Application of Near Infrared Spectroscopy (NIRs), PCA and PLS models for the analysis of dried medicinal plants

J. Gajdoš Kljusurić, **D. Valinger**, **A. Jurinjak Tušek**, **M. Benković** and **T. Jurina**

Department of Process Engineering, University of Zagreb, Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10 000 Zagreb, Croatia

In traditional medicine, botanicals and medicinal plants in their natural and processed form are widely used [1] due to their medicinal and antioxidant properties. Numerous analytical methods have been developed for the analysis of chemical composition of medicinal plants extracts like gas chromatography (GC), mass spectrometry (MS), thin layer chromatography (TLC), UV spectrometry, and high performance liquid chromatography (HPLC). All these methods are precise but expensive, time-consuming and require many reagents. As an alternative, near infrared spectroscopy (NIRs), as a simple, selective, and environmentally friendly method [2], can be used.

NIR spectroscopy is a non-destructive measurement method that allows intact measuring, without any additional sample preparation or pre-treatment. Use of spectroscopy in the near infrared region allows a wide range of applications in the food chain production, from control of raw materials to intermediary and final products [3] in order to provide a quality guarantee for consumers.

NIR spectroscopy is based on the electromagnetic absorption in the near infrared region. Spectral analysis has to be assisted with various chemometric techniques, such as multiple linear regression analysis (MLRA), principal component analysis (PCA) and partial least squares regression (PLSR) [4]. Chemometric techniques and chemometric modelling have become an integral part of spectral data analysis which also includes pre-processing of NIR spectra. The pre-processing objective is removal of physical phenomena in the spectra in order to improve the subsequent multivariate regression, classification model or exploratory analysis [5].

In this work, most widely used pre-processing techniques including (i) scatter-correction methods and (ii) spectral derivatives are explained through analysis of spectra of dried medicinal plants collected during the size reduction process (milling), as well as during analysis of the kinetics of the solid-liquid extraction process using water as a solvent [6]. In order to identify patterns in large set of data and express the data to highlight similarities and differences among them, PCA was used. PCA presents the pattern of similarity of the observations and the variables by displaying them as points in maps [7]. PLS regression was used to predict or analyse a set of dependent variables from a set of independent variables or predictors. The predictive ability of a PLS model is expressed as one or more statistical measures. Which parameter should be used is described by R-Squared Coefficient, Ratio of standard error of Performance to standard Deviation (RPD) and Range Error Ratio (RER).

Keywords: near infrared spectroscopy; data pre-processing, modelling, principal component analysis, partial least squares regression

1. Introduction

Our fast-paced modern lifestyle leaves us very little time for regular nutrition and care for our health leading to increased occurence of chronical disseses such as cancer, vascular diseases and neurodegenerative diseases. Medicinal plants have been used since ancient times for treatment of a wide range of diseases and are the richest bioresource of drugs for traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [8]. Since medicinal plants represent a rich source of biologically active compounds that have a beneficial effect on health, a wide range of medicinal plants can be found on the market today. Studies have been carried out globally to verify their efficacy and some of the findings have led to the production of plant-based medicines. Some of the health benefits of medicinal plants include antimicrobial and antioxidant efficacies, which can be of great significance in therapeutic approaches of many diseases [9]. Up to this day, there have been several studies documenting the antibacterial, antifungal, antiviral, anticancer and anti-inflammatory properties of plant ingredients [10, 11, 12].

With the rapid increase in sale and variety of medicinal plants there is a growing concern for quality of the products present on the market. Although there are many methods for quality control such as HPLC, GC, MS, TLC etc., there is still demand for less expensive, consistent, rapid and non-destructive method. A significant number of literature reports have emerged over the past 20–30 years regarding the use of near infrared spectroscopy (NIRS) as both a research tool for formulation development and as a quality control technology for monitoring unit operations for product manufacturing [13].

