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Original Research

Determination of Heavy Metals in Aerial Part of Hyssop (*Hyssopus officinalis* L.) Using High Performance Ion Chromatography with the Aid of a Linear and Non-Linear Weighted Least-Squares Regression Model

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Abstract

Heavy and transition metals are of special importance from the ecotoxicological point of view, both because of the high toxicity of compounds containing in these metals and because of their accumulation in various organisms. Information on their content in plants and animals is of great botanical, nutritional and environmental interest. Some of the heavy and transition metals are toxic when their concentrations exceed certain values.

After applying a microwave-assisted high pressure digestion system, a highly sensitive analytical technique based on ion chromatography under isocratic eluent flow-rate conditions (0.3 mL/min) was used. A bifunctional ion-exchange column (IonPac CS 5A, Dionex, Sunnyvale, CA, USA) together with PDCA (pyridine-2.6 dicarboxylic acid) eluent was applied. Detection of all metal ions was carried out by UV-Vis spectrophotometry (at 530 nm) after a post-column derivatization reaction applying 4-(2-pyridylazo) resorcinol (PAR). The selected method of digestion of plant sample and the application of the optimized chromatographic system to heavy metal analysis of *Hyssopus officinalis* L. are presented in this work.

Linear and non-linear calibration curves for all ions were constructed using a weighted least-squares (WLS) regression model in order to estimate properly detection and quantitation limits obtained during the analysis on IonPac CS 5A column. The LODs for all the chromatographic tests were determined using Hubaux-Vos' method.

Keywords: ion chromatography, heavy metals, mineralization, hyssop, WLS regression model

Introduction

For ages plants have played a vital role for people, serving as both nutrition and medicines. Thousands of years ago the ancient Assyrians, Arabs, Chinese, Egyptians, and Greeks were already experts in their medicinal properties, and were aware of some dose-related effects of plants and their extracts [1].

It is well known that despite the significant development of the modern medicine, investigations of new drugs from natural products are continuously of great importance.

The determination of trace elements, particularly heavy metals, in real matrices has received increasing attention in

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recent years. Their accumulation and distribution in a plant depends on the plant species, type of element – its chemical properties and bioavailability, soil characteristics (e.g., pH, amount of dissolved oxygen and trace elements) as well as on weather conditions. Excessive levels of heavy metals can be hazardous to plants as well, e.g. more than 100 ppm of Ni develops symptoms of toxicity; 60 ppm of Cu and 120 ppm of Zn are the critical toxic levels for plants that are not unusually sensitive to both metals. Peanuts, for example, are extremely sensitive to Zn, and toxicity has been seen at levels as low as 12 ppm [2]. Effects of heavy metals on plants result in growth inhibition, structural damage, and a decline of physiological and biochemical activities as well as of the function of plants [3, 4].

Many metals are essential to life, but in excess, these same chemicals can be poisonous. The basic tenet of toxicology – "the dose makes the poison" – is still actual. Too little can lead to a deficiency; too much can result in adverse health effects. Much of the pertinent work on the subject has been summarized by the World Health Organization [5].

Heavy metals are dangerous because they tend to bioaccumulate. Similarly, chronic low exposures to heavy metals can have serious health effects in the long run [6]. Due to the increasing interest in phytotherapy it is necessary to perform the analysis of plants used in medicine and plant-derived drugs in order to assess potential health risks for consumers [7].

All herbal medicines placed on the market should undergo examination in order to ensure their quality and safety.

Chemical analyses of heavy metals have traditionally been carried out mostly by atomic absorption spectrometry (AAS), graphite furnace- atomic absorption spectrometry (GF-AAS) possessing better detection capabilities, and currently, new high-cost technologies like inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma mass spectrometry (ICP-MS) that are slowly replacing other analytical techniques. Unfortunately, the newest (the most expensive) analytical instruments are often beyond the reach of Polish laboratories.

Chromatographic methods have become the main powerful analytical tools available for analyzing heavy metals [8] and have been recommended to the quality control of phytotherapeutical products with many advantages over other methods [9]. High Performance Ion Chromatography (HPIC) is a useful analytical tool and offers several advantages for the determination of metals, including the ability for oxidation state speciation and multi-elemental capability in a single analysis [10-12].

