

Research Article

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Relationships between flavonoids and selected elements in infusions of medicinal herbs

Abstract: The aim of the studies was to establish relationships between flavonoids and elements important for human health. Therefore, total contents of flavonoids and phosphorus were determined by UV/Vis methods, flavonoids by HPLC, and Ca, Mg, Fe, Mn, Zn and Cu by FAAS in 68 infusions of medicinal herbs. Total flavonoids content in the aqueous extracts were in the range of 0.26 - 16.40 mg per 100 mL. The mean flavonoid contents (in mg per 100 mL of aqueous extract) were 2.24, 2.01, 1.83, 1.88 for rutin, myricetin, quercetin and kaempferol, respectively. The concentrations of Ca, Mg, P were determined in mg per 100 mL, and of Fe, Mn, Zn and Cu in μg per 100 mL. Total content of flavonoids was weakly correlated with quercetin ($r = 0.41$), kaempferol ($r = 0.53$), Cu ($r = 0.43$), and Ca ($r = -0.30$). Statistically significant correlations were also found among Cu, Ca, Mn, Zn and Fe. Cluster analysis grouped the studied herbs based on total flavonoids, also four flavonoids and essential elements contents, extracted from the whole population of herbs *Sambuci flos*, *Betulae folium*, and *Sylbi mariani semen*. Principal component analysis confirmed these findings and enabled identification of quercetin, kaempferol, Cu and Fe as the factors responsible for differentiation of the studied material.

Keywords: flavonoids, essential elements, infusions of medicinal herbs, statistical analysis

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1 Introduction

Flavonoids are ubiquitous secondary metabolites occurring in the plant kingdom. These compounds play an important role in prevention and self-medication of many

diseases associated with modern civilization. First of all, flavonoids have protective activity for human organism as radical scavengers (antitumor properties) [1]. They offer health benefits through a strong antioxidant activity owing to their polyphenolic structure. Other benefits for human health are protective effects against cardiovascular diseases, anti-inflammatory activity, and inhibition of hydrolytic and oxidative enzymes, such as phospholipase A₂, cyclooxygenase and lipoxygenase [2].

Medicinal herbs rich in flavonoid compounds encompass several botanical plant species, e.g. *Viola tricoloris* L., *Solidago virgaurea* L., *Hypericum perforatum* L., *Betula* sp. Ehrh., *Crataegus oxyacantha* L. and many others. To study the benefits of medicinal plants for human organism, water-extractable species of essential elements should be taken into consideration. Infusions of medicinal herbs can be regarded as natural “cocktails”, in which all water-soluble active organic compounds and water-extractable metals can interact with one another. For example, Fe acts as cofactor or activator of enzymes involved in biosynthesis of phenolic compounds in medicinal plants, moreover Zn, Mn and Cu ions modulate the initial stages of phenolic compound biosynthesis [3]. It was found that often positive correlations existed between such pairs of elements, as N-P, Fe-Cu, Zn-Mn and Zn-Cu [4-6]. On the other hand, negative correlations were found between pairs: Mg-Mn, Mg-Cu and Mn-Cu [7,8].

Interactions of flavonoids with metals were studied by conductometric method and fluorescence spectroscopy [9]. It was found that Mg^{2+} , Al^{3+} , Pb^{2+} and Zn^{2+} enhanced the fluorescence of quercetin, whereas Cu^{2+} , Ni^{2+} and Co^{2+} quenched the fluorescence emission. Other researchers have studied complexes of naringin with Co^{2+} ions by FT-IR and UV-Vis spectrometry to show that the Co^{2+} ion is bonded to two flavonoid molecules [10].

Another research with the use of UV/Vis spectrometry, has shown that quercetin, rutin, kaempferol, flavanol and catechin interact with Cu(II) and Fe(III) [11]. Interactions of the metal – flavonoid type were also studied by Malesev and Kuntic, and about 40 color complexes of this type were detected using UV/Vis spectrometry [12]. It was reported that often a bathochromic effect was associated

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with metal-flavonoid interaction, and important influence had also the acidity of buffer solution. The stoichiometry of the metal – flavonoid complexes was in the majority of cases 1:1 and sometimes 1:2 [12].