It is well known that NIR spectra contains both chemical and physical information (such as particle size and bulk density) [14, 15, 16] thus regular or in-line particle size measurement can be one of the ways to control the quality of certain products where medicinal plants are used as raw materials or as extracts for their production. The aim of this

study was to examine the possibility of differentiating particle size of the same plants and the different particle size of different plants and also to show the potential of Partial Least Squares Regression (PLSR) models for prediction of different parameters such as conductivity, dry matter, total disolved solids (TDS) and total polyphenols (TP) based on NIR spectra. The non-invasive spectroscopy in the near infrared range of 904 - 1699 nm (NIR) was used to record spectra of five different medicinal plants (sage, lavender, lemon balm, thyme, mint) for seven different particle sizes (<100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm and 1000 μm) that were further used for diferentiation of different particle sizes by Principal Component Analysis (PCA). Except crude medicinal plants, water extracts of medicinal plants were also investigated (marigold, sage, lemon balm, thyme, St. John's wort and lavender) by PCA. PLS regression modelling was performed on lavender water extract as part of study for the project „Application of microreactors in the analysis of antioxidant activity of medicinal plants (MICRO-AA)", project number HR.3.2.01-0069, which was funded by the European Social Fund (ESF) through the Human Resources Development program.

Results have shown that NIR spectroscopy was successful in differentiating particle sizes of different plants and highly sensitive to different particle sizes of the same plants. PLS regression modelling results show a very promising potential in monitoring certain physical and chemical properties of medicinal plants water extracts by NIR spectroscopy.

2. Materials and methods

2.1 Materials

2.1.1 Plant material

Dried plant materials of sage (*Salvia officinalis* L.), lavender (*Lavandula angustifolia* L.), lemon balm (*Melissa Officinalis* L.), thyme (*Thymus vulgaris* L.), mint (Mentha piperita L.), marigold (Calendula officinalis) and St. John's wort (Hypericum perforatum) collected in the north-western part of Croatia during the flowering season of 2015, dried and properly stored, were purchased from a specialized herbal store (Suban d.o.o., Strmec, Croatia).

2.1.2 Chemicals and reagents

Folin-Ciocalteu's reagent and sodium carbonate were purchased from Kemika (Zagreb, Croatia). Trolox (6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid) and potassium peroxodisulphate were obtained from Fluka (Buchs, Switzer land). ABTS•+ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt), gallic acid (3,4,5-trihydroxybenzoic acid) were obtained from Aldrich (Sigma–Aldrich Chemie, Steinheim, Germany), and ethanol (96%) was obtained from Carlo Erba Reagents (Cornaredo, Italy).

2.2 Methods

2.2.1 Milling

Dried plant material of sage, lavender, lemon balm, thyme and mint was milled using IKA control Tube mill (IKA-Werke, Staufen, Germany). Milling conditions were adjusted as follows: 20 000 min^{-1} for 40 seconds. Since the plant material had different organic structures and thus different hardness, for some samples milling time was reduced to 20 seconds. After milling, samples were kept in a desiccator until further used.

2.2.2 Separation of particle size fractions

In order to separate the particle size fractions milled plant material was subjected to sieving. Used sieves had 100 μm, 250 μm, 355 μm, 500 μm, 800 μm and 1000 μm pore opening. Seven different particle size fractions were obtained marked as <100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm and 1000 μm which were then subjected to NIR analysis.

2.2.3 Extraction procedure

An amount of 2 g of dry plant material of marigold, sage, lemon balm, thyme, St. John's wort and lavender was placed in a 150 mL glass with 100 mL of deionised water and heated to 80 °C using Ika HBR4 digital oil-bath (IKA-Werk GmbH & Co.KG, Staufen, Germany). Selected temperature and extraction conditions (t = 90 min, rpm = 500 min^{-1} and T = 40 °C) were chosen for water extracts of six medicinal plants (marigold, sage, lemon balm, thyme, St. John's wort and lavender) on which seven parameters: pH, conductivity, dry matter, TDS, TCP, AO and BR-AO were measured. For the PLSR models of lavender selected temperature and extraction conditions were: t = 90 min, rpm = 500 min^{-1} and T = 60 °C. After the extraction process samples were immediately cooled in the water-ice mixture, then filtered through

a 100 % cellulose paper filter (LLG Labware, Meckenheim, Germany) with 5 – 13 μm pore size and stored at T = 4 °C until analysed.

