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IDENTIFICATION OF SIMILARITIES IN METALLIC CONTENT OF HERBAL INFUSIONS USING NON-LINEAR APPROACH

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Abstract: The content of Cd, Cr, Cu, Fe, Pb, Mn, Ni and Zn was determined, using flame atomic absorption spectrometry in infusions made from commercial herbal mixtures readily available in herbal stores. ANOVA test revealed that the metal contents did not differ considerably in most of infusions. The scatter plot of principal component analysis (PCA) designated infusions characterized by similar contents of trace elements. It was possible to indicate distinctive metal concentrations in Pyrosan, Septosan and Urosan infusions as they vastly contrasted with others. The levels of Mn, Zn, Cd were characteristic for Pyrosan infusions, while Cr, Fe, Cd were specific to Septosan, and Mn, Zn, Fe to Urosan. Self-organizing maps did not fully correspond with PCA results because PCA classified samples relying mainly on apparent similarities in metal contents.

Keywords: herbal infusions, principal component analysis, self-organizing maps, trace metals

Herbal remedies have been used for centuries in order to sustain good health, cure common illnesses or treat various health conditions. Nowadays, even though conventional medicine is developing at a high rate with a growing number of drugs obtained by chemical synthesis, the interest in herbalism and phytopharmaceuticals has not diminished (1). The increased use of herbal medicines is based on the belief that they are natural and therefore safe, so fewer side-effects will be produced. In addition, most of them are freely available (2).

One of the most popular forms of herbal drugs to be taken by patients are the mixtures composed of several medicinal plant raw materials coming from various species of plants. They are usually available in small sachets and administered by making a tea to be drunk twice or three times a day in order to cure various ailments such as: infections, diabetes, rheumatism, inflammatory diseases, influenza etc. (3). However, apart from active organic ingredients responsible for therapeutic activities (4), medicinal herbs also contain trace elements. Some of them have potential synergistic or antagonistic properties to pharmacological effects of organic compounds, but others are simply toxic for human health (5).

In recent years, many papers have been published on analysis of trace elements in various herbal products, mostly for quality control reasons (6–9) according to WHO recommendations (10). However, in many cases, along with determination of metals levels in herbals, different chemometric techniques were involved (11–17). The reason for that was the fact that samples were described by a large number of metal concentrations, so it was difficult to identify any intercorrelations between those metals straightforward. The most commonly used method was principal component analysis (PCA) (12, 14), most often accompanied by cluster analysis (CA) (11, 13, 16) and at times, by linear discriminant analysis (LDA) (15, 17). These chemometric techniques have been applied to investigation of the contents of heavy metals in herbs or herbal teas in order to classify and examine any relationships between these metals (15), identify similarities regarding mineral and trace metal contents of spices and herbs (17), quantify the content of various elements possibly responsible for some therapeutic properties of *Echinacea purpurea* (11), recognize potential anthropogenic contamination sources of herbal drugs (12, 13), establish standardization and quality control procedures for crude drugs (14), and differentiate herbal raw materials belonging to different plant families according to metal contents (16).

After reviewing the above literature, it was concluded that mostly applied chemometric meth-

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ods used for identification of similarities or correlations between metallic content in herbals were based on linear modeling. Having assumed that interrelationships between chemical constituents occurring in nature are rarely of linear character, it was decided to explore relationships in elemental content of herbal infusions using non-linear modeling. In this paper, nonlinear approach was proposed using self-organizing maps (SOMs) also known as Kohonen neural networks.

SOMs are intended for unsupervised learning tasks similarly to PCA and CA. Contrary to supervised learning, the training data set contains only inputs and SOM attempts to learn the structure of the data on the basis of these variables. SOM is capable of recognizing clusters of data and relating classes similar to each other, which makes understanding of the data feasible. Once classes of data are identified, they are tagged and the network becomes ready for classification tasks. Kohonen networks can be also applied for classifications where the output classes are known in advance. In such circumstances, they are able to highlight similarities between groups (18, 19).