Therefore, the aim of the studies was to determine relationships between the level of flavonoids and the contents of essential elements (P, Mg, Ca, Fe, Zn, Mn and Cu) in the investigated group of medicinal herbs. Moreover, the goal of the research was also to identify the differences and similarities in chemical composition of the studied herbs, based on total flavonoids and particular flavonoid compounds, as well as on selected elements taking into consideration the origin of the studied samples from various botanical species and different morphological plant part.

2 Experimental procedure

2.1 Plant material

The analyzed medicinal herbs originated from Polish herbal companies (Kawon, Herbapol, Flos, Dary Natury) and are listed below (Latin names and sample numbers are given in parentheses):

Herbs of: wild pansy (*Viola tricoloris herba*, 1-4), European goldenrod (*Solidaginis virgaureae herba*, 5-7), St John's wort (*Hyperici herba*, 8-12), knotgrass (*Polygoni avicularis herba*, 13-17), common horsetail (*Equiseti herba*, 18-22); leaves of birch (*Betulae folium*, 23-28); flowers of: common hawthorn (*Crataegi inflorescentia*, 29-34), common elder (*Sambuci flos*, 35-40), linden (*Tiliae inflorescentia*, 41-44), everlasting (*Helichrysi inflorescentia*, 45-50), wild chamomile (*Chamomillae anthodium*, 51-56); fruits and seeds of: common elder (*Sambuci fructus*, 57 and 58), common hawthorn (*Crataegi fructus*, 59-62) and blessed milkthistle (*Sylibi mariani semen*, 63-68).

2.2 Methods

Infusions of medicinal herbs were obtained by pouring boiling redistilled water onto 2.0 g of a plant material. After 15 min the infusions were filtered through paper filters (Macherey-Nagel MN 640, Germany) and diluted to 100 mL with redistilled water. Redistilled water obtained in Heraeus (Switzerland) distillation apparatus was used throughout.

For all spectrophotometric measurements, a Metertek SP-870 (South Korea) UV/Vis apparatus was used. Total

flavonoid contents were directly determined at 510 nm in infusions based on reaction with $AlCl_3$ using external calibration graph based on the rutin standard (Across Organics, Belgium).

To determine the contents of rutin, myricetin, quercetin and kaempferol, a HPLC chromatograph (Merck-Hitachi LaChrome, Darmstadt, Germany) was used. The 1 mg per mL standard solutions (Across Organics, Belgium) were injected onto chromatographic column, and the concentration of the flavonoids was obtained based on external calibration graphs. The analytical parameters were as follows: chromatographic column Hypersil Gold C18 250×4.5 mm, 5 μ m particles (Thermo Scientific Runcorn, UK) maintained at 30°C, the mobile phase: redistilled water + 0.05% solution of trifluoroacetic acid (Sigma Aldrich, Germany)/ methanol (HPLC-ultra gradient, POCh, Poland), injection volume: 20 μ L, mobile phase flow rate: 1 mL min^{-1} , detection at 370 nm.

For the assay of the essential elements, standard analytical conditions (air/acetylene) were used employing a flame program of the Atomic Absorption Spectrometer 250 Plus (Varian, Australia) and the analytical wavelengths (nm) were as follows: 285.2 (Mg), 422.7 (Ca), 279.5 (Mn), 248.3 (Fe), 213.9 (Zn) and 324.8 (Cu).

2.3 Validation of the methods

Table 1 shows the validation parameters obtained for the analytical procedures used for quantitation of total flavonoids, rutin, myricetin, quercetin, kaempferol, phosphorus and essential metallic elements. The validation values are satisfactory, and all methods had a high linearity, and can be applied for qualitative and quantitative analysis of plant materials rich in flavonoids. Limits of detection (LOD) and quantification (LOQ) were calculated according to the following formulas: $LOD = 3 \times s_0/a$ and $LOQ = 10 s_0/a$, where: s_0 – standard deviation of a blank sample based on six replicates, a – slope of the calibration curve.