2.2.4 Light microscopy

Light microscope imaging was performed on the milled plant material samples in order to identify size, structure and type of particles present in the samples. Samples were viewed by a Motic B1 Series microscope (Motic, Kowloon, Hong Kong) at 4x magnification and photographed by a Moticam 3 microscope camera (Motic, Kowloon, Hong Kong).

2.2.5 Determination of pH, conductivity, total dissolved solids (TDS) total phenol content (TPC), dry matter and antioxidant capacity measured by DPPH method (AO) and Briggs-Rauscher method (BR-AO)

All the analyses were performed as described in the work of Jurinjak Tušek et al., 2016 [17] except Briggs-Raucher method for antioxidant capacity which was performed as described in the work of Gajdoš Kljusurić et al., 2005 [18].

2.2.6 NIR spectroscopy

The NIR spectra (range extends from λ = 904 nm to λ = 1699 nm) of medicinal plants for different particle sizes and water extracts were collected with the setup for NIRS studies that included: a laptop, NIR-128-1.7-USB/6.25/50μm scanning monochromator from Control Development, Inc., provided with Spec32 software, the polychromatic source of light, optical cables, and a hemispherical cup that serves as a sample tray. Complete NIR instrument setup has been previously described in detail by Valinger et al. (2011) [19]. For dried plant material, small metal vessel was used while the water extracts were poured in quartz cuvette of 1 mL volume. No mechanical or chemical treatment of the samples was needed prior to NIRS measurements.

2.2.7 Spectral analysis and chemometric models

Principal Components Analysis (PCA) was used for identifying patterns in data and expressing the data to highlight similarities and differences. PCA also represents the pattern of similarity of the observations and the variables by displaying them as points in maps [20, 21, 22]. Data obtained by NIRS were used to perform principal component analysis (PCA) by means of statistical software StatSoft STATISTICA v. 10 (StatSoft Inc., Palo Alto, USA) and were plotted in 3D using Wolfram Mathematica v10 (Wolfram Research, Champaign, USA).

PLS regression was used to predict a set of dependent variables from a set of independent variables or predictors. This prediction is achieved by extracting from the predictors a set of orthogonal factors called latent variables which have the best predictive power. PLS regression is particularly useful when we need to predict a set of dependent variables from a (very) large set of independent variables (i.e., predictors) [23]. PLSR modelling was carried out using Unscrambler® X 10.4, trial version software (CAMO software, Oslo, Norway).

3. Results and discussion

3.1 Near infrared (NIR) spectra

For five different medicinal plants (sage, lavender, lemon balm, thyme, mint), 6 recordings of NIR spectra were made for each particle size (<100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm i 1000 μm), which resulted altogether in 42 recordings per medicinal plant (210 spectra in total). Example of unprocessed spectra for different particle sizes of lavender are presented in Figure 1.

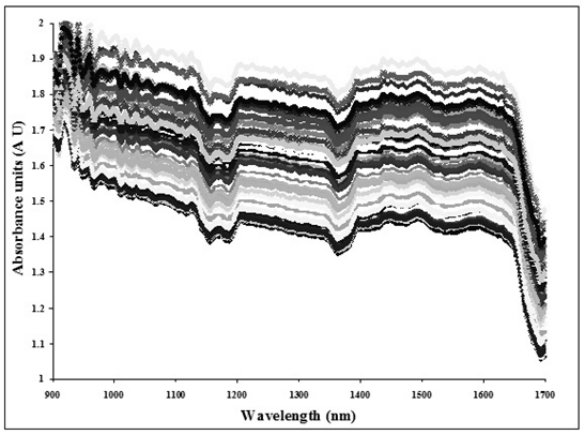

Fig. 1 NIR spectra of different particle size fractions (<100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm i 1000 μm) of lavender.

