constant	24.0	78.1	102.1
U16	20	90	45	constant	25.8 defg	83.8 defg	109.6 defg
Plasma-treated samples							
P1	20	constant	45	1.5	24.5 defg	83.3 defg	107.8 defg
P2	10	constant	35	1	21.1	74.3	95.5
P3	10	constant	45	1.5	21.9	78.7	100.7
P4	30	constant	55	1	23.8	85.4	109.2
P5	20	constant	35	1.5	21.6 dg	78.6 dg	100.1 dg
P6	20	constant	45	1.5	22.8 dfg	77.6 dfg	100.4 dfg
P7	20	constant	45	2	22.9 e	80.5 e	103.4 e
P8	30	constant	45	1.5	22.0 f	79.1 f	101.1 f
P9	10	constant	35	2	21.2	75.9	97.1
P10	20	constant	45	1	22.5 e	81.9 e	104.4 e
P11	30	constant	35	2	21.4	74.1	95.4
P12	30	constant	55	2	22.4	78.7	101.1
P13	10	constant	55	2	22.5	78.8	101.3
P14	30	constant	35	1	22.3	77.8	100.1
P15	10	constant	55	1	23.3	84.0	107.3
P16	20	constant	55	1.5	21.1 dg	75.4 dg	96.5 dg
Samples treated by Soxhlet extraction							
S1	180	constant	constant	constant	20.6	51.4	71.9
S2	360	constant	constant	constant	20.0	50.0	69.9

Ultrasound-treated samples: means followed by small letters in a vertical line differ from each other according to Tukey's test ($p \leq 0.05$), significant influence of: a – time; b – amplitude; c – temperature; significant influence of two factors: d – time and amplitude; e – time and temperature; f – amplitude and temperature; g – significant influence of three factors: time, amplitude and temperature.

Plasma-treated samples: means followed by small letters in a vertical line differ from each other according to Tukey's test ($p \leq 0.05$), significant influence of: a – time; b – flow; c – temperature; significant influence of two factors: d – time and flow; e – time and temperature; f – flow and temperature; g – significant influence of three factors: time, flow and temperature.

increase caused by ultrasound, the extraction temperature was kept constant and controlled by immersing a glass beaker to ice bath.

Plasma source and gas-phase plasma treatments

In its basic form, the non-thermal plasma jet is produced using a gas-fed dielectric tube, which houses an internally powered electrode. The application of a high voltage gives rise to formation of an ionization front, which emanates from the electrode and travels along the jet of existing gas, ionizing and exciting the working gas along the way. The plasma source used was a single-electrode atmospheric jet (end-field jet type), designed at the Institute of Physics (Zagreb, Croatia) (Fig. 1) [32]. It consisted of polytetrafluoroethylene body, to which a glass capillary tube of 7.5 cm length, 0.15 cm outer diameter and 0.1 cm inner diameter was attached. A copper wire of 1×10^{-4} m in diameter was placed inside the capillary tube. The end of the wire was placed about 1 mm from the orifice. The tube was connected to the high voltage source through the vacuum-tight connector. An alternating current high-voltage source of nominal power of 6 W provided 2.5 kV at 25 kHz of quasi-sinusoidal shape [31]. The actual current through the electrode was measured by Pearson current monitor (Model 8590C; Pearson Electronics, Palo Alto, California, USA) to be typically 3 mA. The actual power of plasma was about 4 W as determined from the voltage and current waveforms. The capillary tube was also connected to the argon gas input, the flow of which was regulated by means of a flow meter (rotameter). Argon (purity 99.996%; Messer, Sulzbach, Germany) was used as operating gas for the generation of atmospheric-pressure non-thermal plasma.

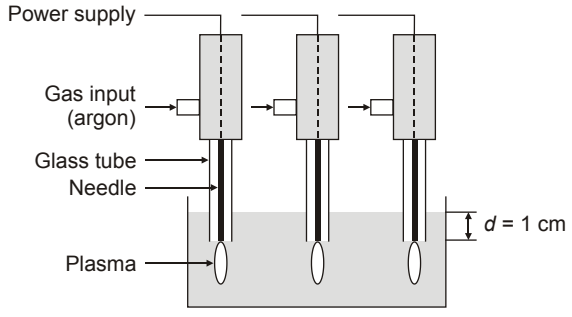

Fig. 1. Schematic description of the used plasma source.

d – distance between the plasma electrode and the surface of the liquid.