In this study, output classes (herbal mixtures) and individual samples were known, therefore, the subject was to discover the characteristic differences between the classes and similarities within the classes with regard to the concentrations of some trace elements. Therefore, the heavy metals content (Ni, Cr, Cu, Fe, Mn, Zn, Pb and Cd) was determined using flame atomic absorption spectrometry in infusions prepared from commonly available herbal mixtures. Furthermore, ANOVA, PCA and SOMs were employed to verify if there are any similarities regarding the concentration of the metals. It was decided to establish if the presence of certain herbal constituent in a mixture can be assigned to the levels of some trace elements. The results of the mapping of the infusions performed by SOMs were compared to widely applied PCA.

EXPERIMENTAL

Herbal material

Herbal mixtures produced by "Herbapol" commercially available in drug or herbal stores were involved in the study. The total number of infusions

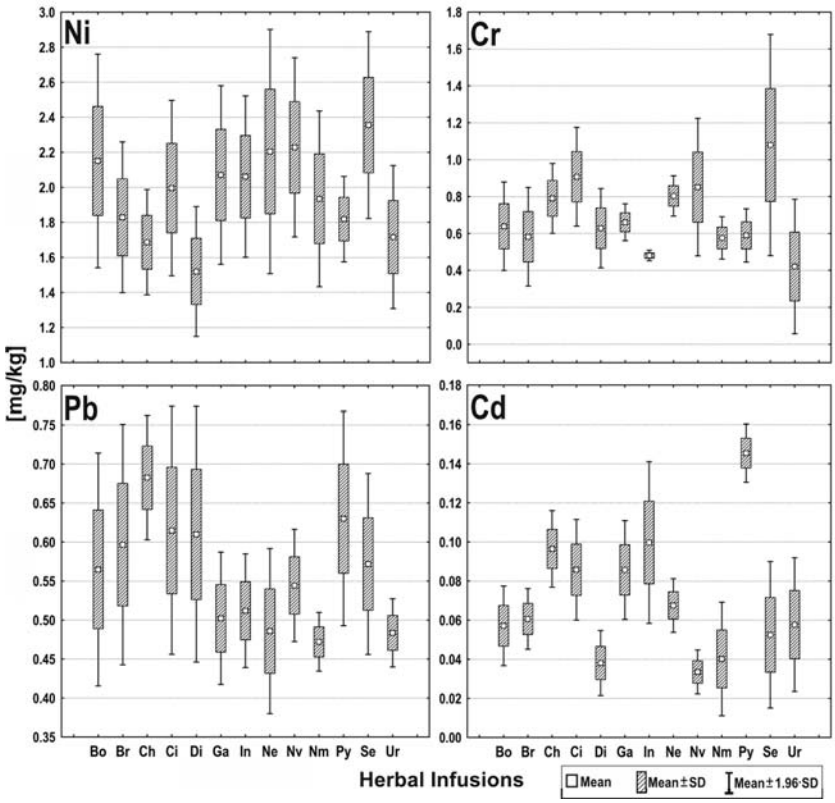

Figure 1. The box-whiskers plot of Ni, Cr, Pb, Cd content in herbal infusions

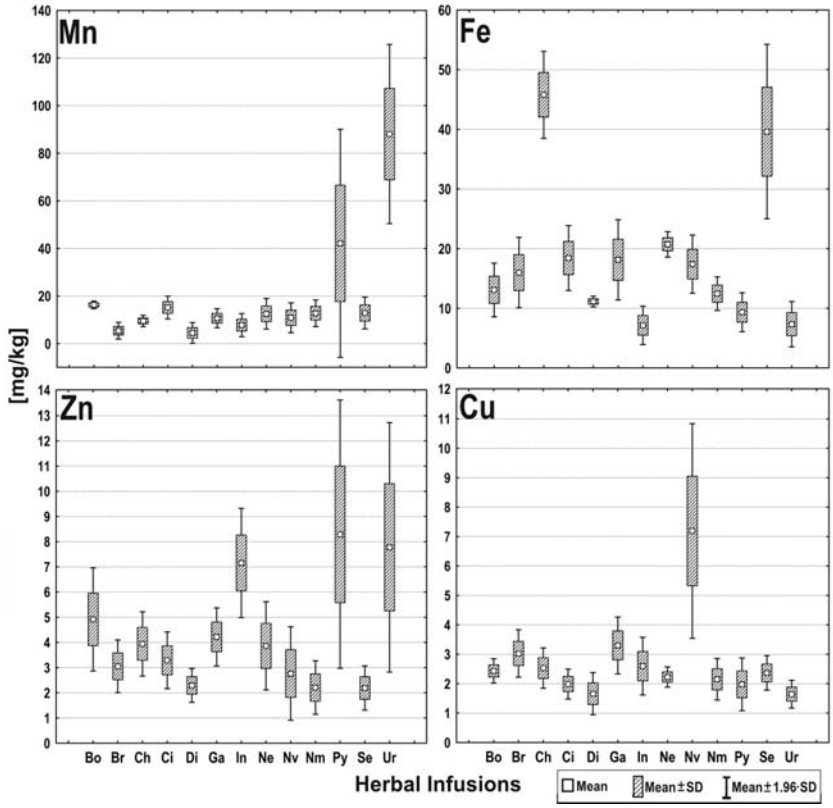

Figure 2. The box-whiskers plot of Mn, Fe, Zn, Cu content in herbal infusions

was 73, which were prepared from 13 different mixtures. The analyzed herbal preparations are listed below in alphabetical order with two letter abbreviations and samples numbers provided in parentheses: Bobofen (Bo, no. 1–4), Bronchial (Br, no. 5–12), Cholagoga II (Ch, no. 13–16), Circulosan (Ci, no. 17–20), Diabetosan (Di, no. 21–24), Gastrosan (Ga, no. 25–32), Infektoten (In, no. 33–35), Nervinum (Ne, no. 36–38), Nervosan (Nv, no. 39–44), Normosan (Nm, no. 45–53), Pyrosan (Py, no. 54–56), Septosan (Se, no. 57–64) and Urosan (Ur, no. 65–73).