3 Results and discussion

3.1 Total contents of flavonoids

The mean content of total flavonoids in infusions prepared from the analyzed herbal samples was 4.90 mg per 100 mL, which corresponds to 2.45 mg g^{-1} of dry mass of the herb. The total amount of flavonoids in all samples ranged

Table 1: Validation parameters of the applied analytical procedures (n = 6).

Parameter of validation	Total flavonoids	Rutin	Myricetin	Quercetin	Kaempferol	Ca	Mg	Fe	Zn	Mn	Cu	P
Range	1.8 – 800.0	25.0 - 75.0	15.0 - 100.0	15.0 - 100.0	15.0 - 100.0	0.4 - 3.2	0.1 - 1.2	0.4 - 2.0	0.2 - 2.4	1.0 - 5.0	0.2 - 1.2	1.6 - 7.2
[$\mu\text{g mL}^{-1}$]												
Slope (a)	0.00114	14036	47535	75115	58750	0.05907	0.73942	0.0585	0.14278	0.14700	0.06892	0.00414
Intercept (b)	0.07171	2380.8	865000	1270000	1070000	0.05401	0.11796	0.0012	0.0094	0.0404	-0.0011	0.00498
Linearity (r)	0.9885	0.9958	0.9765	0.9842	0.9820	0.9991	0.9887	0.9992	0.9984	0.9989	0.9989	0.9980
LOD [$\mu\text{g mL}^{-1}$]	0.5	8.3	2.5	2.0	2.2	0.1	0.03	0.03	0.01	0.01	0.03	0.4
LOQ [$\mu\text{g mL}^{-1}$]	1.8	25.3	7.6	6.2	6.6	0.2	0.1	0.1	0.05	0.03	0.1	1.4
Precision [%]	8.4	4.5	5.0	6.7	1.5	12.5	5.7	2.7	1.5	5.1	5.0	2.8
Recovery [%]	89.2	102.2	96.9	101.2	98.5	102.9 ¹⁾	100.7 ²⁾	83.0 ²⁾	95.6 ²⁾	118.5 ²⁾	138.7 ³⁾	86.4 ²⁾

1) NIST SRM 1573a (Tomato Leaves), 2) INCT – MPH-2 (Mixed Polish Herbs), 3) INCT-TL-1 (Tea Leaves)

between 0.26 - 16.40 mg per 100 mL (all results were expressed as mg per 100 mL of the aqueous extract).

The highest concentration of total flavonoids, 16.40 mg per 100 mL, and 14.56 mg per 100 mL was detected in two *Hyperici herba*. The mean value for all 5 samples of *Hyperici herba* was 10.95 mg per 100 mL. Similar level of total flavonoids was found also in three samples of *Solidaginis virgaureae herba*, with the mean of 10.38 mg per 100 mL. The lowest concentration of total flavonoids was found in *Betulae folium*, 0.26 mg per 100 mL. A relatively low level of total flavonoids, around 0.41 mg per 100 mL, was also found in the samples of *Viola tricoloris herba*, which is the lowest concentration in all of the studied herbal infusions.

An analysis of the contents of total amounts of flavonoids in infusions obtained from different morphological plant parts (Fig. 1), has shown that the highest levels were in the fruits and flowers, 6.50 mg per 100 mL, and 5.88 mg per 100 mL, respectively. The lowest contents of total flavonoids were found in anthodium. These findings were confirmed by Student *t*-test ($\alpha < 0.05$). Statistically significant differences of the mean values of total flavonoid contents were found between the leaves and flowers ($t = -4.85$), inflorescences ($t = -2.68$), and fruits ($t = -3.36$), as well as between anthodium and flowers ($t = 7.51$), inflorescences ($t = 3.43$), and fruits ($t = -3.61$).

Total contents of the flavonoids in the same plant species, did not differ generally. For example, in infusions from *Sambuci fructus*, total flavonoid contents fell in the range of 6.53 – 7.82 mg per 100 mL. These results are generally lower than those given in the literature [12-15]. The reason for this can be various, for example one of them is the fact that other researchers analyzed plant materials derived from different botanical plant species than ours. Besides, the plants originated from different geographical locations, were cultivated in various types of soil under other environmental conditions, *etc.*, as well as that total flavonoids were determined by different analytical techniques.