Three identical plasma sources used in the present experiment were each driven by separate high voltage source but connected to argon gas supply via a common flow meter. The plasma sources were geometrically placed in corners of an equilateral triangle with separation of about 4 cm. The sample (5 g ± 0.0001 g) of milled leaves of stevia was suspended in 100 ml distilled water pre-heated to the chosen temperature extraction in a glass (250 ml). While immersing capillary tube into the liquid, the first step was to provide gas flow in order to keep the capillary tube dry. The second step was to move capillaries into liquid. During the extraction process, three electrodes were immersed as a source of plasma into the extraction vessel, at a depth of 1 cm from the surface (Fig. 1). At given gas flows, bubbles were continuously formed at the orifice of the capillary and the electrode was never in contact with liquid. When high voltage was provided, plasma jets were formed extending from the capillary tube through the bubble and penetrating into liquid. The treatments were carried out at a constant power of 12 W, with three variable parameters: duration of treatment was 10 min, 20 min or 30 min, gas flow was 1 $dm^3 \cdot min^{-1}$, 1.5 $dm^3 \cdot min^{-1}$ or 2 $dm^3 \cdot min^{-1}$, and temperature of extraction was 35 °C, 45 °C or 55 °C, according to the design of the experiment (Tab. 1).

HPLC analysis

Water was purified in Direct-Q 3 apparatus (Millipore, Bedford, Massachusetts, USA). All solutions were prepared in acetonitrile : water (3 : 7) diluent. Two calibration solutions were prepared by weighing approximately 20 mg and 30 mg of rebaudioside A and dissolving in 25 ml of diluent. Identification solution of stevioside was prepared by weighing approximately 2 mg of stevioside and dissolving in 5 ml of diluent. System suitability solution was prepared by weighing approximately 5 mg of System Suitability Mix and dissolving in 5 ml of diluent. Steviol glycosides sample extracts were diluted with diluent in ratio 1 : 10. Mobile phase A consisted of 5 $mmol \cdot l^{-1}$ KH_2PO_4, pH adjusted to 3.0 with phosphoric acid. Mobile phase B was acetonitrile.

The liquid chromatographic system was TSP Spectra system consisting of P2000 binary pump, SCM1000 solvent degasser, AS1000 autosampler and UV2000 detector (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Separation was performed on Hypersil GOLD C18 (5 μm, 250 mm × 4.6 mm) column (Thermo Fisher Scientific). Chromatographic column was maintained at 25 °C, with a flow of 0.75 $dm^3 \cdot min^{-1}$,

using a gradient as follows: linear gradient 10 % to 35 % B in 10 min, 15 min of constant ratio 65 % A : 35 % B, linear gradient 35 % to 75 % B in 10 min, 15 min of constant ratio 25 % A : 75 % B, return to initial conditions 90 % A : 10 % B in 2 min, column re-equilibration for 15 min, total 67 min. An injection volume of 20 ml was used. Steviol glycosides were detected at 210 nm. Calibration solutions were injected in bracket, before and after sample injections. Stevioside identification solution and system suitability solution were injected at the beginning of each sequence. On the basis of calibration injections, calibration curves were created using linear regression analysis with lines forced through the origin. Steviol glycosides sample extracts were analysed in ten sequences.

Near-infrared spectroscopy of steviosides

Near-infrared spectroscopy (NIR) was applied across the range 904–1 699 nm (11 062–5 885 cm^{-1}) with a spectral resolution of 6.25 cm^{-1}. Three different spectral measurements were conducted for each sample and the average spectrum was used for analysis. Measurements of the spectra were performed using NIR spectrophotometer NIR128L-1.7 (Control Development, South Bend, Indiana, USA) with installed Control Development software Spec32 using a halogen light source (HL-2000). The first and second derivative NIR spectra were also obtained for each sample giving a total of 105 NIR spectra (raw, 1st and 2nd derivative for 35 different samples). Measurements were done under controlled temperature and humidity.

