

ANALYSIS

ELECTROCHEMICAL FINGERPRINT STUDIES OF SELECTED MEDICINAL PLANTS RICH IN FLAVONOIDS

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Abstract: The combination of a size-exclusion column (SEC) with electrochemical (voltammetric) detection at a boron-doped diamond electrode (BDDE) was applied for studying the correlations between electroactive Cu and Fe species with phenolic groups of flavonoids. For comparison with electrochemical results, SEC-HPLC-DAD detection was used. The studied plant material comprised of: *Betula verrucosa* Ehrh., *Equisetum arvense* L., *Polygonum aviculare* L., *Viola tricolor* L., *Crataegus oxyacantha* L., *Sambucus nigra* L. and *Helichrysum arenarium* (L.) Moench. Based upon the results, high negative correlation was found for the chromatographic peak currents at 45 min with the sum of Cu and Fe for the aqueous extracts of *Sambucus*, *Crataegus* and *Betula* species, and for the peak currents at 65 min of the aqueous extracts of *Sambucus*, *Crataegus*, *Helichrysum* and *Betula* botanical species. This behavior confirms that it is mainly the flavonoids with easily oxidizable phenolic groups which are strongly influenced by the presence of Cu and Fe. Moreover, the electrochemical profiles obtained thanks to the use of HPLC hyphenated with voltammetric detection can be potentially applied for fingerprint studies of the plant materials used in medicine.

Keywords: electrochemical profiling, medicinal plants, voltammetric detection, boron-doped diamond electrode (BDDE), redox-active metals (Fe and Cu)

Herbal medicines are often in focus of extensive research. The reason for this is the fact that medicinal plants are rich sources of not only biologically active substances, and also of vast pool of macro- and microelements. These essential elements together with the organic compounds create natural complexes, in which all constituents are balanced, and can act simultaneously on the human organism. Therefore nowadays, an important problem for researchers is to recognize not only total concentration of essential elements or separated secondary metabolites present in herbal medicines, but rather their interactions and the species, which are eventually absorbed from natural drugs by human organism (1, 2).

An alternative for the complete identification and monitoring of potentially relevant chemical species present in medicinal plants, so called “targeted approach”, can be the non-targeted metabolic profiling (fingerprinting) approach. This non-targeted approach is particularly important for detecting effects, which are based upon interactions of inorganic and organic plant constituents, because neither

the identity of interested species nor the exact mechanism of interaction is previously known. A typical example of such an interaction is the interaction of electro-active metals with flavonoids, which could have an impact on therapeutic effects of plants.

In plant kingdom, flavonoids are ubiquitous secondary metabolites with numerous positive impacts on human organism. Among them are scavenging effects on superoxide radicals (3), antioxidant and antimutagenic activities, and reducing the risk of cardiovascular disease and stroke (4). Flavonoids, among other polyphenolic compounds, can also be preventive agents against cancer, because they have ability to modulate enzyme activities resulting in decreased carcinogenicity of xenobiotics that are responsible for oxidative stress induced cancer (5). The main sources of flavonoids in human’s diet constitute vegetables, fruits, juices from fruits, red wine, as well as black and green tea (6). However, herbal infusions prepared from several medicinal plant, drunk by patients regularly, can be rich sources of flavonoids, too.

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The investigations leading to establishing the metabolic fingerprints of plant extracts using ESI-MS hyphenated technique have already been done (7), as well as there were some studies based on HPLC fingerprinting of traditional Chinese herbal drugs (8, 9). The application of HPLC fingerprint based on selected phenolic acids analysis in *Melissa officinalis* L. medicinal herb has recently been reported (10). Several studies were also conducted, in which electrochemical techniques were applied in order to study the metabolic profiles of herbal drugs. Among them were potentiometric fingerprint techniques used for the analysis of liquid extracts of *Eurycoma longifolia*, a medicinal plant originating from Malaysia (11), and cyclic voltammetry used for studying of interactions among flavonoids and Cu(II) and Fe(III) species in the roots of Brazilian plant *Dioclea grandifolia*, used in folk medicine (12). Cyclic and differential pulse voltammetry were also applied as analytical tools for characterization of electrochemistry of flavonoids in order to detect their interactions with Cu (13). The working electrodes used in this investigation were pencil graphite electrodes.