The herbal mixtures consist of various herbal raw materials. Some of them are present in different mixtures e.g., melissa leaf in 5 mixtures, peppermint leaf and chamomile in 4 and hop in 3. Several mixtures include one common raw material e.g., birch leaf – Urosan and Pyrosan, yarrow herb – Cholagoga II and Nervosan, lime inflorescence – Bronchial and Pyrosan etc. The mixtures are mostly used as anti-inflammatory, analgesic, diaphoretic, sedative, antipyretic, diuretic, spasmodic, diastolic and cholagogic.

Sample preparation

All batches of herbal mixtures were homogenized in a water-cooled grinder Knifetec 1095 (Foss Tecator, Höganäs, Sweden) at 20°C for 30 s. After that, an accurately weighed homogenized sample in the amount of 10 g was put into a beaker and poured onto with 200 mL of boiling deionized water. The beaker was covered with a watch glass and left for 15 min to steep. After that time, infusion was cooled, strained using MN 640d ashless filter paper (Macherey-Nagel, Duren, Germany) into a 200 mL volumetric flask and filled up with deionized water. Each infusion was prepared three times.

Trace elements determination

The content of Cd, Cr, Cu, Fe, Pb, Mn, Ni and Zn was determined using spectrometer SpectrAA 250 Plus (Varian, Australia) in flame atomic absorption mode. The accuracy and precision of the procedure were established on the basis of several measurements of the Certified Reference Material CTA-VTL-2 (Virginia Tobacco Leaves), manufactured by The Institute of Nuclear Chemistry and Technology

(Warszawa, Poland). The recovery levels were in the range of 89.1–96.0% depending on the element. The lowest recovery was obtained for Cr and the highest for Zn.