3.2 Contents of rutin, myricetin, quercetin and kaempferol

All investigated flavonoids (rutin, myricetin, quercetin and kaempferol) were determined only in 3 out of 14 infusions of the studied botanical plant species, namely those of *Sambucus nigra*, *Helichrysum arenarium* and *Matricaria chamomilla*. In *Tiliae inflorescentia* characteristic is the presence of three flavonoids, rutin, myricetin and quercetin. Only the first one was assayed

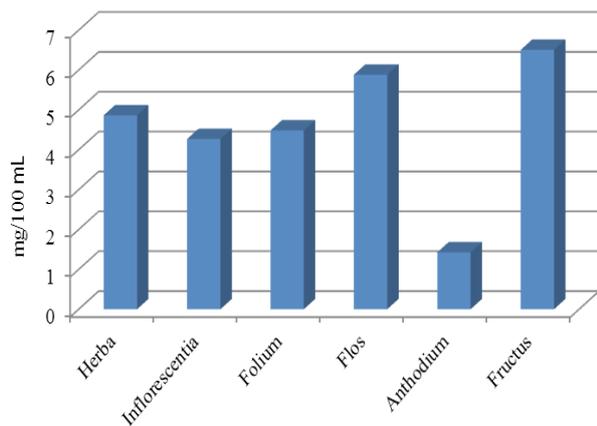


Figure 1: The contents of flavonoids in herbal infusions.

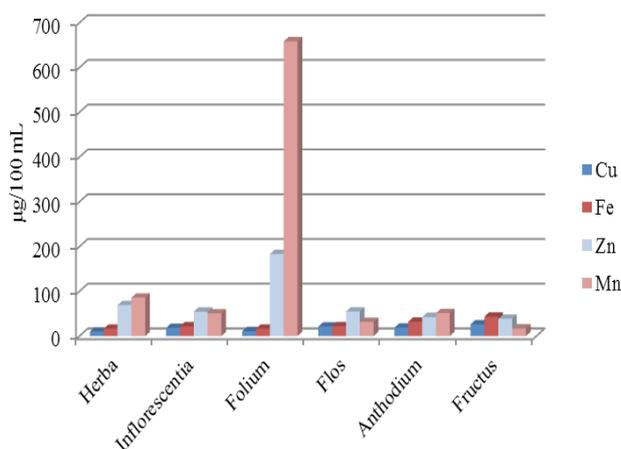


Figure 2: The contents of microelements in herbal infusions.

in the following herbs: *Violae tricoloris herba*, *Betulae folium*, *Crataegi fructus* and in *Sylibi mariani semen*. It is worth admitting that in *Sambuci fructus* only myricetin was determined, which is a characteristic feature of this plant material.

The mean content of rutin in all samples was 3.22 mg per 100 mL of infusion. The range covers the values from 2.01 µg per 100 mL to 6.29 mg per 100 mL, so it is quite large. The highest rutin concentration, 6.29 mg per 100 mL, was found in one of *Helichrysi inflorescentia*, and in *Crataegi fructus*, 0.21 mg per 100 mL. The lowest rutin level was characteristic for *Sylibi mariani semen*, 2.01 µg per 100 mL of the infusion.

The mean contents of myricetin in the infusions was 2.01 mg per 100 mL. The highest myricetin concentration, 3.10 mg per 100 mL was found in one infusion of *Helichrysi inflorescentia*. The lowest myricetin level, 1.82 mg per 100 mL was assayed in *Crataegi fructus*. It was noticed that often the samples originating from the same herbal company contained similar levels of myricetin, as it was in

the case of *Sambuci flos* purchased from the *Kawon* herbal company. For instance, infusions of this herb contained 1.83 mg per 100 mL of myricetin, and another 1.82 mg per 100 mL.

The mean contents of quercetin was 1.83 mg per 100 mL. The range in which quercetin was determined in the studied materials, extended from 1.69 mg per 100 mL in one of *Polygoni avicularis herba*, to 2.49 mg per 100 mL in *Helichrysi inflorescentia*. As far as the kaempferol assays are concerned, its mean level was 1.88 mg per 100 mL of infusion, and the range of concentrations extended from 1.83 mg per 100 mL to 2.62 mg per 100 mL.

