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Near-Infrared Spectroscopic Analysis of Total Phenolic Content and Antioxidant Activity of Berry Fruits

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Summary

This study evaluates the feasibility of using near-infrared (NIR) spectroscopy as a rapid and environmentally friendly technique for validation and prediction of the total phenolic content (TPC) and antioxidant activity (AOA) indices (as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, inhibition time (IT) of the Briggs-Rauscher oscillating reaction, and relative antioxidant capacity (RAC)) of berry fruit extracts. The analysed berry samples originated from Croatia (blackberries, wild blueberries, raspberries, red currants and strawberries) and Bulgaria (wild blueberries, raspberries and strawberries). Principal component analysis and partial least squares (PLS) regression were used from the set of chemometric tools in distinguishing and validating the measured berry fruit extract. ANOVA and PCA showed no significant impact of the origin and freshness of the samples. PLS models were developed to validate the relationship of NIR spectra with TPC and AOA of berry fruits. Representativeness of the models was expressed with the R^2 and the ratio of performance to deviation. Calculated R^2 values were above 0.84 and the ratio of performance to deviation was between 1.8 and 3.1, indicating adequacy of the PLS models.

Key words: berry fruits, antioxidant activity, NIR spectroscopy, PLS models, total phenolic content

Introduction

Generally, berries have short shelf-life and are widely processed into juices, jams and fruit concentrates used in production of ice cream, yoghurt and confectioneries (*1*). The most commonly consumed cultivated berries are strawberries (*Fragaria* × *ananassa*), raspberries (*Rubus idaeus*), blackberries (*Rubus* spp.), blueberries (*Vaccinium corymbosum*), black currants (*Ribes nigrum*) and red currants (*Ribes rubrum*). On the market some crosses between raspberries and blackberries, such as loganberry (*Rubus loganbaccuus*) are also available. Bilberry (*Vaccinium myrtillus*), also known as wild blueberry, and lingonberry (*Vaccinium vitis-idaea*), are the most important wild berry species in Europe (*2*).

Different bioactive substances in berries such as anthocyanins, flavanols, flavonols and phenolic acids have biological effects such as antioxidant, antimutagenic, anti-inflammatory, antiproliferative and antimicrobial activities (*1–3*). A great number of *in vitro* studies show that berry polyphenols are powerful dietary antioxidants, a

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property based on easy donation of a hydrogen atom from an aromatic hydroxyl group to the free radicals (*4*). In addition, the ability to chelate transition metal ions involved in radical-forming processes such as Fenton reactions could also contribute to the antioxidant efficacy of these compounds (*5*).

The antioxidant activity depends on the berry species and, for example, wild berries are known to have generally higher total antioxidant capacity than the cultivated ones (*6*). However, extensive variations in the phenolic content in different wild berry populations have been observed (*7,8*), indicating a strong influence of environmental conditions, especially the effect of processing and subsequent storage. Therefore, a rapid and reliable method is required to discriminate berry samples, both raw materials and processed food products, with high content of polyphenols as well as to assess their antioxidant capacity.

Near-infrared (NIR) spectroscopy has been proven to be a suitable technique for rapid food analysis that does not require sophisticated sample preparation (*9*). NIR spectroscopy is based on the absorption or reflection of electromagnetic radiation in the near-infrared region and spectral analysis needs to be assisted with various chemometric techniques. NIR measurements have been successfully used in food quality evaluation (*10,11*) and prediction (*12*). Recently, the combined application of NIR spectroscopy and chemometrics has been used in the control of wild berry fruit freshness (*13*).

This study aims to investigate the applicability of the NIR spectroscopy in verifying the grouping of samples according to the species, regardless of the origin and freshness (*14,15*). To achieve the set goal, modelling techniques must be applied; for potential grouping, the principal component analysis (PCA) was used, and to evaluate the feasibility of using NIR spectroscopy in validation and prediction of the observed parameters, partial least squares (PLS) regression was applied. Total polyphenolic content and the examined antioxidant activity indices of berry fruits were used as validation and prediction parameters. For the first time the potential of sample separation with respect to the growing location (different regions) and their freshness status has been examined. The separation, calibration and validation are based on the polyphenolic data associated with their NIR spectra, followed by the PLS modelling. The Croatian berry samples were used as the calibration sets and the validation was performed on Bulgarian samples.