Software

All calculations were made by means of a statistical software – STATISTICA 9.0, (Statsoft Inc., USA).

RESULTS AND DISCUSSION

Results of analysis of variance

Categorized box plots showing the mean contents (small squares) of particular elements in the herbal infusions are presented in Figures 1 and 2. The mean value is surrounded by rectangle, which denotes ±1 times the standard deviation (SD), while whiskers represent a 95% confidence interval defined as the mean ±1.96 times the SD, in view of the fact that the distribution is normal.

As it is shown in the plots, most of infusion samples demonstrate similar levels of 8 analyzed elements, in spite of the fact that commercial herbal mixtures contain different medicinal plant raw materials in varied proportions. However, some water extracts prepared from Pyrosan, Septosan and Urosan mixtures were presented as exceptions. The observations were statistically confirmed by ANOVA test ($p < 0.05$), which pointed to significant differences between mean contents of certain metals. In order to determine which group means differ from one another, Tukey's multiple comparison test, for unequal number of cases, was used. The results indicated that the fewest statistically significant differences between mean contents of elements in infusions were observed mainly in the case of Ni and Cr. The levels of Pb, Cu, Mn and Zn did not present a considerable number of meaningful dissimilarities as well. The elements which differentiated between infusions to the largest extent were Cd and Fe.

The analysis of Ni and Cr in infusions showed that only Septosan infusions statistically differed from other extracts (mean content 2.35 mg Ni/kg and 1.08 mg Cr/kg). The levels of Pb distinguished extracts of Normosan and Cholagoga II with the