A bifunctional ion-exchange column (IonPac CS 5A, Dionex, Sunnyvale, CA, USA) was found to be the most effective analytical column to separate heavy and transition metals. The column has both anion- and cation-exchange capacity and metals can be separated by both cation- and anion-exchange mechanisms. Most heavy metal ions, i.e., Fe^{3+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, and Mn^{2+} can be well separated in one single run using pyridine-2.6-dicarboxylic acid (PDCA) [13]. Metal ions can be well separated under isocratic conditions, and detected at 530 nm using 4-(2-pyridylazo) resorcinol (PAR) as post-column reagent, which forms stable chelates with all ions of interest.

Some recent papers reported in literature [14-16] prove that the nature of data in bioanalytical calibration curves is such that the data are rather heteroscedastic. Therefore, in the present work the weighted regression model (WLS) was applied instead of the conventional ordinary least-squares model (OLS). The WLS method enables better estimation of the LODs because it takes into account the variability of the standard deviation of the analytical signal as a function of concentration. The statistical theory of the WLS model described in details in literature [17] was applied for constructing the calibration curves for all ions of interest.

Hyssopus officinalis L., F. Lamiaceae is a perennial undershrub which grows wild and is cultivated in temperate regions of Asia, Europe and America [18]. The essential oil is the main constituent of hyssop, which possesses antibacterial, antifungal and antiviral properties [19]. The spasmolytic action of this oil has been described in literature [20]. Essential oil obtained from hyssop is widely used in the food, pharmaceutical and cosmetic industries. Hyssop extract reduces postprandial hyperglycemia because of its inhibitory effect on intestinal α-glucosidase; and therefore on the digestion of dietary carbohydrates [21]. Furthermore, it can be used to alleviate chronic catarrhal bronchitis and bronchial asthma.

The present work involved two experimental steps:

(i) sample preparation of plant material, and

(ii) qualitative and quantitative HPIC analysis of heavy metal ions present in the sample. The experimental data were analyzed statistically.

Experimental Procedures

Materials and Methods

Since the trace level analysis of transition metals is limited by the purity of water and the reagents, precautions must be taken at every step of sample and standard preparation to minimize contamination.

All reagents were of analytical-reagent grade. Eluents were filtered and degassed before use. All mobile phase components and PAR monosodium salt (post-column derivatization reagent) were obtained from Sigma-Aldrich, Steinheim, Germany.

Double distilled water was purified (deionized) with an Easypure system (Barnstead, USA) (18 MΩcm).

Preparation of Standard Solutions

Appropriate concentrations of standards were prepared from 1 g/L stock standards solutions (Merck, Darmstadt, Germany). All standards, samples and reagents were stored in polyethylene bottles.

The solutions used to determine the calibration curves were prepared by weight dilution (i.e. both the solution being diluted and water were weighed on an analytical balance) using standard solutions of 1000 ppm concentration (Merck, Darmstadt, Germany). Weight dilution reduces the uncertainty of concentrations obtained to such levels that it may be assumed that any error due to the calibration lines does not contribute to the error of measured peak areas.

First, the solutions of the cations of about 100 ppm concentration were prepared, from which the solutions of 10 ppm concentration were prepared by their dilution. From the 10 ppm solutions the solutions of 1-9 ppm concentrations differing by 1ppm were obtained. The 1ppm solutions were applied to prepare standard solutions of 0.1, 0.2, 0.4, 0.6, and 0.8 ppm. From the 0.1 ppm solutions 0.01, 0.02, 0.04, 0.06, and 0.08 ppm solutions were prepared. Thus, for each cation a series of 20 standard solutions in the range of 0.01-10 ppm was obtained. This method, however, causes the prepared solutions to be not statistically independent from each other. The whole dilution procedure (from stock solution 1000 ppm) was carried out three times; obtaining for each concentration three statistically independent solutions (statistically independent solutions are essential for later statistical analysis). Total 420 standard solutions were prepared.

The method of weight dilution reduces the uncertainty of concentrations of solutions prepared; however, it does not permit preparation of solutions of exactly identical concentrations. The concentrations were close to the nominal ones, but were minimally different.

In the next stage the peak areas were determined within individual series by interpolation (for extreme concentrations, if necessary, by extrapolation) for exactly identical assumed concentrations. It was found that the data are of the heteroscedastic type, therefore, the weighted regression method was applied. The weights for the individual points of the curve were calculated by the standard method [17]. The parameters of the correlation equations were determined using the TableCurve 2D v5.0 (ASIN Software Inc.) program.