Materials and Methods

Plant material

Berry fruit samples originating from Croatia (blackberries, wild blueberries, raspberries, red currants and strawberries) were purchased on the local market from manufacturers who sell only fruits from their orchards (Velika Gorica, surroundings of Zagreb). Bulgarian samples (wild blueberries, raspberries and strawberries) were hand-picked from their natural habitats (south-western part of the Rhodope Mountains). All fresh berry fruits were sorted to eliminate unripe, overripe or damaged fruits and placed in polyethylene bags (approx. 200 g) and frozen (−20 °C). These two regions were selected because of the lack of scientific data comparing berry fruit originating from them. It is expected to find certain differences based on their origin.

Sample preparation and storage

Frozen berry samples (blackberries, wild blueberries, raspberries, red currants and strawberries) were defrosted, pitted and homogenized in a blender at room temperature. A mass of 2.5 g of the homogenized fruit was mixed with 10 mL of 96 % methanol in order to prepare the basic solution. The reaction mixture was stirred at the reflux temperature for 10 min. Extractions were performed in triplicates. The solution was cooled at room temperature and the content was filtered using a Büchner funnel. The mother liquor was decanted in the flask and 12.5 mL of methanol were added to the remaining sample, and the refluxing was repeated. The second mother liquor was added to the flasks and methanol was refilled to 25 mL.

All extracts were analysed as fresh berry fruit extracts and then placed in a refrigerator at the average temperature of 5 °C. They were analysed again after 30 days, without exposure to light and additional moisture.

Total phenolic content

Total phenolic content was determined following the modified method of Singleton and Rossi (*16*). The values are expressed in mg of gallic acid equivalents (GAE) per 100 g of fresh mass using gallic acid standard curve (0–500 mg/L).

Inhibition time during Briggs-Rauscher reaction and relative antioxidant capacity

Briggs-Rauscher (BR) oscillating reaction is based on the reactions of three colourless solutions that are prepared and mixed as proposed by Cervelati *et al.* (*17,18*). All three solutions are mixed in equal volume ratios at room temperature (*18–20*). A volume of 0.5 mL of the fruit extract was added to the total volume of the BR mixture (15 mL). BR reaction is potentiometrically monitored using a combined platinum electrode and the inhibition time (IT) is recorded (using LabX® Software; Mettler Toledo, Greifensee, Switzerland). The IT presents the oscillation inhibition that linearly depends on the concentration of the added antioxidant (*18*). When the oscillation quenching is recalculated using a standard solution (in our study – gallic acid), the result is presented as relative antioxidant capacity (RAC) (*21*), which is the ratio of the concentration of the standard and the concentration of the sample added to the mixture at a certain inhibition time. BR method is presented as a potential *in vivo* antioxidant activity method (*17*), because the pH value of the reaction mixture is around 2, which corresponds to the acidic conditions of the stomach (*18*).

Antioxidant capacity as DPPH radical scavenging activity

The antioxidant capacity of the samples was evaluated by 2,2-diphenyl-1-picrylhydrazil radical ($DPPH^{\cdot}$) using the method proposed by Brand-Williams *et al.* (*22*).

The phenolic fruit extracts were also used for DPPH assay. Final results are expressed in mmol of Trolox equivalents (TE) per 100 g of fresh mass.

All analytical experiments were carried out in triplicate including all spectrophotometric measurements, which were performed by the UV-Vis spectrophotometer (Spectronic Unicam, Cambridge, UK).

Near-infrared spectroscopy

Near-infrared spectroscopy (NIR) was conducted in the range from 904 to 1699 nm, using the spectrophotometer NIR-128-1.7-USB/6.25/50μm (Control Development, Inc., South Bend, IN, USA) with installed CDI software Spec32 (Control Development, Inc). NIR spectroscopy was used as a nondestructive technique of sample measurement in which the radiation reflected by the berry fruit extracts was measured. Each absorbance spectrum was recorded in triplicate and the mean spectra were calculated.

Modelling

Statistical analysis was carried out using the STATISTICA v. 10.0 software (StatSoft, Inc, Tulsa, OK, USA). NIR spectroscopy data were evaluated using multivariate analysis (chemometrics) to identify patterns in the experimental data (*23*). The principal component analysis (PCA) was used to examine the interrelations among the observed set of variables to identify the underlying structure (*24*) and to screen the possible outliers as well as to identify patterns in experimental data and to express the data based on their similarities and differences (species, origin, freshness). To reduce the analytical work and predict some parameters based on NIR spectra, partial least squares regression was applied (*25,26*). Partial least squares regression feature is used to model multivariate data that are noisy and redundant as NIR spectra *vs.* other characteristics (as total phenols and antioxidant activity and/or species, origin, freshness) (*27,28*). The model performance was evaluated with R^2 and the regression point displacement (RPD). RPD value is the ratio of the standard error of performance and the standard deviation (*29*). Models with the prediction classified as excellent have RPD>3, while RPD<1.5 indicates that presented calibration model is not suitable for prediction, values between 1.5 and 2 show a possibility to distinguish high and low levels with the presented model, and values in the range of 2–2.5 and 2.5–3 show approximate quantitative prediction that is possible and prediction that is classified as good, respectively.