3.2 Application of chemometric tools

From the raw spectra, it is impossible to extract information without using at least one of the chemometric methods. Based on the final aim, most common used chemometric tools are Principal Component Analysis (PCA) and the Partial Least Squares (PLS) regression. PCA analysis is a variable reduction technique that is used when the main aim is grouping or detection of similarities/differences between the observe variables, while PLS regression is used when the main aim is bearing of some relation between the response and independent variables. Both of the most commonly used multivariate tools were applied to present their usefulness. As an independent data set, NIR spectra of different medical plants were used.

3.2.1 Effectiveness of use of Principal component analysis

To investigate the effectiveness of distinction of medical plant(s) based on their particle size, and to determine the detection limit, PCA analysis was applied. Using Principal Component Analysis (PCA) for each medicinal plant and all the particle size fractions, cluster analysis was made, and example of results for two medicinal plants (sage and lemon balm) are presented in Figure 2A and 2B. Results are presented in term of first two principal components where the pronounced effect of the first two eigenvalues is observed. For the results presented in Fig 2 A) and 2 B) cumulative effec[illegible] the first two principal components.

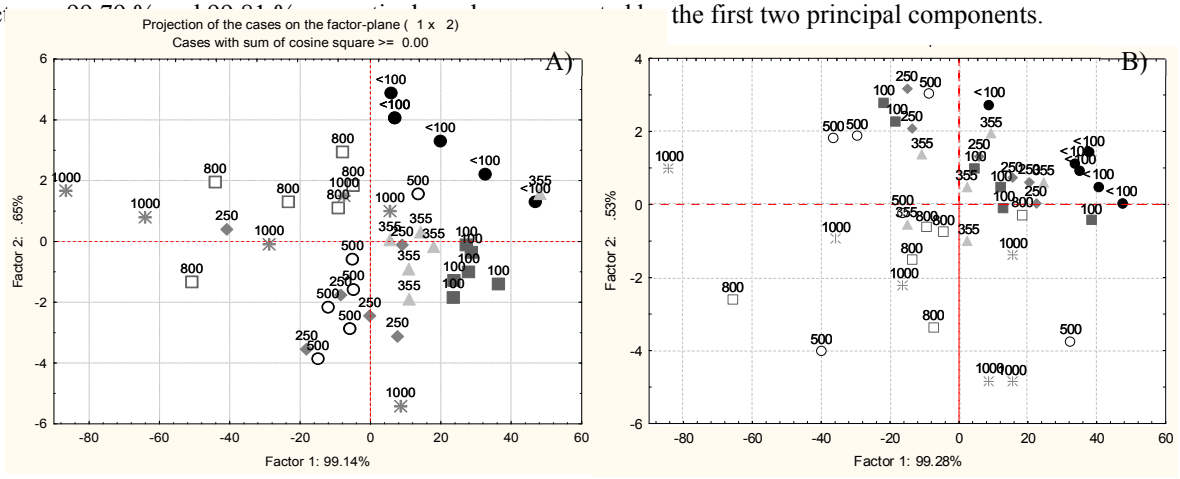

Fig. 2 Principal Component Analysis of NIR spectra presented by first two factors for particle size fractions (<100 μm, 100 μm, 250 μm, 355 μm, 500 μm, 800 μm i 1000 μm) of A) sage and B) lemon balm.

Figures 2A) and 2B) show that NIR distinguished different fractions of the same medicinal plant for some medicinal plants with better precision (lemon balm) while others with lesser precision (sage). Similar results were obtained in the work of Valinger et al. [24], where distribution of different particle size fractions of medicinal plants (chamomile, dandelion, nettle, broadleaf plantain and yarrow) was investigated. As in this case, smaller particle fractions (<100 μm, 100 μm) showed good uniformity and clustering, while overlaping of larger particle size fraction was observed. To explain this overlaping microscopic images of larger particle sizes fractions were made and example of two medicinal plants (lavander and sage) for particle sizes fractions of 800 μm are shown if Fig. 3A) and 3B).