Experimental design and statistical analysis

Experiments were conducted as randomized central composite design (with factorial points, axial points and central point) with a single replication of central point. They included 16 experimental trials listed in Tab. 1 and were performed in triplicates according to the random number generated by software. All independent variables were considered at three levels, low (–1), central (0) and high (1). The experimental design was set by Statgraphics Centurion software (StatPoint Technologies, Warrenton, Virginia USA).

Software Statistica v.10 (StatSoft, Tulsa, Oklahoma, USA) was used for all statistical analyses. Factorial analysis of variance (ANOVA) with use of Tukey's test and least significant difference (LSD) test was applied to determine the equal variance assumption among the treatments (Soxhlet vs plasma treatment and ultrasound). Factorial ANOVA was used to determine if there were interactions that implied that differences in one of the factors depended on differences in the other factor, showing the mutual influence.

All results (NIR spectra and content of observed steviosides, as well as conditions of sample treatment before extraction of steviosides) were subjected to principal component analysis (PCA). Use of PCA was aimed to identify patterns in experimental data and to group or separate the data, as well as to identify possible outliers in the observed data set [33]. In this study, a scatter plot of 1st principal component (PC1) versus 2nd principal component (PC2) of PCA analysis was presented.

Based on NIR spectra, the expected stevioside content using chemometric partial regression model construction (partial least square, PLS) and validation (Statistica) were modelled. All developed PLS models were evaluated regarding the following parameters: rootmean square error of cross validation (*RMSEC*), coefficient of determination (R^2) and ratio of performance to deviation (*RPD*). More acceptable models have lower *RMSEC* and root mean square error of prediction (*RMSEP*) values, and higher R^2 and *RPD*. The models rating according to R^2 and *RPD* are typically interpreted according to guidelines published by Williams and Norris [32], where the interpretation and utility of NIR calibration model is good if R^2 is in the range 0.83–0.9. *RPD* is calculated by dividing the standard error of performance with the standard deviation of the validation set and, for a good model, should be in the range of 5.0–6.4. Values of R^2 and *RPD* higher than previously mentioned show very good and excellent interpretation and the model utility to be used with most and any applications [34].

RESULTS AND DISCUSSION

Extraction of steviosides

The content of rebaudioside A, stevioside and total steviolglycoside in extracts from dry leaves of stevia after ultrasound, plasma and Soxhlet treatment are given in Tab. 1.

Soxhlet extraction was carried out with the aim to compare the yields of steviol glycosides extracted by a classical method (Soxhlet extraction) and modern methods (ultrasound and plasma). In Tab. 1 it is shown that an extension of the extraction time from 3 h to 6 h did not improve the yield. In fact, there was a slight decline in the yield of total steviol glycosides (71.95 $g \cdot kg^{-1}$ and 69.94 $g \cdot kg^{-1}$) after 6 h. However, if the average value of steviol glycosides is compared with average values obtained using modern methods, it is evident that

there was an increase in the yield of these steviol glycosides obtained by ultrasound and gas plasma (59 % and 43 %).

The extracts obtained by an ultrasound-assisted procedure were carried out according to Tab. 1. From the results obtained for total steviol glycoside and the two most abundant compounds, rebaudioside A and stevioside, the average yield of steviol glycosides was 112.78 $g \cdot kg^{-1}$ of dry leaf, rebaudioside A 25.23 $g \cdot kg^{-1}$ and stevioside 87.54 $g \cdot kg^{-1}$. According to the literature, the content of total steviol glycosides can be up to 20 %, which states that the typical yield of stevioside is 5–10 % and of rebaudioside A is 2–4 %, based on the dry matter of leaves [3, 22]. The proportions and contents vary depending on the genotype and cultivation conditions, as well as on the extraction conditions [16, 35–38]. The results give the ratio of rebaudioside A and stevioside 1 : 3.5. A similar ratio of rebaudioside A and stevioside (1 : 3.4) was recorded by Wu et al. [39] for experiments conducted using extraction assisted with high-power ultrasound and water as the solvent.