Results and Discussion

Total phenolic content of berry samples

Results of the determined total phenolic content (TPC) and antioxidant properties are shown in Table 1 expressed as: inhibition time (IT), relative antioxidant capacity (RAC) and antioxidant capacity (DPPH) of different berry fruit extracts, analysed as fresh samples and after one month of storage (at 5 °C), originating from two regions.

Table 1. Total phenolic content (TPC) and antioxidant activity, expressed as inhibition time (IT), relative antioxidant capacity (RAC) and antioxidant capacity (DPPH) of different berry fruit extracts

Extract	*w*(TPC as GAE)	IT	RAC as GAE	*b*(DPPH as TE)
	mg/100 g	s	μg/mL	mmol/100 g
CRC_F	149.8±11.2	291.9±8.2	0.4± 0.1	2.4±0.4
CBB_F	303.0±20.1	1328.0±25.3	1.7±0.4	8.8±1.1
CS_F	251.5±17.1	569.0±33.8	0.7±0.2	(6.4±1.2)[a]
CWB_F	473.0±6.5	2198.5±41.5	2.9±0.7	8.1±1.1
CR_F	299.0±14.6	392.6±21.2	0.5±0.2	(9.2±0.9)[a]
BS_F	263.5±7.2	1412.9±22.7	1.9±0.2	2.8±0.8
BWB_F	(258.7±10.1)[b]	1934.2±11.0	2.5±0.1	3.6±0.4
BR_F	(207.4±9.4)[b]	683.6±5.9	0.9±0.0	2.4±0.2
CRC_S	132.1±7.7	295.9±7.7	0.4±0.1	2.3±0.7
CBB_S	302.3±11.8	1328.1±37.2	1.7±0.5	7.9±1.3
CS_S	61.1±21.7	285.1±11.6	0.4±0.1	(5.4±1.2)[a]
CWB_S	422.2±10.1	1374.8±39.4	1.8±0.6	8.1±1.9
CR_S	218.0±8.3	413.3±21.2	0.5±0.1	(8.2±1.0)[a]
BS_S	208.9±9.4	1200.7±10.1	1.6±0.2	4.5±1.2
BWB_S	(233.8±10.1)[b]	1377.7±8.3	1.8±0.2	2.5±0.7
BR_S	(146.3±11.0)[b]	255.2±12.2	0.3±0.1	2.3±0.6

Values are the average of three replications±standard deviation. Letters in superscript in the same column indicate statistically significant differences ($p<0.05$) between Croatian and Bulgarian samples as well as the differences between fresh (F) and stored (S) samples: Croatian samples: CRC=red currants, CBB=blackberry, CS=strawberry, CWB=wild blueberry, CR=raspberry; Bulgarian samples: BS=strawberry, BWB=wild blueberry, BR=raspberry; GAE=gallic acid equivalent, TE=Trolox equivalent

The obtained TPC in the fresh samples from Croatia ranged from 149.8 mg of GAE per 100 g in red currants, to 473 mg of GAE per 100 g in blueberries. The same pattern remained in the samples stored for one month, where blueberries were the richest in TPC (422 mg of GAE per 100 g), but the TPC loss during storage was the greatest in strawberries, where it was 61.1 mg of GAE per 100 g. These findings for fresh berry fruits are in accordance with other studies (*30–32*). When the retention was expressed in percentage, the losses were between 0.3 % (blackberry) and 75 % (strawberry). To establish significant differences in the data set (Table 1), one-way ANOVA was carried out with the significance level of $p<0.05$. The ANOVA revealed no significant change of TPC in raspberries ($p=0.5$), regardless of their origin (Croatian samples 27 % and Bulgarian 29 % loss), but significant differences were determined in the TPC in strawberries (75 *vs.* 20 %, respectively). The observed differences are to be expected because studies have shown that the content of total polyphenols is related to many factors such as variety, farming area, climatic conditions, degree of maturity and method of cultivation (*33,34*).