3.3 The contents of essential elements

The concentration ranges of Ca, Mg, P (mg per 100 mL) and of Fe, Mn, Zn and Cu (µg per 100 mL) (Fig. 2) in herbal infusions were as follows: 1.70 – 14.40 (P), 83.88 - 245.54 (Ca), 2.07 - 34.42 (Mg), 9.2 - 71.77 (Fe), 4.57 - 950.98 (Mn), 15.80 - 242.80 (Zn), and 5.73 - 51.57 (Cu). It was possible to identify characteristic plant species with high levels of the studied elements determined in their infusions, such as *Sylibum marianum* with high concentration of Fe and Cu, *Sambucus nigra* (high level of Fe), *Betula pubescens* (Mn and Zn), *Hypericum perforatum* (Zn), *Equisetum arvense* (Mg and Ca) and *Solidago virgaurea* (high level of P). These data are consistent with those reported in the literature [16-19]. Especially high levels of water-extractable Zn and Mn were confirmed in *Betula* species [20].

3.4 Statistical interpretation of the results

3.4.1 Relationships among the flavonoids and essential elements

The results of correlation analysis [21] are presented in Table 2. Among 66 pairs of correlations, 11 of them were statistically significant ($\alpha < 0.05$). However, it can be noticed that only 8 relationships out of 11 are high, considering the value of correlation coefficient higher than 0.5 [22]. The total flavonoid content is weakly correlated with quercetin ($r = 0.41$), and kaempferol ($r = 0.53$). The contents of rutin and myricetin are not related to total flavonoids in a statistically significant way. Other higher relationships were found for the pairs of quercetin - myricetin ($r = 0.64$) and quercetin - kaempferol ($r = 0.58$).

Total contents of flavonoids is weakly correlated also with water-extractable Cu ($r = 0.43$) and negatively correlated with water-extractable Ca ($r = - 0.30$). As for

Table 2: The results of correlation analysis. The statistically significant ($\alpha < 0.05$) correlation coefficients are printed in bold font.

	Σ Fl	Rutin	Myricetin	Quercetin	Kaempferol	Fe	Mn	Zn	Cu	Mg	Ca	P
Σ Fl	1											
Rutin	0.05	1										
Myricetin	-0.01	-0.06	1									
Quercetin	0.41	0.16	0.64	1								
Kaempferol	0.53	0.30	-0.14	0.58	1							
Fe	0.22	-0.21	-0.06	0.08	-0.01	1						
Mn	-0.16	-0.23	-0.13	-0.17	-0.12	-0.24	1					
Zn	-0.01	-0.19	0.08	0.28	0.11	-0.20	0.85	1				
Cu	0.43	0.08	0.22	0.35	0.21	0.71	-0.27	-0.21	1			
Mg	-0.15	0.07	-0.13	0.10	-0.20	-0.16	0.01	0.03	-0.30	1		
Ca	-0.30	0.01	-0.16	0.02	0.27	-0.55	0.12	-0.22	-0.55	0.51	1	
P	0.11	-0.08	0.22	0.15	0.12	-0.19	-0.13	-0.06	-0.12	0.02	0.02	1

Σ Fl - total contents of flavonoids

the correlations with Cu, it can be stated that Cu being an electro-active metallic element, can be bound by OH groups of the flavonoid compounds structure. Moreover, Cu(II) is involved in interaction of flavonoids with DNA, as reported recently by Temerk *et al.* [23], and this can also explain the positive relationship between the total flavonoids and Cu. On the other hand, it is not easy to explain the negative correlation between the sum of flavonoids and Ca in infusions, which is however weak, as indicated by the small correlation coefficient.

The statistically significant correlations were obtained for the following pairs of metallic elements: Fe - Cu, Fe - Ca, Mn - Zn, Cu - Ca and Mg - Ca. Some of them are positive, such as those between Mn and Zn or Fe and Cu, and this demonstrates that these metallic elements probably stimulate biochemical transitions in plant organism, as given in literature [3]. On the other hand, there are negative correlations as well, for example in the pair Fe - Ca, which reveals an antagonism between the elements. These correlations can be confirmed by our earlier investigations of inter-relations among the elements in aqueous extracts obtained from plant materials [4].