Among different electrodes used in electrochemical profiling studies, one of particular importance has been boron doped diamond electrode (BDDE), which was applied for estimation of total phenols in food (tea and fruit juice samples) (14). BDDE as working electrode has several advantages, such as its high sensitivity to electrochemically active natural substances, high electrochemical and mechanical stability and minimized fouling effects, which assures good reproducibility. This latter point is advantageous for the application to medicinal plant extracts, because they contain high amounts of compounds that normally, when glassy carbon electrode is used, result in electrode fouling by irreversible adsorption to the electrode. Interactions of flavonoids with electro-active species of metals have been studied previously in leaves of *Ginkgo biloba* L. (15) Several complexes of selected flavonoids with Cu(II), Fe(II) and Fe(III) were also identified by Malesev and Kuntic (16).

Therefore, the main objectives of the investigation were to establish an electrochemical metabolic profiling (fingerprint) method for medicinal plants rich in flavonoids, and to apply this method to plants with different flavonoid and electro-active metals (Fe and Cu) contents. Moreover, the goal was also to find correlations between electrochemical profiles and these metals, in order to reveal possible interactions which potentially could be related to biological activity of the studied plants.

EXPERIMENTAL

Plant material

The studied plant material comprised of the following botanical species of medicinal plants: *Betula verrucosa* Ehrh., *Equisetum arvense* L., *Polygonum aviculare* L., *Viola tricolor* L., *Crataegus oxyacantha* L., *Sambucus nigra* L. and *Helichrysum arenarium* (L.) Moench. All of them were bought at a pharmacy and originated from "Kawon" Polish herbal firm. Prior to analysis, they were ground using Knifetec (Foss Tecator, Denmark) sample mill and were kept in plastic containers.

Extraction

The samples of 1.0 g of each plant material were weighed using the analytical balance. Then to each of the samples 25.0 mL of deionized water at 90°C was added, stirred with electromagnetic stirrer during 20 min, and filtered immediately through Selecta "falten filter". The obtained filtrate was used for HPLC determinations always freshly prepared.

Ultrafiltration

The samples were ultrafiltered by centrifugation at 3000 rpm for 20 min, then 3900 rpm for 60 min, then 4500 rpm for 120 min using Rotixa/P centrifuge (Hettich, Germany) with Centriprep Millipore YM-10 (10,000 nmwl) filters and analyzed by SEC-HPLC-DAD.

SEC-HPLC-DAD detection

For dilution of the filtrates obtained as described above, 0.1 mol/L phosphate buffer of pH = 7.0 was used. It was prepared each week by dissolving of appropriate amount of $\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$ in deionized water.

For the separation, the size-exclusion chromatographic column Shodex OHPak SB-802HQ with 20 μL sample loop, and with the Merck-Hitachi L-4500 Diode Array Detector, were used. The same SEC column was applied in our earlier investigations of aqueous extracts of medicinal plants (17). The plant sample (filtrate) was diluted (1 + 1) with 0.1 mol/L phosphate buffer, and before injection to the chromatographic column, it was filtered through Osmonics Cameo 3N syringe filter of 0.22 micron.

SEC-HPLC-ELC detection

Two electrochemical detectors of the same type: Metrohm 641-VA, were used, connected to the same chromatographic column, as in case of HPLC-DAD. Knauer HPLC K-1001 pump was used. Electrochemical cell comprised of working elec-

trode – 3 mm Boron Doped Diamond Electrode (Windsor Scientific, UK), reference electrode – Ag/AgCl/Cl electrode, and auxiliary electrode – gold electrode. The potentials applied were as follows: +0.4 V, +0.5 V, +0.6 V, +0.7 V, +0.8 V.

Microwave digestion

In the preparation of the plant material before determining Fe and Cu, microwave digestion of accurately weighed plant samples (circa 1.0 g) was performed by the use of the following mixture: 30% H₂O₂ (POChem, Poland)/concentrated 65% HNO₃ (Selectipur, Merck, Germany) (3 : 5, v/v). The digestion process was done in the Uniclever BM-1z (Plazmatronika, Poland) unit applying temperature from 250 to 350°C and pressure from 31 to 45 atm. After this process, the samples were transferred to 50 mL volumetric flasks and diluted with redistilled water obtained from the quartz-glass system (Heraeus, Switzerland). Accuracy (92.1 and 96.1%) and precision as RSD (5 and 9%) of FAAS technique used for the determination of Fe and Cu, respectively, were tested earlier using IC-CTA-VTL-2 certified reference material and they were satisfactory (18).