Antioxidant performance of berry samples

Antioxidant activity (AOA) of berry samples, expressed as IT, RAC and DPPH radical scavenging, was

also investigated (*21*). The highest value of AOA was measured in Croatian raspberries using the DPPH method, regardless of whether they were fresh or stored for one month. Minimal value of AOA was detected in the fresh Croatian red currant and Bulgarian raspberry samples (2.3 mmol of TE per 100 g), inhibition time during BR oscillating reaction, used as a measuring method in determination of AOA (*20*), had the highest values in the blueberry samples (IT(fresh sample)=2198.5 s and IT(stored sample)=1377.7 s). The IT is proportional to the AOA of the examined samples, showing the same trend of changes in IT as in TPC (*23,26*). Expressing the AOA as the ratio of the concentration of the standard (RAC), over 50 % of the berry samples had RAC values greater than 1, which means that their AOA equals or is greater than the AOA of the same mass of gallic acid.

Results presented in Table 1 indicate good AOA regardless of the origin, also supporting the findings of Pantelidis *et al.* (*31*), who determined a strong relationship between the TPC and AOA. Consumption of food rich in TPC and AOA has a positive impact on the health and well-being, with emphasis on regular fruit and vegetable consumption (*35*). The research of Lugasi *et al.* (*36*) showed that the AOA of berry fruits is related to the variety, soil type, climatic conditions and water and nutrient supply to the plants, which explains significant differences in TPC and AOA in Croatian and Bulgarian strawberries. However, the applied ANOVA test did not reveal any significant differences based on the origin or freshness of the samples (except strawberries), so the data set of berry fruits from the two regions could be observed as one set, which was applied here.

Near-infrared spectroscopic measurements

Spectroscopic measurements in the near-infrared (NIR) range were conducted to determine the chemical and physical properties of berry fruit extracts, based on molecular overtone and combination vibrations. NIR spectra of the berry fruit extracts are shown in Fig. 1.

Complex chemical composition of fruits and vegetables results in a large set of overtones and combination bands, which can be observed in the NIR spectra (*13,23,26*). Usually used NIR spectrum range is from 400–2500 nm (*37*), but to ensure the data differentiation between the examined samples, the range of the used NIR instrument (904–1699 nm) can be considered as sufficient if specific molecular overtone and combination vibrations of TPC and AOA are observed (*38*). Significant differences were observed in the NIR spectra from 904 to 928 nm and from 1399 to 1699 nm (highlighted sections in Fig. 1). The important NIR spectrum ranges identified for berry fruit extracts are in accordance with other studies (*23,36*). Vibration differences in the range from 1399 to 1699 nm have been identified for fruit products such as grape juices, wine and oranges (*9,23,38*), showing the vibration range of the C-H and O-H bonds corresponding to the water and phenolic absorbance (*9*). More detailed analyses of the obtained spectra indicated changes at specific wavelengths: 1434–1635 nm, corresponding to the vibrations of C–H bands (the first overtone), and the vibrations in the range from 904 to 928 nm are related to the third CH overtone, second overtone of the OH stretch of H_2O, and a combination of stretch and determination of the OH group in H_2O (*37,39*), which are the bands and groups expected in phenols and antioxidants.

These findings show the complexity of assigning specific absorption bands to a specific functional group. Thus, multivariate statistical techniques are required to extract information from the recorded NIR spectra (*38*). A new data matrix was formed that contains information on TPC and AOA of each sample, with which the corresponding NIR spectrum is associated.

Applied chemometrics

The application of PCA derives a small number of independent linear combinations (principal components, PCs) for a set of variables (*25*). In this study, scatter plot of the first principal component *vs.* second principal component is presented with related loadings (Fig. 2).

Use of chemometrics helps to detect similarities or differences of the studied samples and to exclude their fingerprint using the analysed TPC and AOA with their corresponding NIR spectra (*40*). The differentiation of the examined samples based on TPC, IT, RAC and DPPH is shown in Fig. 2, where the PCA was used as statistical classification method.