Statistical analysis of the data in Tab. 1 (Tukey's test) showed that certain parameters, namely, time, amplitude and temperature significantly influenced the yields of rebaudioside A, stevioside and total steviolglycoside for the following pairs of factors: time × amplitude; time × temperature; amplitude × temperature, and for the triplet of factors time × amplitude × temperature. Highest effect was observed for treatment by time of 30 min, amplitude of 90 μm and temperature of 45 °C. Regarding other parameters of ultrasound-assisted extraction, differences were due to a combination of conditions: time 20 min × amplitude 90 μm; time 20 min × temperature 45 °C, amplitude 90 μm × temperature 45 °C; time 20 min × amplitude 90 μm × all temperatures.

The reason for these results was in the combination of the properties of acoustic waves with the chemical changes caused by imploding cavitation bubbles and subsequent hydrolysis of water to form free radicals, which participate in chemical reactions. Ultrasound can induce rapid and complete degassing, enhance a variety of chemical reactions of free ions (radicals), enhance the reaction of polymerization/depolymerization, and improve the rate of extraction due to tissue breakage because of microstreaming effect [40]. The mechanism by which ultrasound acts can be explained by two competing theories. These theories explain chemical effects due to cavitation, namely, the hot-spot theory and the electrical theory. The hot-spot theory postulates that when the bubbles cavitate, localized hot spots are formed which reach temperatures and pressures in excess of 5 000 K and 101 325 kPa. Under extreme conditions, there is huge mixing and deterioration of tissue, which liberate extractive compounds to surrounding media. The electrical theory postulates that an electrical charge is created on the surface of a cavitation bubble, forming enormous electrical field gradients across the bubbles, which are capable of bond breakage [41–43].

For plasma treatment, significant influences of the folowing two factors were determined: time × flow; time × temperature; flow × temperature. Significant influence of three factors was also observed: time × flow × temperature. The yields were slightly lower than those determined for ultrasound-assisted extraction, and a significant influence of combinations of plasma treatment parameters was observed: time 20 min × flow 1.5 $dm^3 \cdot min^{-1}$; time 20 min × temperature 45 °C; flow 1.5 $dm^3 \cdot min^{-1}$ × temperature 45 °C; time 20 min × flow 1.5 $dm^3 \cdot min^{-1}$ × all temperatures.

Non-thermal plasma is generated by subjection of a process gas to a strong electric field, so it presents a partially ionized gas. Apart of ionized gas molecules, this process also leads to formation of other reactive chemical species, radicals, heat and UV light, which are all together potentially involved in different reactions. In case of the atmospheric pressure plasma treatment, plasma gas is mixed with ambient air, so formation of reactive oxygen and nitrogen species is inevitable. Similar to ultrasound processing, cavitation, shock waves, rapid mixing and accelerated diffusion are also presnet at plasma-assisted extraction. On the other side, antioxidants present in stevia are protecting the cells against the damaging effects of reactive oxygen and nitrogen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite. It was reported that irradiation treatment induces stress responses in plants foods, which in turn may lead to an increase in the antioxidant synthesis [44].

NIR spectra of stevia extracts

In Fig. 2, NIR spectra of stevia extracts treated with ultrasound and plasma-jet vs NIR spectra for samples where steviosides were extracted by use of Soxhlet method (3 h and 6 h) are shown. According to presented raw NIR spectra (Fig. 2) it can be seen that the peaks are mainly enriched in the wavelength range 900–950 nm; around 1 200 nm and in the wavelength range 1 400–1 700 nm, which is in accordance with the previous findings on leaves of *Stevia rebaudiana* Bertoni [45, 46] and on the extracts from them [47], although in the total NIR range from 400 nm to 2 500 nm. However,