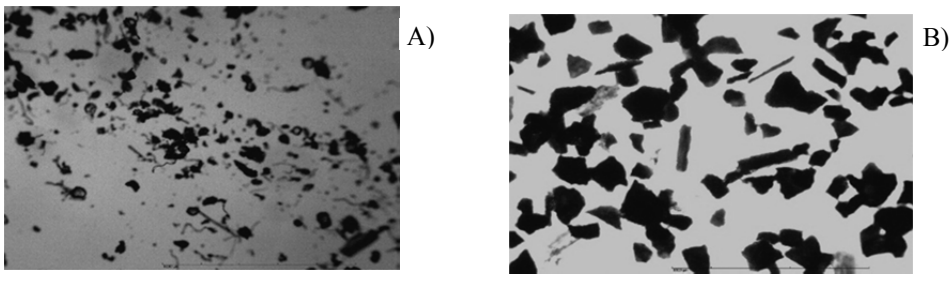

Fig. 3 Images of ground samples for particle sizes of 800 μm for A) lavender and B) sage (magnification 4x)

In Figs. 3A) and 3B) it is visible that samples of sage and lemon balm fractions of 500 μm were not homegenous because there are smaller and larger particles present in desired size fraction. That is the reason why PCA did not cluster every fraction separately. If there is uniformed particle size distribution with the use of NIR spectroscopy it is possible to completely separate different particle size fractions as described in work of Valinger et al. [25]. Because of the NIRS sensitivity, if there are few larger/smaller particle sizes in certain fraction NIRS will remove this sample from the

cluster which shows its potential in the quality control and falsification control of different food products. If, for example, the calibration was done with two different particle size fractions in term of the experiments with different ratios of fractions ranging from 0 % of fraction A and 100 % of fraction B to 100 % of fraction A and 0 % of fraction B it could be possible to determine how much of one fraction was present in the mixture.

To investigate the effectiveness of PCA analysis in distinguishing different medical plants of the same particle size, NIR spectra (NIRS) of 5 different medical plants (sage, lavender, lemon balm, thyme, mint) was analysed. Those plants are often used in Croatia as tea preparations in prevention of different diseases such as gums and throat problems, gastritis and stress. PCA was also used to confirm that NIRS can distinguish different medicinal plants that have the same fraction size. In Figure 4 A) and 4 B) PCA of same particle size fractions (<100 μm and 100 μm) for five different medicinal plants (sage, lavender, lemon balm, thyme, mint) is presented in term of first two principal components (Factor 1 & Factor 2).

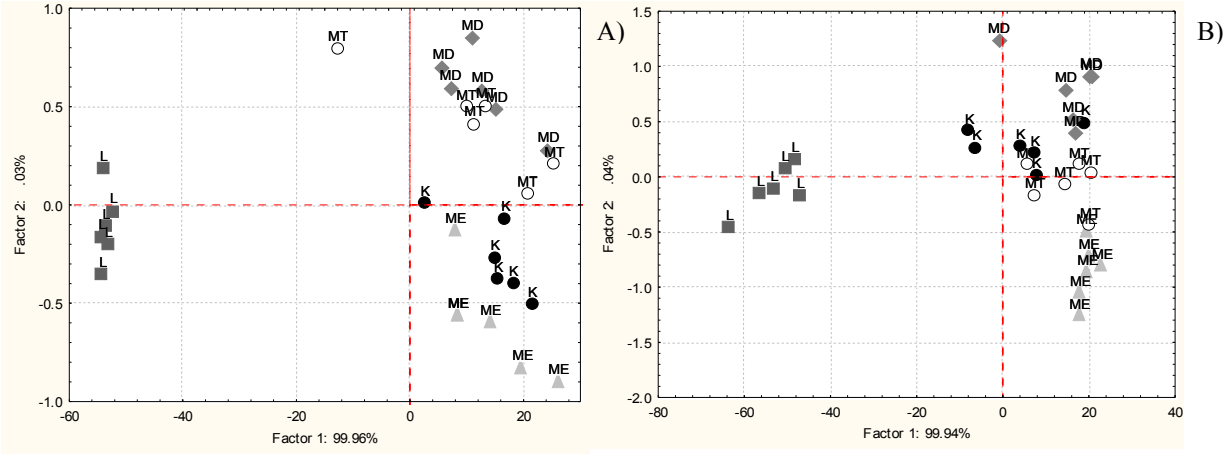

Fig. 4 Images of ground samples for particle sizes of <100 μm A) and 100 μm B) for five different medicinal plants (K - sage, L - lavender, MD – lemon balm, ME – thyme, MT – mint)