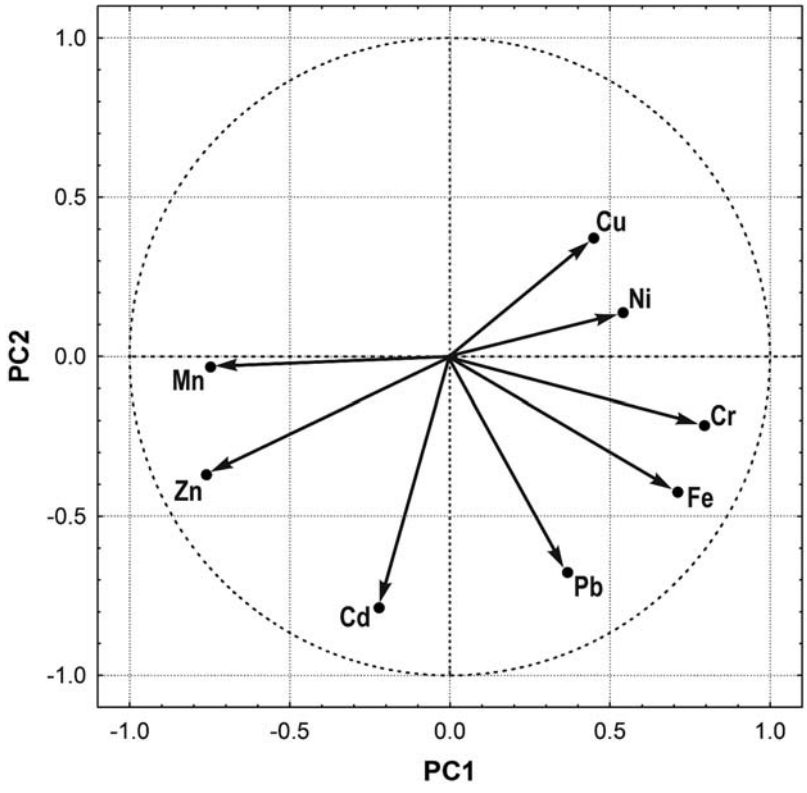

Figure 3. The plot of component loadings of trace elements

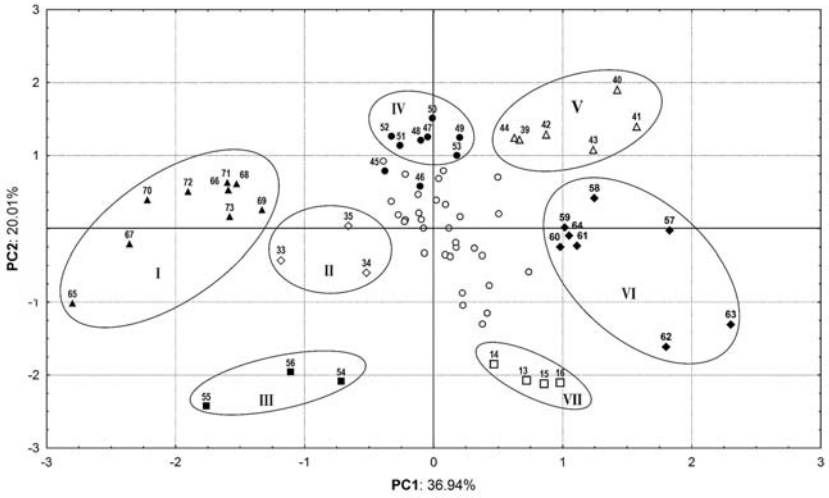

Figure 4. The scatter plot of PCA scores (PC1 *vs.* PC2) for herbal infusions

concentration of Pb amounted to 0.472 and 0.682 mg/kg, respectively. With regard to Cd, it was noted that despite numerous statistically significant differences between concentrations of both metals in various infusions, the most noticeable one was observed in water extracts prepared from Pyrosan (0.145 mg Cd/kg). Gastrosan, Cholagoga II and Infektoten infusions showed no differences between each other considering Cd content, while being dissimilar to other extracts. The mean contents of Cd were 0.086, 0.096 and 0.099 mg/kg, respectively.

The mean levels of Mn were found to be characteristic for Pyrosan (ca. 42 mg/kg) and Urosan infusions (88 mg/kg), while Fe for Septosan and Cholagoga II, containing on average 39.61 and 45.78 mg/kg. The amount of Zn clearly distinguished Infektoten, Urosan and Pyrosan. The mean concentrations of Zn were varied between 7.17 and 8.29 mg/kg. Cu pointed to the extracts of Nervosan (ca. 7 mg/kg).

Results of principal component analysis

The results of PCA showed that PC1 explained 36.94% of overall variance, PC2 – 20.01% and PC3 – 14.12%. Taking into consideration first two PCs, the amount of lost information was equal to 43.05%. The degree of influence on the interpretation of two-dimensional distribution of infusions is determined by loadings values (Fig. 3), which characterize relations between the levels of metals and principal components. The loadings indicate that extracts with higher concentration of Cu and Ni are to be located in upper right side of the PCA scatter plot, as shown in Figure 4. The extracts described by higher levels of Cr, Fe and Pb were shifted towards its lower right side, while the amount of Mn, Zn and Cd was responsible for directing infusions to the lower left side of the plot. In the distribution of analyzed infusions in PCA plot (Fig. 4), seven separated groups were distinguished, denoted by Roman numerals from I to VII. These groups contain infusions prepared from mixtures of the same composition. The closer the samples are located to each other, the more similar are the levels of trace metals determined in them.