Sample Preparation

Standardized dry plant material (taken from the pharmacognostic garden of the Medical University of Lublin) was ground in a laboratory grinder with corundum elements. After that procedure the material was standardized again. The optimized procedure of mineralization was as follows: a 1.0 g amount of powdered sample was placed in a closed polytetrafluoroethylene (PTFE) beaker and wetted with a small amount of water. Then, 7 mL of deionized water and 3 mL of concentrated (65%) nitric acid (Merck KGaA, Darmstadt, Germany) were added. A microwave-assisted high pressure digestion system (UniClever BM-1, Plazmotronika, Poznań, Poland) was applied. The digestion program consisted of the following four high-pressure stages:

(i) 17-20 atm for 3 min (60% power of microwave generator)
(ii) 27-30 atm for 5 min (80% power of microwave generator)
(iii) 42-45 atm for 8 min (100% power of microwave generator)
(iv) a cooling process for 10 min.

After removal of nitrogen oxides and evaporation of the sample to 1 mL, the residue was dissolved to 10 mL with deionized water.

Solutions obtained after mineralization were ten-fold diluted with deionized water and analyzed using an ion chromatograph.

Chromatographic Analysis

Chromatographic analyses were performed on a Dionex DX-500 ion chromatograph (Dionex, Sunnyvale,

Table 1. Operating conditions.

Chromatograph	DX-500 ion chromatograph (Dionex Co., Sunnyvale, CA, USA)- IP25 isocratic pump, AD20 (UV/Vis) detector, along with a Dionex PeakNet chromatography workstation for instrument control and data acquisition.
Metal ions	Fe^{3+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}
Stationary phase	Ion –exchange column IonPac CS5A (Dionex Co., Sunnyvale, USA); 2 x 250 mm. Guard column IonPac CG 5A (Dionex Co., Sunnyvale, USA); 2 x100 mm.
Composition of the eluent concentrate*	7.0 mM PDCA (pyridine-2.6dicarboxylic acid) 66 mM Potassium hydroxide 5.6 mM Potassium sulphate 74 mM Formic acid (pH= 4.2 ± 0.1)
Composition of post-column reagent	0.5 mM PAR (4-(2-pyridylazo)resorcinol) 1.0 M 2-dimethylaminoethanol 0.50 M ammonium hydroxide 0.3 M sodium bicarbonate (pH= 10.4 ± 0.2)
Detection	absorbance, λ=530 nm
Eluent flow rate	0.3 mL·min^{-1}
Post column reagent flow rate	0.15 mL·min^{-1}
Sample loop	25 µL
Temperature	29 ± 1°C
Operating pressure	1900 psi

* This concentrate simplifies eluent preparation and improves reproducibility. It was 5 times diluted with deionized water and used as the mobile phase during chromatographic analysis.

CA, USA) equipped with an IP 25 isocratic pump, an IonPac CG 5A guard column, an IonPac CS 5A analytical column (250x4.6 mm I.D., 9 µm bead diameter ethylvinylbenzene functionalized with both quaternary ammonium and sulfonate functional groups), a 25 µL injection loop and a Dionex absorbance detector with PC 10 Pneumatic Controller post-column reactor. Before injection, the column was equilibrated by applying PDCA eluent.

All samples were injected at least in triplicate. All measurements were made at 29 ± 1°C.

The eluent flow-rate was set at 0.3 mL/min; the flow-rate of post-column reagent was 0.15 mL/min. A Dionex PeakNet chromatography workstation was used for instrument control and data acquisition.

Results

Fig. 1 presents the blank chromatogram, ion chromatogram of heavy metals in synthetic standard solution, and chromatogram of heavy metal ions occurring in the aerial part of hyssop analyzed using IonPac CS5A column and PDCA eluent under the optimized conditions (listed in Table 1).

To test the type of distribution of the values of the data obtained, the values of the correlation coefficient R^2 (between standard deviation and concentration) were calculated (given in Table 2). All values of R^2 indicate a strongly heteroscedastic character of the distribution of the values. Therefore, weighted regression methods should be applied. The weights were determined by the method described in literature [17].

A frequently used measure of goodness of fit of the curve to experimental points is the determination coefficient (R^2). Its value always increases with the number of equation parameters. Therefore, another measure of goodness of fit was introduced, the F-statistics, which takes into account the number of these parameters. It is defined as the ratio of the determination coefficient to the indetermination coefficient multiplied by the ratio of the number of degrees of freedom to the number of parameters of the equation (the formula is given under Table 3).

For all cations investigated four correlation equations were tested on the basis of F-statistics: two linear of the $y = ax+b$ and $y = bx$ types and two nonlinear ones, i.e. $y = a+bx+cx^2$ and $y = bx+cx^2$, choosing those for which

Table 2. Retention times and squares of correlation coefficients of standard deviation vs concentration.