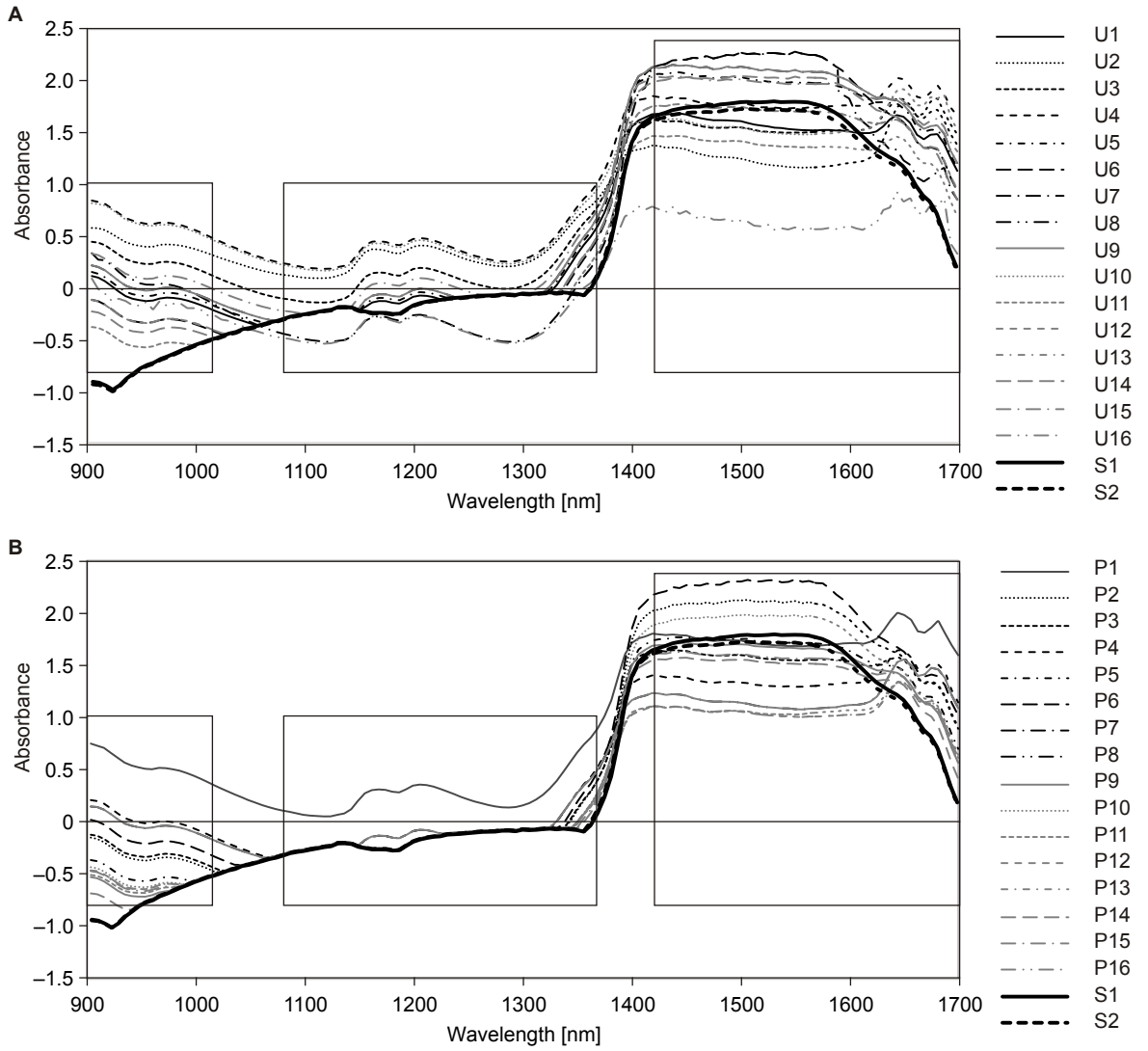

Fig. 2. Near-infrared spectra of stevia extracts.

A – spectra of extracts treated with ultrasound or extracted by the Soxhlet method; B – spectra of extracts treated with plasma jet or extracted by the Soxhlet method.
Rectangle area shows important spectral ranges that significantly differ for the observed stevia extracts.
U1–U16 – stevia extracts treated with ultrasound; P1–P16 – stevia extracts treated with plasma jet; S1, S2 – stevia extracts obtained by Soxhlet extraction for 3 h and 6 h, respectively.

our results provide first information on extracts obtained with assistance of ultrasound and plasma jet. To optimize the selection of the most informative spectral region and the spectral bands that were in correlation with content of steviosides, the first and second derivative of the spectra were calculated (data not shown).