The most numerous group of extracts, made from Urosan mixture, was situated on the left side of the plot (group I). The distinctive feature of these samples was a high concentration of Mn, above 64 mg/kg. The extracts (no. 65) and (no. 67) were separated from other samples as a result of the greatest content of Zn, 13.24 and 9.89 mg/kg, respectively. Close to the center of the plot, in group II, there are three infusions of Infektoten (no. 33–35). No strong dominance of any element was noticed. The samples had similar levels of Cr (0.470–0.497 mg/kg), Zn (above 5.95 mg/kg) and Cd (above 0.076 mg/kg). Group III is composed of Pyrosan extracts (no. 54–56), with the highest concentration of Cd, exceeding 0.138 mg/kg, and relatively high level of Mn, above 19.44 mg/kg. Although Mn content is the largest when related to other extracts (excluding Urosan samples), the differences between individual samples are too great (ca. 1.5–3.5-fold). Therefore, it is difficult to regard the concentration of Mn as a characteristic attribute of Pyrosan infusions.

The infusions found in groups I–III are in many cases characterized by similar concentrations of trace elements. It comes to attention that mutual medicinal plants raw materials are included in their composition. Infektoten and Pyrosan contain black elder flower and willow bark, while the common ingredient for Pyrosan and Urosan is birch leaf.

Group IV represents Normosan infusions (no. 45–53). The position of the samples was caused by comparable amounts of Fe (10.56–14.45 mg/kg), Cr (0.474–0.667 mg/kg) and Pb below 0.49 mg/kg. Unfortunately, three samples representing other infusions are characterized by similar concentrations of those metals. In near vicinity group V was positioned. This group includes Nervosan infusions (no. 39–44), which are characterized by the highest level of Cu, in the range of 4.01–9.35 mg/kg, and low content of Cd, less than 0.042 mg/kg.

The extracts of Septosan created group VI. Their most distinctive attribute is high concentration of Fe (28.48–50.09 mg/kg). In two samples of Septosan (no. 62 and 63), the highest levels of Cr – 1.240 and 1.721 mg/kg, pushed them to the lower side of the PCA plot. Cholagoga II infusions, characterized by the highest concentration of Fe (41.44 to 50.49 mg/kg), formed group VII located in lower right side of PCA plot. Both Septosan and Cholagoga II mixtures contain peppermint leaf. This plant raw material is present in Gastrosan and Normosan as well. However, in PCA they were placed in the middle of the plot together with infusions prepared from other mixtures. Cholagoga II mixture also comprises yarrow herb similarly to Nervosan samples, which are on the same side of PCA plot.

Results of self-organizing maps

In further step of the research, the results of PCA were juxtaposed with those obtained by application of self-organizing maps. The mapping of