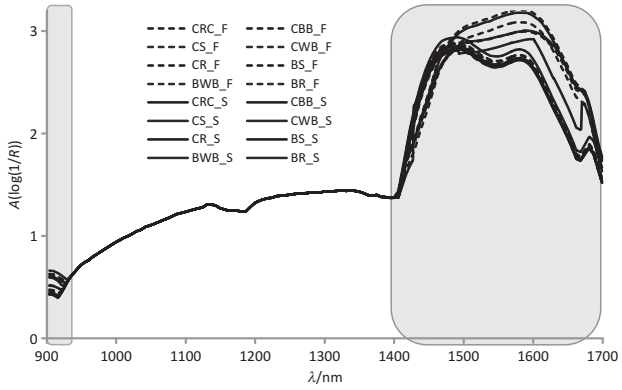

Fig. 1. NIR spectra of different berry fruit extracts. For sample abbreviations see Table 1

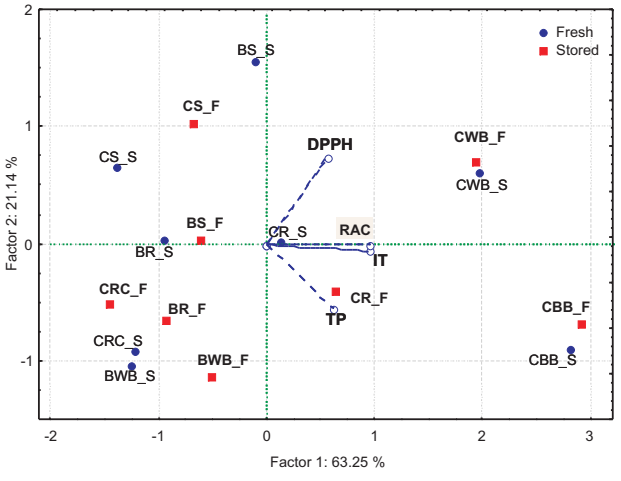

Fig. 2. Principal component analysis used for differentiation of the berry fruit extracts with additional loadings. For sample abbreviations see Table 1

First two PCs explain 84.39 % of the total variance in the observed data set. The first factor (PC1) is influenced by the relative antioxidant capacity and the antioxidant activity expressed as inhibition time of the Briggs--Rauscher reaction (63.25 %), while the TPC and DPPH are responsible for 21.14 % of variation in the data set.

Application of the PCA was effective because the examined data were separated according to the berry species. The separation of samples according to their freshness was not as successful as in the work of Georgieva *et al.* (*13*), but they used only NIR spectra without the association with the TPC and AOA data, and the set of berry samples was limited. The blackberry and blueberry samples are positioned in the 1st and 4th quadrant of the coordinate system (Fig. 2), demonstrating the abundance of the observed parameters (TPC and AOA). Although the PCA was not successful in data separation according to the sample freshness or origin, we assumed that the data set will be a good input matrix for calibration and validation of some analytical parameters (*24,40–42*). The origin was not a parameter provided for calibration or validation, because the ANOVA analysis did not show significant difference between samples grown in Croatia and Bulgaria. As a multivariate method that is a successful tool in calibration, validation and prediction, we applied the partial least squares (PLS) regression, which is a classic multiple linear regression model (*24*). In the PLS regression, an orthogonal basis of latent variables was constructed, one by one (*37*). PLS regression can perform particularly well when various X variables express common information, like for a large amount of correlation, or even co-linearity, as for spectral data of biological materials such as fruits. The variables we used in calibration and validation based on the NIR spectra are: TPC, antioxidant activities (DPPH and IT) and RAC. Croatian samples were used for the model calibration and Bulgarian samples were used for validation. The standard error of calibration (SEC), coefficient of determination in calibration and the standard error in cross-validation were obtained for the calibration set, as well as for the validation set (Table 2). The regression point displacement (RPD) was expressed for the validation set.

Efficiency of the PLS regression models can be observed in Fig. 3. The obtained models (Table 2) were satisfactory for most of the five observed parameters, showing highly acceptable coefficients of correlations (R^2>0.971), with the exception of the correlation when the freshness was predicted (R^2=0.881). The cross-validation supports the prediction of TPC and AOA using the NIR spectra as input set. Determined correlation coefficients of the calibration set outperform those coefficients of prediction models (R^2_c>R^2_p), which is substantiated with the values of root mean square error of calibration (RMSEC) and validation (RMSEP), showing lower values in the calibration set, and exactly such trend is expected (*10,24*). The RPD value (*29*) was included in the evaluation of the PLS models where a good validation model should have a lower RMSEP and higher R^2 and RPD. Studies of Cervellati *et al.* (*17,18*) have shown linear relationship between total phenols and antioxidant activity (measured with the DPPH method and BR reaction), and our study confirms good correlations of the mentioned parameters with the NIR spectra that are also in a positive relation with the RAC and IT. Our conclusions are based on highly preferable RPD values. Good prediction of total phenolic content resulted in the RPD over 3, which is classified as excellent prediction, in good prediction of the AOA expressed as RAC (RPD=2.68), while the prediction of the