Sometimes using PCA for first two factors overlapping of samples may occur and for that reason third factor, although sometimes with low eigenvalue, can help in better separation of clusters. Example of PCA for first three factors of five medicinal plants ground samples for particle sizes of <100 μm can be seen in Figure 5. 3D PCA is very useful in working with large sets of experimental data and is in intensive use nowadays.

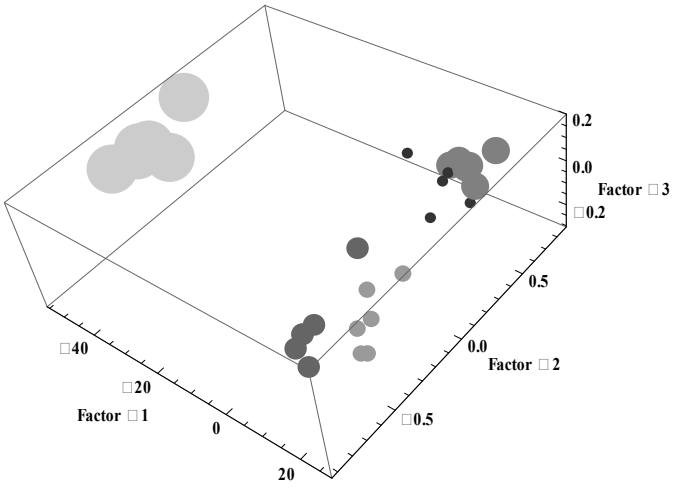

Fig. 5 Principal Component Analysis of five medicinal plants ground samples for particle sizes of <100 μm by first three factors (● sage, ● lavender, ● lemon balm, ● thyme, ● mint)

In Figure 5, better separation of size clusters was visible in comparison to 2D PCA. E.g. lemon balm, mint and sage, which were overlaping in 2D PCA are clearly separated with introduction of the third factor in 3D PCA. This confirmed the usefullnes of 3D PCA application when working with large datasets and its intensive use in data analysis.

Based on the NIR spectra of medical plants, the effectives of PCA application in distinguishing particle size (<100, 100, 250, 355, 500, 800 & 1000 μm) for the same plant, (Fig. 2., for 2 different medical plant), was confirmed. Then the same method (PCA) was applied for successful differentiation of different medical plants (Figure 4). The data matrix consisted of 796 points of the absorbance at NIR light, per one scan. Each medical plant extract was scanned at least 3

times. For the investigation of the effect of different particle sizes for one plant, the matrix consisted of 21 rows (different particle sizes and 3 repetitions) and 796 columns (absorbance at corresponding wavelengths starting from 904 to 1699 nm). In distinguishing different medical plants, the data matrix consisted of the same number of columns as previously stated, but the number of rows was the number of repeated scans (3x) multiplied with the number of investigated medical plants (5x), what resulted in total of 15 rows.

The next aim was to present the effectiveness of application of the same method on a much smaller data matrix, comprising of 7 different parameters measured for 6 medical plants (data matrix of 7 rows and 6 columns). In this case, PCA (Figure 6) was applied for determination of similarities and differences among six different medicinal plant extracts (marigold, sage, lemon balm, thyme, St. John's wort and lavender) depending on observed parameters (pH, conductivity, dry matter, total dissolved solids (TDS), total polyphenol content (TPC) and antioxidant capacity measured by DPPH method (AO) and Briggs-Rauscher method (BR-AO)). Water extracts were prepared at following conditions: $T = 40$ °C, $t = 90$ min and $rpm = 500$ min^{-1}.