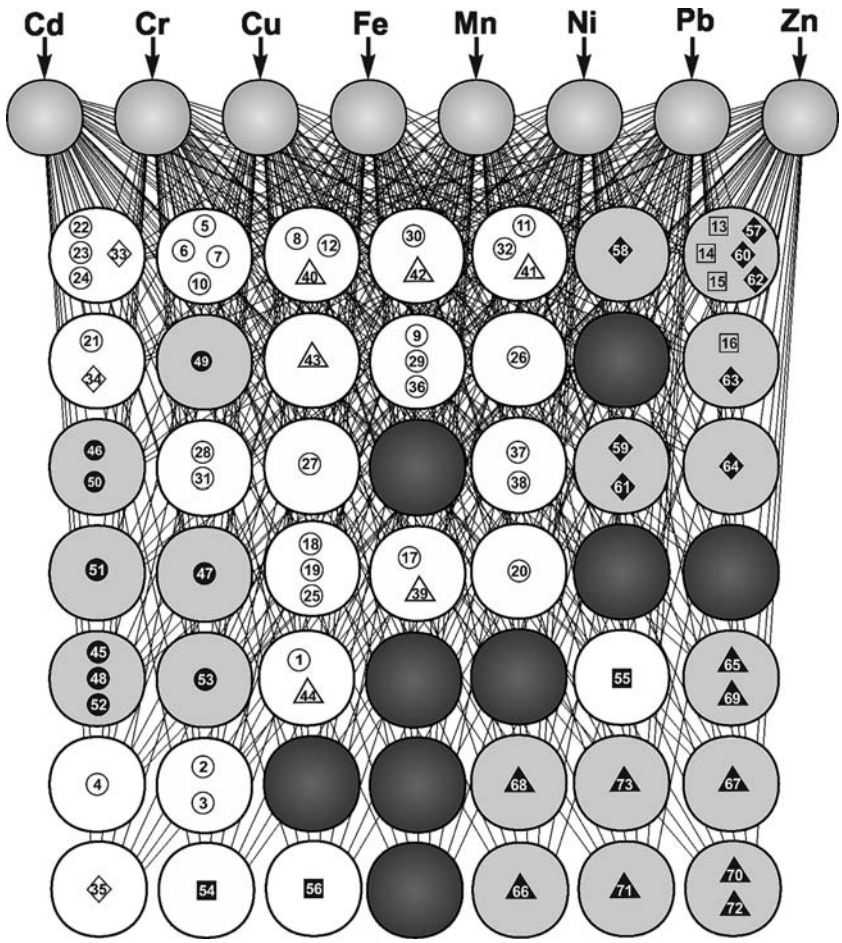

Figure 5. The distribution of herbal infusions on Kohonen map. (Neurons with no samples are marked dark grey).

infusion samples shown in Figure 5 did not distinguish as many consistent groups as PCA. It turned out that Nervosan extracts placed together in group V in PCA plot, were scattered all over Kohonen map, and its samples can be found in different distant neurons. It proves that despite the same composition of herbal mixture, there are substantial dissimilarities in the concentration of some elements. Furthermore, the level of Cu, quite different in comparison to other mixtures, was not recognized as a special attribute of all Nervosan extracts. A similar case was observed with regard to Pyrosan and Infektoten infusions, which formed two separate groups II and III in PCA score plot.

Although ANOVA test indicated differences in Cd levels in the extracts of both mixtures, the concentration of remaining elements was comparable. Two groups of infusions prepared from Cholagoga and Septosan, containing the highest amount of Fe, as opposite to PCA, remained unseparated on the map. They were put together in neurons located in upper right corner of SOM (grey neurons). The difference in mean concentrations of Cd and Pb caused separation of these two groups of mixtures in PCA. Kohonen map, however, while taking into consideration the levels of all analyzed metals, did not identify significant distinctions, and placed them together.

Normosan infusions presented themselves as an interesting case. The analysis of variance did not reveal differences between Normosan and extracts of other mixtures. In spite of this fact, PCA distinguished these samples as group IV, though situated closely to other extracts. In contrast, infusions of Normosan were gathered in adjacent neurons on the left side of the Kohonen map, which clearly suggests a considerable resemblance within the group in regard to metals levels.

The infusions of Urosan, similarly to PCA, were also differentiated from others by SOM. They can be found in lower right corner (grey neurons). Undoubtedly, trace elements whose concentrations greatly affected such allocation of these extracts were Mn, Zn and, to a lesser extent, Fe.

CONCLUSIONS

The research showed that metals composition of water extracts, prepared from commercial herbal mixtures, is vastly similar. ANOVA test indicated that only in a small number of infusions, the content of elements considerably differed from each other. PCA indicated that the metals content can be a characteristic feature for some infusions. It was demonstrated by the formation of several consistent groups. The concentrations of Mn, Zn, Cd in Pyrosan, Cr, Fe and Cd in Septosan and Mn, Zn, Fe in Urosan extracts, particularly varied from extracts prepared from the rest of herbal mixtures. Infusions, in which the mean content of only one metal determined their distinction from others, were also identified. It was Cu contents in Nervosan, and the levels of Fe in Cholagoga II samples.

Non-linear modeling performed by Kohonen map managed to reveal more subtle similarities in metal contents in Urosan and Normosan, and to some extent – in Septosan and Chologoga II extracts. In this way, the results of ANOVA test were confirmed, pointing out to some simplifications made by PCA method, which considered only evident similarities in metal contents.

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