3.4.2 Cluster and principal component analysis

Cluster analysis (CA) [21] is a very useful statistical tool leading to grouping of the studied objects into clusters with similar properties. The CA dendrogram (Fig. 3) illustrates the grouping of the studied infusions of medicinal herbs based on similarity of their chemical composition, namely on total flavonoids, contents of rutin, myricetin, quercetin

and kaempferol, as well as the concentrations of macro- and microelements. The calculations were done for the standardized experimental matrix using the Ward method and the Euclidean distance.

It is possible to notice the characteristic groups of the samples. For example, in the lower part of the dendrogram, there are all *Betulae folium* (cluster I). Moreover, there is a long distance, based on almost 100% of maximum distance measure, between clusters I and remaining other clusters II, III and IV, which indicates the different composition of *Betulae folium* from the other analyzed infusions of herbs.

It is also possible to identify the clusters, which contain the infusions obtained from the herbs originating from the same botanical species and characterized by similar level of flavonoids and the elements. For example, cluster II consists of *Sylbi mariani semen*, *Crataegi fructus* and *Tiliae inflorescentia*, cluster III *Sambuci flos* and *Crataegi inflorescentia*, whereas cluster IV includes *Violae tricoloris herba*, *Hyperici herba* and *Helichrysi inflorescentia*.

Principal component analysis (PCA) [21] has often been applied for interpretation of huge databases [24-26]. Preliminary experimental matrix with the dimensions 68×12 was created based on the results of total flavonoids, rutin, myricetin, quercetin, kaempferol and water-extractable species of P, Mg, Ca, Fe, Mn, Zn and Cu in 68 infusions of medicinal herbs. The matrix was standardized before PCA calculations. The new matrix of principal components contained new variables (principal components, PCs) where three first PCs explained together 62% of the variability among the infusions. However, in an effort to obtain as much clear interpretation as possible,

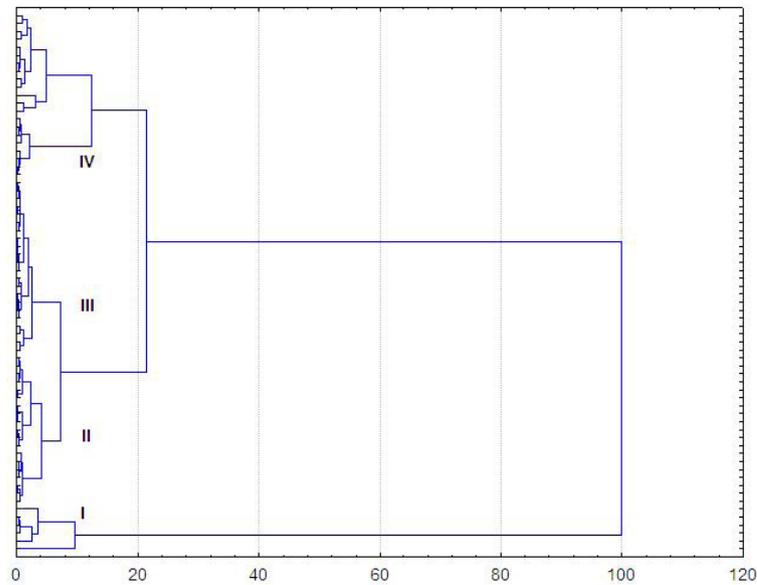


Figure 3: Dendrogram of the studied herbal infusions.