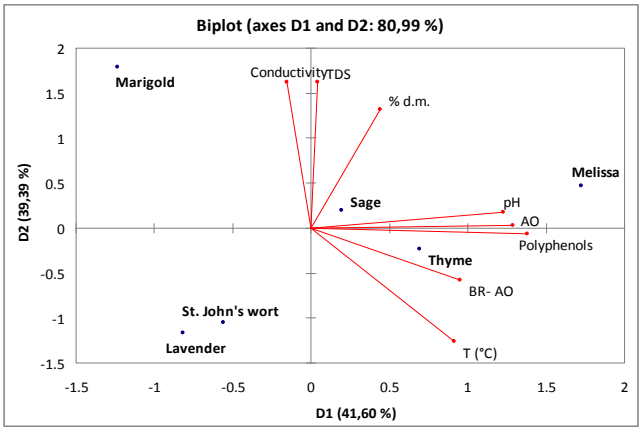

Fig. 6 PCA for water extracts of six medicinal plants (marigold, sage, lemon balm, thyme, St. John's wort and lavender) and seven observed parameters (pH, conductivity, dry matter, TDS, TCP,AO and BR-AO)

As presented in Fig. 6, water extracts of medicinal plants were distributed in all four quadrants. Water extract of marigold, which was situated in the second quadrant, had the highest conductivity, while extracts of St. John's wort and lavender, which were situated in the third quadrant, showed the lowest content of all measured parameters above the X-axis (conductivity, TDS, dry matter and pH). This confirmed the potential of NIRS in sample identification, in accordance with claims of other researchers [26, 27]. By using PCA, one can determine which parameters had significant influence on distribution of samples. Total polyphenols and antioxidant capacity measured by DPPH method and Briggs-Rauscher method had most influence based on first principal component, which explained 41.6 % of all the data interactions. Second principal component explained 39.39 % of variance where conductivity, TDS and dry matter were dominant. Totally 81 % of variance was explained for observed data (PC1 + PC2 = 80.99 %).

3.2.2 Effectiveness of use of Partial least squares regression

As mentioned previously, PLS regression is also a multivariate tool. It presents the projection on latent structures and its goal is to separate a set of dependent variables from a set of independent variables or predictors. The quality of the prediction obtained by the PLS regression model is evaluated with cross-validation techniques. PLS models for one medical plant (lavender) are presented, where the independent variables were the NIR spectra of lavender water extract at wavelengths 904-1699 nm, and the depended variables were observed experimental data for total dissolved solids (TDS), conductivity, dry matter, antioxidant activity (AOA) and the content of total polyphenols.

Considering that NIR spectroscopy is a non-destructive method, the aim of developing such PLS models was to predict the expected value or concentration of the parameter which was treated as a dependent variable in the model based on the NIR spectrum of the investigated sample. Such approach saves time, chemicals and workforce, which increases the efficiency of application of such models and the real sector.

Coefficient of determination (R^2) for an applicable prediction of a parameter based on the PLS model, should be higher than 0.9. Calculated coefficients for water extract of lavender are presented in Table 1. From five observed parameters, four of them were very well predicted based on the input data (NIR spectra): total dissolved solids (R^2 >0.98), conductivity of the extract (R^2 >0.99), dry matter (R^2 >0.96) and the content of total polyphenols (R^2 >0.98).

Table 1 Determination coefficients (R^2) for PLS regression models for different parameters of lavender extracts.

Plant	Parameter (depended variables of the PLS model(s))				
	Total dissolved solids	conductivity	dry matter	Antioxidant activity	content of total polyphenols
lavender	0.987	0.993	0.968	0.318	0.985

The antioxidant activity of lavender water extracts has not results in a successful model (R^2 < 0.9), which is an implication of a weak relation between the input and output variables.
The general outline of the PLS model equation in this example was:

$$y_i = b + a_1 \cdot x_1 + a_2 \cdot x_2 + a_3 \cdot x_3 + \cdots + a_n \cdot x_n \quad (1)$$

Where:
y_i = observed parameter (i = 1, for total dissolved substance (TDS); i = 2, for conductivity; i = 3, for dry matter content; i = 4, for antioxidant activity and i = 5, for concentration of total polyphenols)
xj = wavelength of the spectrum (j = 904 to 1699)

To present the effectiveness of "acceptable" models for prediction based on NIR spectra, the results were presented graphically in Fig. 7.