Metal ion	t_R (± 1 SD)	R^2
Fe^{3+}	4.83 (0.07)	0.7020
Cu^{2+}	6.63 (0.08)	0.7183
Ni^{2+}	7.45 (0.09)	0.9620
Zn^{2+}	8.39 (0.09)	0.9741
Co^{2+}	9.48 (0.10)	0.4843
Cd^{2+}	10.31 (0.09)	0.2537
Mn^{2+}	10.60 (0.15)	0.9097

* t_R, retention time [min]; SD, standard deviation; R- correlation coefficient (between standard deviation and concentration).

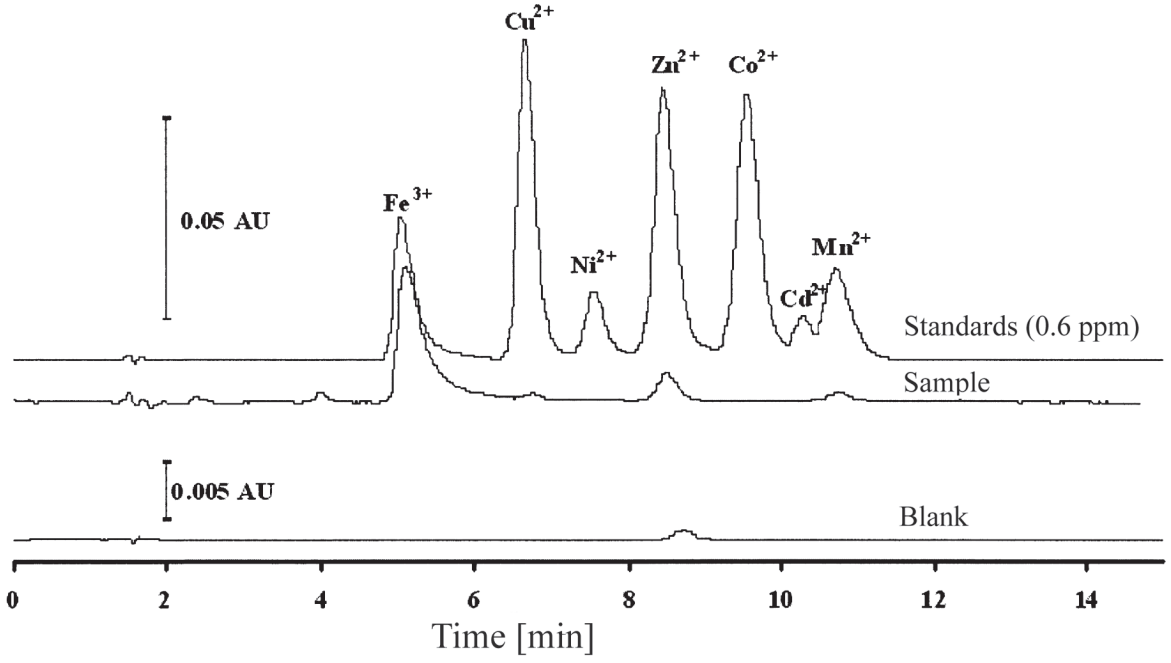

Fig 1. Separation of heavy metal ions by High Performance Ion Chromatography with spectrophotometric detection (530 nm) after post-column derivatization reaction with PAR. All operating conditions are described in Table 1.

Table 3. F-values for four tested equations.

	F*-value						
Equation	Fe^{3+}	Cu^{2+}	Ni^{2+}	Zn^{2+}	Co^{2+}	Cd^{2+}	Mn^{2+}
y=bx	5680	622	360	370	13632	3791	3859
y=a+bx	7523	813	448	857	15458	3824	3757
$y=a+bx+cx^2$	5618	555	511	937	12267	9981	2755
$y=bx+cx^2$	6012	600	779	622	21972	19845	4074

* $F = \frac{R^2}{1-R^2} \cdot \frac{n-k-1}{k}$, where *n*- total numer of data values, and *k*- number of coefficients in the model.

Table 4. The values of parameters (with chosen statistical data) of some equations of calibration lines for individual cations; LOD and LOQ values.