When the sample is irradiated with NIR light, it absorbs the light with frequencies matching characteristic vibrations of particular functional groups, whereas the light of other frequencies will be transmitted or reflected and various fundamental molecular vibrations, including those generated from C–H, O–H, N–H, C=O, and other functional groups can be detected [48]. The spectral range of the used NIR instrument (904–1699 nm) facilitated observation of vibrations generated from C–H, O–H and other functional groups. The C–H stretch 3rd overtone including relation to either $C–H_3$ stretch 3rd overtone or to compounds containing C–H aromatic groups is related to the absorption band at 900–950 nm, where our spectra and derivatives indicated important wavelengths. NIR band at 1450 nm is related to the second

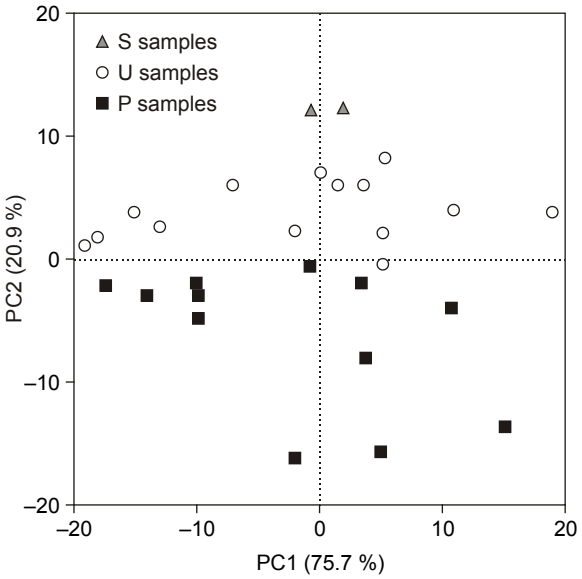

Fig. 3. Principal component analysis of stevia extracts based on the near-infrared spectra and content of steviosides.

S samples – extracts obtained by Soxhlet extraction, U samples – extracts treated with ultrasound; P samples – extracts treated with plasma.

overtone of O–H stretching related to the content of water, but this wavelength is also related to the second overtone region of R–OH stretching. The first overtone of the C–H_3 stretching in studies related to the compounds containing C–H aromatic groups is related to specific vibration at the NIR wavelength at 1 650–1 699 nm [49]. Although visual differences among the extracts could be seen as for differently treated samples (ultrasound and plasma) and not-treated (Soxhlet – 3 h and 6 h), it was necessary to remove some parts of the spectra that were not informative regarding the content of steviosides. The modelling band range was chosen according to ranges used in observations of sugars in wines and fruits [48, 50] or in a study of water-sugar interactions at increasing sugar concentration by NIR spectroscopy [50].

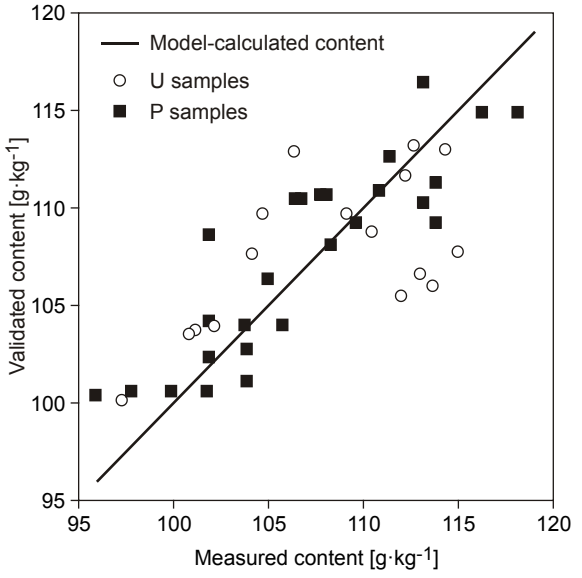

Fig. 4. Validation of stevioside content with near-infrared spectrometry using partial least square models.

U samples – extracts treated with ultrasound; P samples – extracts treated with plasma.