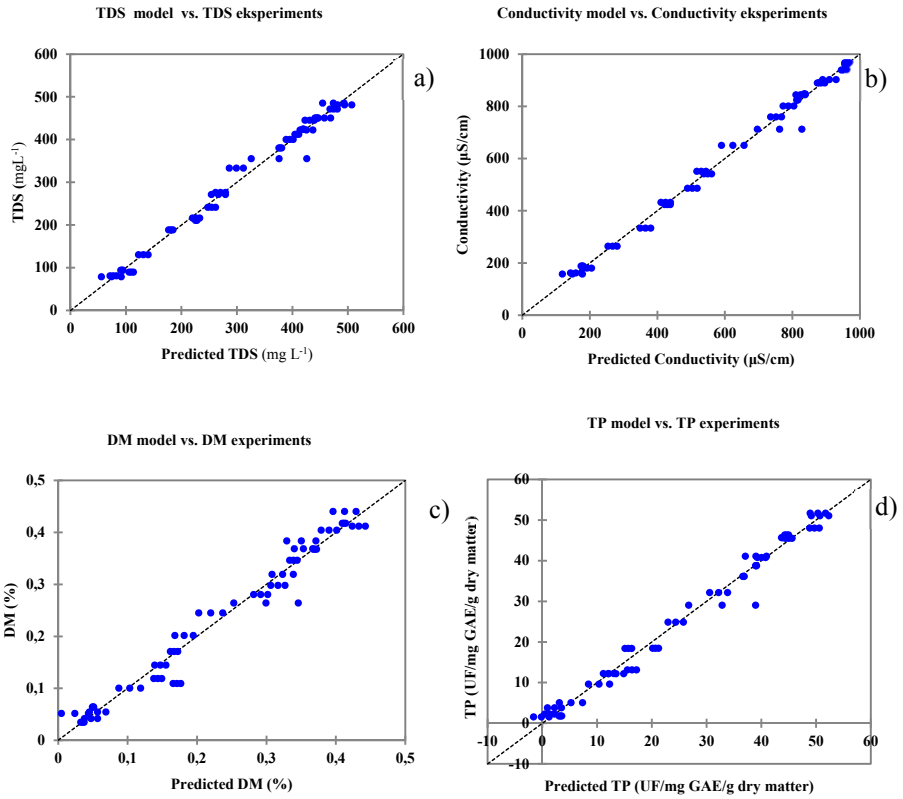

Fig. 7 Results of PLS regression for prediction of (a) TDS, (b) conductivity, (c) dry matter, (d) total polyphenols for water extracts of lavender at T = 60 °C, t = 90 min and rpm = 500 min^{-1}.

For the four models (TDS, conductivity, dry matter and total polyphenols) coefficient of determination and coefficient of correlation were obtained. The coefficient of determination represents the square of the coefficient of correlation and shows the percentage variation for y, which is explained by all the x variables together. For all the models coefficient of determination was higher than 0.96 which showed very good agreement between observer dependent and independent variables. Values for coefficient of determination for TDS, conductivity, dry matter and TP model were 0.987, 0.993, 0.968 and 0.985 respectively. This confirmed the adequacy of PLS models for prediction of physical and chemical properties of water extracts.

4. Conclusions

NIR spectroscopy combined with PCA as a chemometric method showed good potential for monitoring differeniation of medicinal plants and their grounded fractions in term of quality control. Results obtained by PCA and PLS regression models showed a very promising potential in monitoring physical and chemical properties of medicinal plant water extracts by NIR spectroscopy.

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