FAAS determination of Cu and Fe

All determinations were performed using standardized analytical methods. Iron (at $\lambda = 248.3$ nm) and copper (at $\lambda = 324.8$ nm) were determined using flame-AAS with a 250 Plus Atomic Absorption Spectrometer (Varian, Australia), after microwave digestion (total concentration) or directly in the aqueous extracts themselves (extractable concentration).

RESULTS AND DISCUSSION

Results of Fe and Cu determination

The reproducibility of all FAAS measurements presented in Table 1 is good, and the measured con-

centrations are consistent with the expected values in medicinal plants (18). Looking at the relative difference of extractable and total concentrations of Fe and Cu, it is noticeable that only very little (0.3–3.2%) of total Fe level is extractable, which is in agreement with the literature data (19). In the case of Cu, much more (4.6–35.4%) of this metal is water-extractable. In particular, the high percentage of extractable Cu in the sample of *Betula* is interesting, especially because this plant material is the one in which the lowest amount of total Cu was found. This is a first hint that *Betula* behaves quite differently compared to other studied plants.

Application of SEC-HPLC-DAD to the selected medicinal plants

The optimized electrochemical profiling method, i.e., size-exclusion-chromatography with voltamperometric detection at BDDE electrode was applied to all plant extracts, and in parallel the same separation was done with diode-array detection system at 200–600 nm (SEC-DAD).

Analyzing the results obtained thanks to the use of SEC-HPLC-DAD, it can be noticed that the plants can be grouped into three subgroups, which differ significantly in their phenolic and flavonoid contents. These substances were eluted well after the SEC elution range below, which was between 5 and 18 min. The first group comprised of *Crataegus*, *Sambucus* and *Helichrysum* aqueous extracts, which are characterized by several SEC-DAD peaks at the retention times less than 20 min. The second group includes *Polygonum*, *Equisetum* and *Viola* extracts, which have practically no peaks in the retention range less higher than 20 min. The sample of *Betula* can be regarded as intermediate position between the two groups of plants, because it can be characterized by several peaks at retention times higher than 20 min, but not such high as obtained for *Crataegus*, *Sambucus* and *Helichrysum* aqueous extracts.

Table 1. Results of determination of total and water-extractable iron and copper. The arithmetic mean \pm SD is given (n = 10).

Sample No.	Plant sample	Total Fe [mg/kg]	Extr. Fe [mg/kg]	Extr. Fe/total Fe [%]	Total Cu [mg/kg]	Extr. Cu [mg/kg]	Extr. Cu/total Cu [%]
1	<i>Betula</i>	91.3 \pm 0.98	2.64 \pm 0.02	2.9	6.86 \pm 0.41	2.43 \pm 0.30	35.4
2	<i>Equisetum</i>	249.9 \pm 19.4	1.75 \pm 0.10	0.7	7.39 \pm 0.40	0.55 \pm 0.03	7.4
3	<i>Polygonum</i>	233.1 \pm 8.9	0.75 \pm 0.05	0.3	8.77 \pm 0.05	0.47 \pm 0.02	5.4
4	<i>Viola</i>	287.1 \pm 14.6	2.21 \pm 0.09	0.8	6.04 \pm 0.21	0.35 \pm 0.02	5.8
5	<i>Crataegus</i>	122.8 \pm 11.4	2.46 \pm 0.05	2.0	12.7 \pm 0.20	0.62 \pm 0.02	4.9
6	<i>Sambucus</i>	123.7 \pm 3.4	0.51 \pm 0.07	0.4	10.8 \pm 0.11	0.61 \pm 0.02	5.6
7	<i>Helichrysum</i>	98.6 \pm 4.1	3.11 \pm 0.19	3.2	11.8 \pm 0.48	0.54 \pm 0.02	4.6