Parameter	Value of parameter W	Standard deviation s	t-value *t=W/s* t	95% confidence limit		P*>t	LOD** [ppm]	LOQ*** [ppm]
Fe R^2 = 0.9976 F =7523								
a	-0.000159	0.000060	-2.67	-0.000285	-0.000034	0.0155	0.009	0.030
b	0.02587	0.00030	86.74	0.02525	0.02650	0		
Cu R^2 = 0.9783 F =813								
a	-0.000235	0.000091	-2.57	-0.000427	-0.000043	0.0191	0.048	0.16
b	0.02420	0.00085	28.52	0.02242	0.02598	0		
Ni R^2 = 0.9774 F =779								
b	0.00733	0.00033	22.17	0.00664	0.00803	0	0.006	0.020
c	-0.000337	0.000072	-4.69	-0.000487	-0.000186	0.00018		
Zn R^2 = 0.9910 F =937								
a	0.000559	0.000093	6.01	0.000363	0.000755	0.00001	0.056	0.19
b	0.02761	0.00083	33.43	0.02587	0.02935	0		
c	-0.00084	0.00018	-4.68	-0.00122	-0.00046	0.00022		
Co R^2 =0.9992 F = 21972								
b	0.03204	0.00058	55.58	0.03083	0.03325	0	0.026	0.087
c	-0.000303	0.000085	-3.55	-0.000482	-0.000124	0.0023		
Cd R^2 = 0.9991 F = 18845								
b	0.004812	0.000076	62.93	0.004651	0.004973	0	0.022	0.073
c	-0.000106	0.000012	-9.00	-0.000131	-0.000081	0		
Mn R^2 = 0.9951 F = 4074								
b	0.01362	0.00018	76.32	0.01325	0.01400	0	0.006	0.020
c	-0.000071	0.000049	-1.43	-0.000174	0.000033	0.1696		

*- P – probability of insignificance of parameter
**- LOD –limit of detection
***- LOQ- limit of quantitation; LOD = 10/3·LOD

Table 5. Ion chromatography results obtained during the analysis of heavy metals in aerial parts of Hyssop.

Heavy metal	Concentration (± 1 SD*) (mg/L)
Fe^{3+}	5.73 (0.3)
Cu^{2+}	0.340 (0.006)
Ni^{2+}	n.d.**
Zn^{2+}	0.58 (0.02)
Co^{2+}	n.d.
Cd^{2+}	n.d.
Mn^{2+}	0.640 (0.007)

* Standard deviations values related to the triplicate analyses of the sample

** Not detectable (i.e. the concentration is less than the limit-of-detection for that ion)

the value of F-statistics was maximal (underlined values in Table 3).

LOD values (given in Table 4) were estimated by Hubaux-Vos' method (described in detail in [23]) from weighted regression from data up to 0.1 ppm concentration (with 95% confidence limit). The idea of the method permits the determination of the LOD value after earlier assumed risks of errors of the first and second type. Moreover, it can be applied both for linear and nonlinear regressions, and, most important, takes into account the heteroscedasticy of the system (standard deviation of the analytical signal at concentration corresponding to LOD may be different from that of the analytical signal for the blank sample (heteroscedasticy of the system).

Discussion of Results

In all types of chemical analysis which a require sample preparation step it is important to remember about possible sources of contamination of the sample. Applying a closed microwave-assisted high pressure digestion system it is possible to avoid contamination from the laboratory air. Although we used only two reagents for digestion of the plant sample, i.e. nitric acid and deionized water (where other sources of contamination were minimized), the relatively high content of nitric acid in the solution after mineralization could be a problem, and could cause some instability of the baseline. Therefore, the mineralized solution of the sample must be ten times diluted before chromatographic analysis. It is proper to notice that the mass of the plant sample taken for mineralization should be compatible with the used digestion procedure.

In order to check the presence of heavy metal ions in plant sample using HPIC technique and IonPac CS5A column, the complexing eluent (PDCA) was applied. Since the column has both anion- and cation-exchange capacity, metals can be separated by cation- and anion-exchange. When PDCA is used as the eluent, it forms stable anionic complexes with heavy and transition metals. The selectivity of the separation is due to the different degrees of association between the metals and the chelating agent (PDCA), which produces different net charges on the metal complexes [23]. Using PDCA eluent it is possible to separate and determine Fe^{3+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, and Ni^{2+} in one single chromatographic run. However, because of the drastically oxidative environment applied during described digestion procedure it was not possible to detect the presence of iron (II).

The initial chromatographic conditions (except temperature and injection volume) used with this bifunctional mixed-bed column (Table 1) are directly taken from technical literature provided by the column manufacturer.

Analysis time did not exceed 15 min.

The high values of the correlation coefficient R^2 (Table 2) indicate a strongly heteroscedastic character of the distribution of data. Therefore, the method of weight regression was applied; the weights were determined by the method described in literature [17].