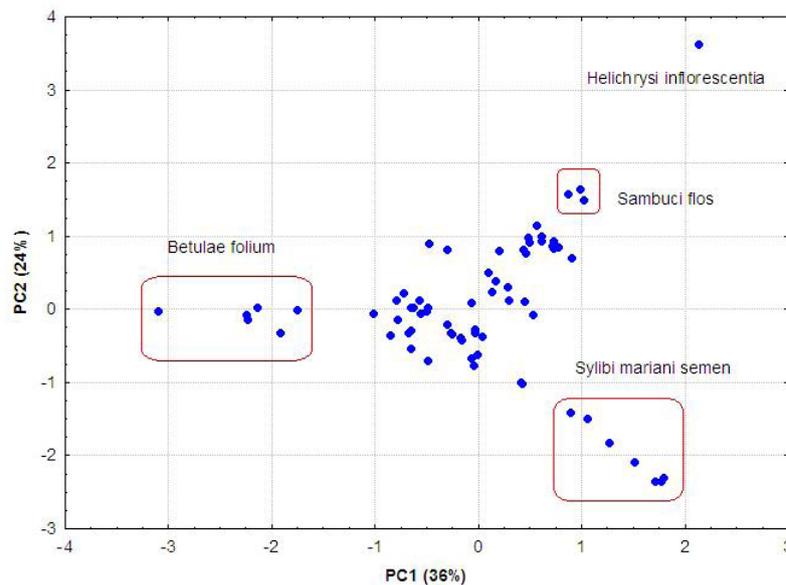


Figure 4: Scatterplot of PC1 vs PC2 for the studied herbal infusions .

it was decided to recalculate PCA taking only 7 variables (total flavonoids, quercetin, kaempferol, Fe, Mn, Zn and Cu), which were correlated with first five PCs. This allowed for obtaining the results in which PC1 explained 36%, PC2 24%, and PC3 20% of variability of samples. The distribution of the infusions in a two-dimensional plot allows to identify some characteristic groups of infusions (Fig. 4). There are birch infusions (*Betulae folium*) in the left side of the plot, in the upper part there is one sample of *Helichrysi inflorescentia* and three of *Sambuci flos*, and

in the right hand area of the plot one can find *Sylbi mariani semen*. The other remaining infusions are concentrated in the central area of the plot and are not well separated from one another.

The characteristic distribution of the infusions in Fig. 4 can be explained by the loadings of the principal components shown in Fig. 5. The highest impact on this distribution have the contents of water-extractable Fe and Cu, correlated with PC1, as well as concentrations of quercetin, kaempferol, which are correlated with PC2.

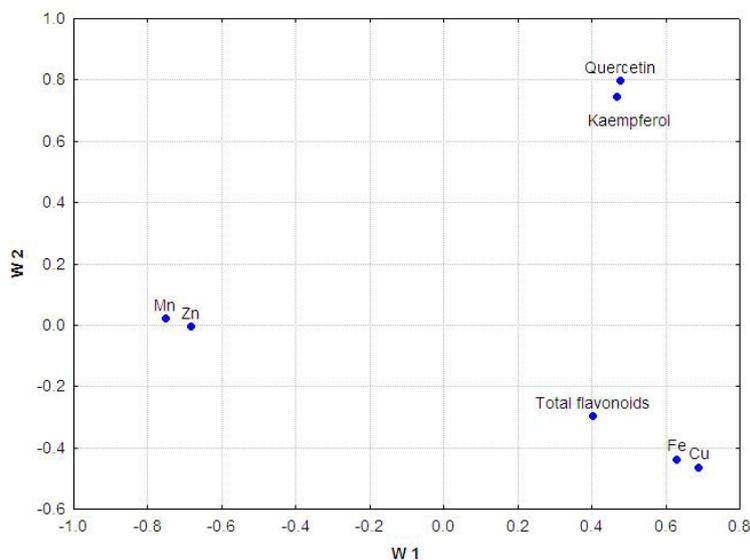


Figure 5: Loading plot of PC1 vs PC2 for the studied herbal infusions .

4 Conclusions

It was found that total flavonoids content was positively correlated with quercetin and kaempferol, as well as weakly with Cu and negatively with Ca water-extractable species. Statistically significant correlations were also found among Cu, Ca, Mn, Zn and Fe, which confirmed their interrelationships.

The use of CA and PCA enabled the grouping of the studied infusions based on the total flavonoids, particular flavonoids and essential elements contents, and has extracted *Sambuci flos*, *Betulae folium* and *Sylbi mariani semen* from the whole population as the herbal infusions with especially high levels of water-extractable species of the elements. Moreover, PCA allowed the identification of quercetin, kaempferol, water-extractable forms of Cu and Fe as the factors responsible for differentiation of the studied herbal materials.

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