Extr. = water-extractable species

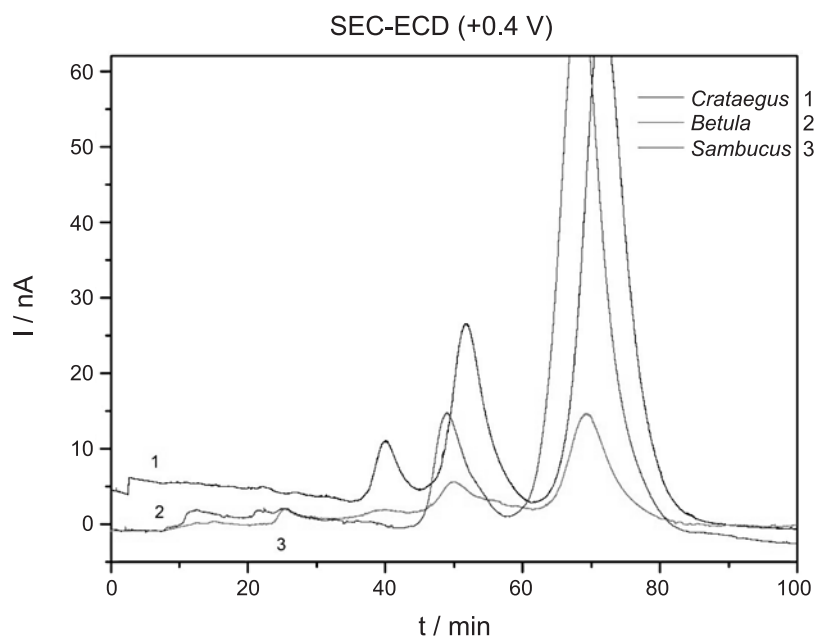


Figure 1. Chromatogram obtained by SEC-HPLC-ELC at +0.4 V

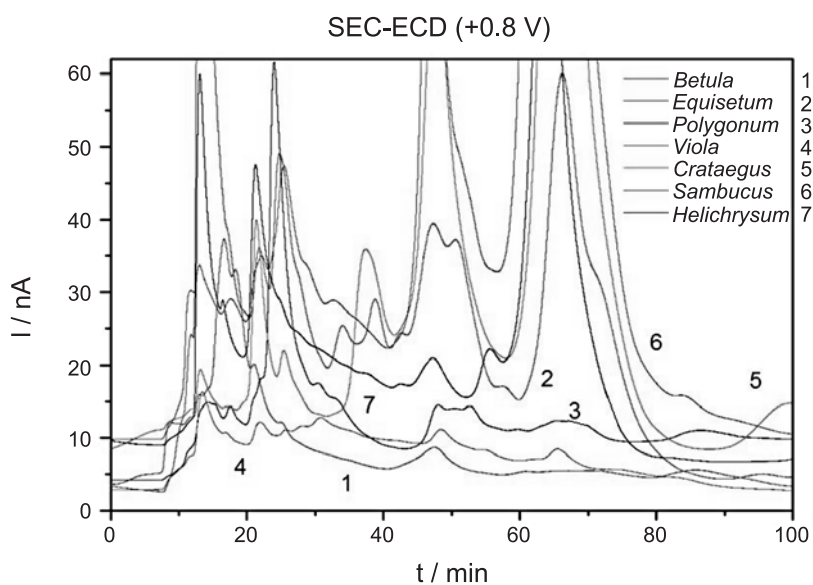


Figure 2. Chromatogram obtained by SEC-HPLC-ELC at +0.8 V

Electrochemical results obtained by using the SEC-HPLC-ELC

The electrochemical SEC-HPLC-ELC data fully confirm the data obtained for SEC-HPLC-DAD. In particular, for *Crataegus*, *Sambucus* and

Helichrysum aqueous extracts, and also for *Betula*, but with lower intensity, high concentrations of flavan- and flavon-compounds were detected. This also confirmed the earlier findings concerning the use of the combination of SEC-

HPLC technique with AAS detection for medicinal plant extracts (17). For the other extracts (*Polygonum*, *Equisetum* and *Viola*), only small peaks were detected in the respective retention range. There are typical chromatograms registered for selected plants at +0.4 V vs. Ag/AgCl, shown in Figure 1, as well as for all plants registered at +0.8 V (Fig. 2).

Figure 1 shows only the samples of *Crataegus*, *Sambucus* and *Betula*, since only for these plants the peaks at retention times higher than 30 min were found. For the other analyzed plant extracts, *Polygonum*, *Equisetum*, *Helichrysum* and *Viola*, practically no peaks were found at this potential. This also indicates the selectivity of SEC-HPLC-ELC at lower detection potential (+0.4 V), which is obvious taking into consideration the fact that these plants contain flavonoids with easy oxidizable phenolic groups (i.e., *o*-diphenolic groups), which are also potential interaction sites for electro-active metals. In contrast to Figure 1, the chromatogram registered for the potential +0.8 V (high oxidation potential) shows the peaks obtained for all plants. So it easily can be concluded that for distinguishing the differences of electrochemical behavior of all the studied plants, lower potentials are more suitable. Moreover, trying to point out the differences between the SEC-HPLC-DAD and SEC-HPLC-ELC, it can be stated that the electrochemical detection is much more sensitive, more peaks are detected using electrochemical detection at +0.8 V, and electrochemical detection at lower potentials, +0.4 V, is more selective.