Application of principal component analysis

Fig. 3 shows grouping of samples based on PCA in the ranges extracted by use of factorial analysis (1 398–1 627 nm). The reason was that those ranges were enriched in the expected absorption bands of steviosides, such as C–H stretch, C–H_3 stretch or C–H stretch of aromatic groups, as well as O–H overtone of water and related stretches of R–OH. The grouping was based on raw NIR spectra (absorbance) revealing 2 outliers. The PCA score plot was used to observe complementary sets of attributes in their grouping based on the properties of the samples, showing differences or similarities. Scores on PC1 vs PC2 with the cumulative contribution of 96.6 % was the first step to visualize the main trends in the sample set. Second principal component presents the contribution of the content of steviosides in the examined extracts (20.9 %), while the first and dominant principal component was related to NIR spectral data and explained 75.7 % of the variations in the data set. The control samples (Soxhlet extraction of steviosides) were presented by a square and placed on the right side of the chart, as a separate group. The seclusion of Soxhlet samples was expected but, from the group of treated samples, segregated two samples already mentioned as outliers.

Modelling for prediction of the content of steviosides

Next, we intended to investigate if it was possible to calibrate and predict the content of steviosides in differently treated samples. PLS regression analysis was carried out to construct calibration models for predicting the content of steviosides (Fig. 4). PLS relates two data matrices X (wavenumbers) and Y (stevioside contents) by a bilinear multivariate relationship [34].

The idea was to apply PLS to the entire range of raw spectra to examine the NIR model for non-destructive prediction of stevioside content. According to Bursać Kovačević et al. [49], we used ranges of NIR spectra proven to correlate

with sugars in wines, fruits and in water-sugar extracts [50]. Included were ranges from the second and third marked section (Fig. 2) because those ranges were enriched in the expected absorption bands (C–H stretch; C–H_3 stretch or compounds containing C–H aromatic groups; O–H overtone of water and related R–OH). More acceptable models have lover *RMSEC* values, where excellent models have a high R^2 (> 0.9) and *RPD* above 5 [51]. In the models for stevioside content (Tab. 2), higher *RMSEC* was detected when NIR wavelength suggested by GIANGIACOMO [50] were used. This also resulted in a higher correlation coefficient R^2. The preffered *RPD* value was not reached but the best performing was the 1st model with samples treated with plasma jet, where this value showed the best accuracy and robustness of the models.

Tab. 2. Quality parameters of the partial least square regression models.

Observe treatment	Calibration		*RPD*
	r_c^2	*RMSEC*	
Ultrasound treatment			
M1	0.999	2.712	3.5
M2	0.997	4.066	2.3
Plasma-jet treatment			
M1	0.998	5.340	4.6
M2	0.989	5.108	4.1

M1 – model according to GIANGIACOMO [49], M2 – model including total spectral range (900–1 700 nm).
r_c^2 – correlation coefficient for validation, *RMSEC* – root mean error of cross validation, *RPD* – ratio of performance to deviation.

CONCLUSIONS

This study showed the capacity of NIR spectroscopy that, combined with multivariate analysis tools (PCA and PLS), was successful in grouping data regarding the sample treatments and potentially can be used in prediction of expected content of steviosides in extracts treated with ultrasound and plasma-jet, respectively. Average value of extracted steviol glycosides, in comparison with average values obtained using classical extraction method (Soxhlet), showed an increase in the yield of steviol glycosides obtained by ultrasound and gas plasma (59 % and 43 %). For plasma treatment, there was a significant influence on extraction yield of two factors that interacted: time × flow; time × temperature and flow × temperature. Significant influence of three factors was also determined for interaction of time × flow × temperature. The yields of investigated parameters were slightly lower than those of ultrasound, and there was a significant influence of combinations of plasma treatment parameters - time 20 min × flow 1.5 $dm^3 \cdot min^{-1}$; time 20 min × temperature 45 °C; flow 1.5 $dm^3 \cdot min^{-1}$ × temperature 45 °C. For the interaction of three factors (time, flow and temperature) were the significant changes determined for samples treated for 20 min, using the flow of 1.5 $dm^3 \cdot min^{-1}$ and for all temperatures (35 °C, 45 °C and 55 °C). Certain parameters of ultrasound treatment, namely, time, amplitude and temperature, significantly influenced the yield of rebaudioside A, stevioside and total steviolglycoside in the interaction of two factors: time × amplitude, time × temperature and amplitude × temperature. Also are determined significant influence of three factors: time × amplitude × temperature. Highest effect of three factors was in treatment by 30 min time, the amplitude of 90 μm and a temperature of 45 °C.

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