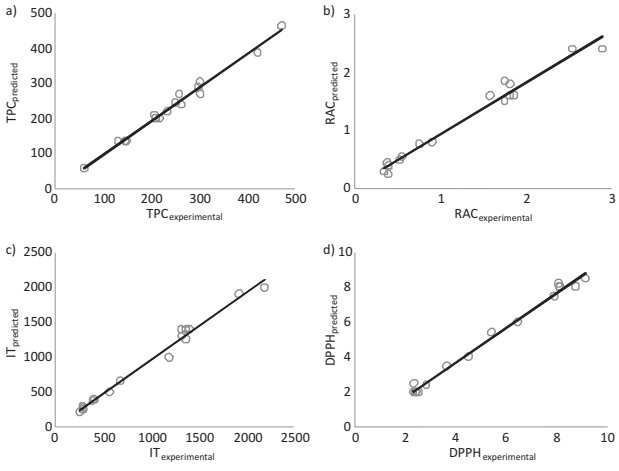

Fig. 3. PLS regression models for prediction of: a) total phenolic content (TPC), b) relative antioxidant capacity (RAC), and c) and d) antioxidant activity (IT and DPPH, respectively) for validation set of berry fruit extracts

Table 2. Partial least squares (PLS) calibration models for antioxidant activities, relative antioxidant capacity and freshness using the NIR absorbance spectra

Observed parameters	PLS				
	Calibration set (*N*=32)		Prediction set (*N*=16)		
	RMSEC	R^2_c	RMSEP	R^2_p	RPD*
Total phenolic content	30.31	0.99	35.48	0.98	3.05
Antioxidant activity (DPPH)	2.14	0.99	2.2	0.99	2.39
Antioxidant activity using BR reaction (IT)	41.63	0.98	41.80	0.97	2.11
Relative antioxidant capacity	0.48	0.99	0.64	0.98	2.68
Freshness	2.1	0.88	2.7	0.84	1.77

*Regression point displacement (RPD)<1.5=calibration model is not suitable for prediction, 1.5–2=it is possible to distinguish between high and low levels, 2–2.5=approximate quantitative prediction is possible, 2.5–3=prediction is classified as good, RPD>3=prediction is classified as excellent (*29*). RMSEC and RMSEP=root mean square error of calibration and validation respectively, R^2_c=correlation coefficient of calibration set, R^2_p= correlation coefficient of prediction model, BR=Briggs-Rauscher, IT=inhibition time

AOA measured by use of the DPPH or BR method is possible (RPD between 2 and 2.5). The freshness is possible to predict, but on a lower level (RPD=1.77), so we decided that this is not enough to be used in validation (therefore, this relation was not shown in Fig. 3). These results indicate the advantage of using NIR spectroscopy coupled with multivariate tools in separation, calibration and validation of some analytical parameters (TPC and AOA) in berry fruit extracts, regardless of their origin and freshness.

The results clearly show the potential of near-infrared spectroscopy for a nondestructive detection of different berry fruit species and to validate and potentially predict their antioxidant activity and content of total phenols. The aim of our following study will be to examine which parameters influence the classification of berry samples according to their freshness, the effect of storage (time and temperature) and to develop PLS regression models that will allow validation of these changes based on the NIR spectra.

Conclusion

Although the samples of berry fruits originate from two different parts of Eastern Europe, they show some similar trends in the changes of total phenolic content and antioxidant activities. ANOVA was applied to test if there was any significant difference based on freshness and origin of berry fruit extracts. Multivariate tools such as principal component analysis (PCA) and partial least squares (PLS) regression were applied to the data set consisting of near-infrared (NIR) spectra and additional analytical parameters (total phenolic content and antioxidant activity). PCA was successful in distinguishing berry samples regarding their species, and PLS models calibrated and validated the observed analytical parameters well. The applicability of the PLS models was additionally evaluated by use of the regression point displacement (RPD). The RPD showed good and excellent accuracy for prediction of the total phenolic content and antioxidant activities respectively, while it was possible to distinguish freshness but not predict it with high accuracy (RPD<2). The obtained results confirmed that NIR spectroscopy coupled with multivariate analysis is a promising technique for rapid and environmentally friendly measurements allowing good calibration, validation and possible prediction of the observed parameters.

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