Correlation of the SEC-HPLC-DAD and SEC-HPLC-ELC detection with Fe and Cu

Firstly, correlation analysis was performed for the investigated electro-active metals and their water-extractable species. It was revealed that a negative correlation of total Cu versus total Fe exists, as shown in Figure 3. It is not clear, if this correlation is somehow related to the flavonoids. It could be due also to some metal uptake and/or transport mechanism. The inverse correlation (if Cu decreases, Fe increases and *vice versa*) could point to some limitation in binding sites or competition for them, also in transport capacity etc. Therefore, the following discussion was not focused in Cu and Fe separately, but the sum (in mol/L) of both metals, too.

As anticipated from the well-known electrochemical properties of flavonoids (15, 16) and other phenolic compounds (12), the SEC-HPLC-ELC analyses confirmed the possibility to detect selectively those phenolic functional groups that are most important for the interaction with metals. These phenolic groups include the easily oxidizable *o*-diphenolic groups at potentials lower than +0.5 V. Together with the chromatographic separation, which separates chemically similar compounds (e.g., flavons and flavans), it should be possible to find statistically significant correlations between metals and different types of easily oxidizable flavonoids. This is demonstrated in Figures 4 and 5, using the "flavan-peak" (at 45 min) and the "flavon-peak" (at 65 min), respectively. In both figures the peak current at the detection potential +0.5 V (Ip in

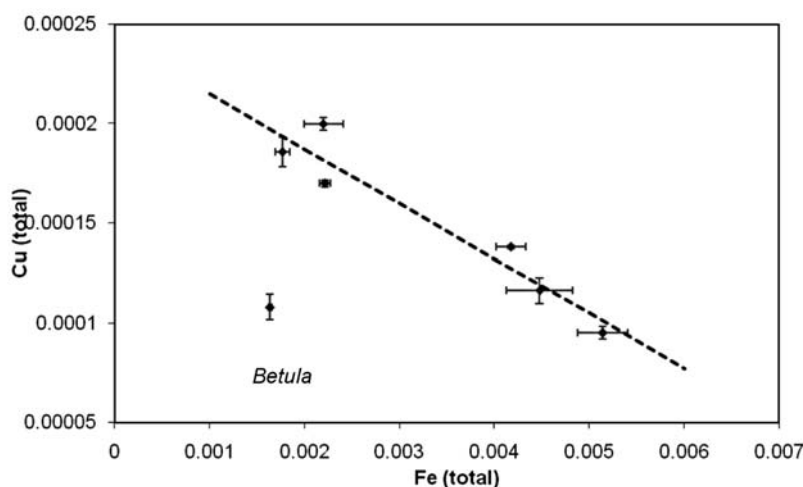


Figure 3. Correlation of Fe and Cu concentrations (both in mol/L) in the studied plants

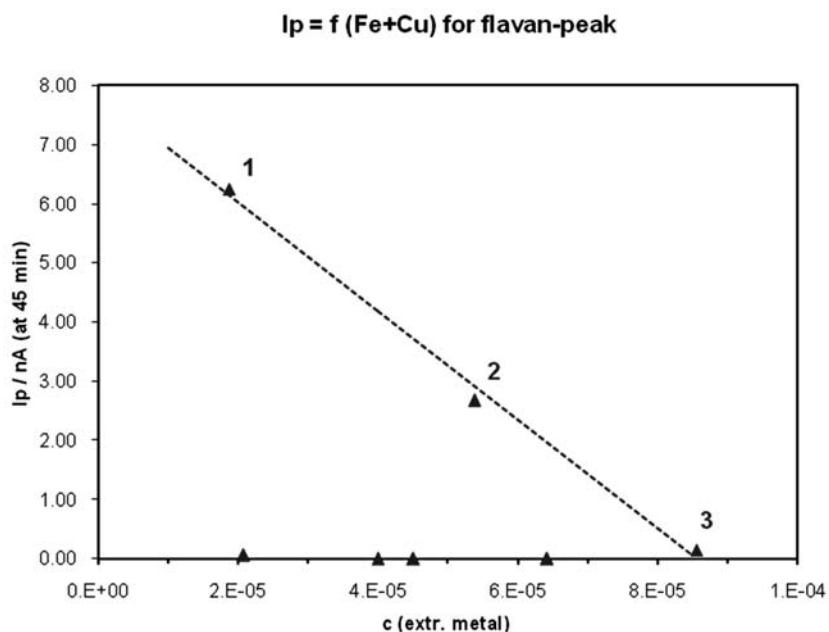


Figure 4. Correlation of the electrochemical detection at 45 min with the sum of extractable Fe and Cu. *Crataegus* (1), *Sambucus* (2) and *Betula* (3)