It follows from the data in Table 3 that only for Fe^{3+} and Cu^{2+} ions was the best fit obtained for linear relationships. For the remaining ions the nonlinearity coefficient (c) was statistically significant. For Mn^{2+}, Cd^{2+}, Co^{2+}, and Ni^{2+} ions the intercept was statistically negligible. For Fe^{3+}, Cu^{2+}, and Zn^{2+} the (a) constants were statistically significant. For Fe^{3+} and Cu^{2+} its value was negative, which is senseless from the physicochemical point of view, while for Zn^{2+} it was positive due to trace amounts of zinc in the demineralized water (see Fig.1).

The LOD values (Table 4) etimated by Hubaux-Vos' method were in the range 0.006 -0.056 ppm. The presence of zinc in the demineralized water is the case of the highest LOD value for zinc (see Fig. 1).

In some recent papers [12, 14, 15] the authors indicate that the so called "zig-zag" pattern of the LODs is consistently observed for lanthanides and some metallic elements, according to which the elements with odd atomic numbers have lower LOD values than the even atomic number neighbor elements. After analysing the LOD data it was not observed that the LODs estimated in this chromatographic work have such a clear systematic "zigzag" pattern. However, authors who described such "odd-even effect" indicate that it is based on at least 30 replicates of the lowest concentration of the standard or the blank [14, 15].

It could be expected that the LOD values of the individual cations should be inversely proportional to the peak heights of the standard mixture (Fig.1). It is known that peak height depends on both the molar adsorption coefficient of a given ion with the derivatization reagent, (PAR) as well as on the retention and the spreading of the peak involved. In the present study no such relationship was observed. One of the causes of this discrepancy may

be due to the interpretation of LOD, since the relationship mentioned should occur when the LOD value corresponds to the instrument detection limit (IDL), but not necessary when it corresponds to the method detection limit (MDL). Since in the work described the standard deviations were determined on the basis of three independently prepared solutions (and not on three repeated measurements of a single solution), the LOD values determined correspond to the MDL values.

The ion chromatogram presented in Fig. 1 confirmed the presence of the following metal ions in hyssop: Fe^{3+}, Cu^{2+}, Zn^{2+}, and Mn^{2+}. The standard deviations (related to the triplicate analyses of the sample – Table 5) of the amounts of the individual metal ions in the hyssop sample do not differ statistically from the standard deviations following from the corresponding concentrations from the calibration lines. It means that the material investigated was well averaged and the sample size being analyzed was sufficient.

The present study confirmed that IonPac CS5A is a suitable column for determining heavy metals at low concentration levels. However, due to a long working use the efficiency of the column was decreasing continuously, and that would have an influence on the detection being obtained. Comparison of the number of theoretical plates, (for instance in zinc ions) a decrease of ca. 600 was observed relative to the number of theoretical plates declared by the producer for the column. Unfortunately, we have no data regarding the duration of the use of the column and the variation of its efficiency.

Conclusions

The content of heavy metal ions in the aerial part of hyssop (*Hyssopus officinalis L.*) estimated by use of ion chromatography technique with the aid of a weighted least-squares regression model is reported. The results obtained confirm the heteroscedastic character of the data distribution. The LOD values were in the range 0.006-0.056 ppm. The presence of zinc ions in the demineralized water is the cause of the highest LOD value for zinc ion in comparison to the remaining cations.

The chosen sample preparation method (use of only two inexpensive reagents, water as one component), i.e. microwave mineralization system has minimized the risk of contamination (through the closed system) and was not time-consuming. The low content of heavy metals, i.e.: 5.73 ppm of Fe^{3+}, 0.34 ppm of Cu^{2+}, 0.58 ppm of Zn^{2+}, and 0.64 ppm of Mn^{2+} enables the use of the investigated plant in medical treatment, in which all its medical properties would be developed.

It is important to estimate the effects of heavy metals on plants since they are also influenced by other elements. These interactions would include antagonism and synergism. The objective of further experiments will be the assessment of the effects of cultivation conditions of the plant on the content of heavy metals.

Abbreviations

HPIC – High Performance Ion Chromatography
PDCA – pyridine-2.6 dicarboxylic acid
PAR – 4-(2-pyridylazo) resorcinol
SD – standard deviation
LOD – limit of detection
IDL – instrument detection limit
MDL – method detection limit
LOQ – limit of quantitation
OLS – ordinary least-squares regression model
WLS – weighted least-squares regression model

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