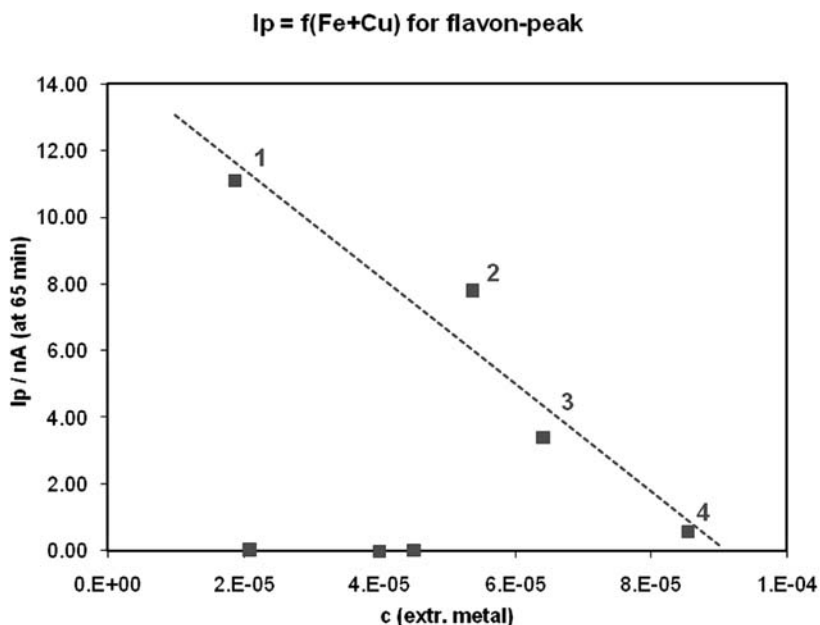


Figure 5. Correlation of the electrochemical detection at 65 min with the sum of extractable Fe and Cu. *Crataegus* (1), *Sambucus* (2), *Betula* (3), and *Helichrysum* (4)

nA) was plotted *versus* the sum of extractable Cu and Fe.

A very good correlation coefficient ($r = 0.99$) was found for the peak currents registered at 45 min

with the sum of Cu and Fe for *Crataegus* (1), *Sambucus* (2) and *Betula* (3) (Fig. 4), and also a good correlation ($r = 0.97$) for the peak currents at 65 min with the sum of Cu and Fe for *Crataegus* (1),

Sambucus (2), *Betula* (3), and *Helichrysum* (4), as shown in Figure 5. For the other plants (*Polygonum*, *Equisetum*, *Viola* and, for Figure 4, also *Helichrysum*) there was absolutely no correlation, and the respective data points are on the x-axis. The difference in the behavior of *Helichrysum* included in the good correlation of Figure 5, but excluded in Figure 4 can be explained by the fact that *Helichrysum* extract has no electrochemically detectable flavonoids at 45 min.

It should be noted that the good correlation for the three (Fig. 4) or four plants (Fig. 5) is not obtained, if the detection potential is raised above +0.5 V or if another chromatographic peak is used. This behavior confirms the assumption that these are mainly the flavonoids with easily oxidizable phenolic groups which are strongly influenced by the presence of Fe or Cu. There is an inverse correlation, which means that the detectability of flavonoids decreases with increased metal's concentrations. This can be explained by the fact that redox interaction with metals would lead to oxidized phenols, and that complexation would lead to less available phenolic groups and higher oxidation potentials. Summing up, both effects result in a decrease of the electrochemical signals for the easily oxidizable phenols.

CONCLUSIONS

Based upon the investigation, it was demonstrated that the combination of a SEC column with electrochemical (voltammetric) detection at a BDDE is capable of detecting correlations with Fe and Cu. These correlations can be explained by the presence of flavonoids containing easily oxidizable phenolic groups. As these flavonoids are important for antioxidant effects (4, 6), it is very likely that the metal-flavonoid interaction influences biological effect of the studied plants. Hence, it can be concluded that electrochemical profiling (fingerprint analysis) is a valuable tool for indirect detecting the metal-flavonoid interactions in medicinal plant extracts. This can be helpful in future studies of medicinal plants profiling analysis, including joint research of their electrochemical and spectral properties.

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