Assessment of active ingredients and metal impurities in phytoestrogen-containing food and dietary supplements

TAJANA BUHAČ – DANIELA AMIDŽIĆ KLARIĆ – ILIJA KLARIĆ – BILJANA NIGOVIĆ – EDVIN BRUSAČ – MARIO-LIVIO JELIČIĆ – ANA MORNAR

Summary

The success of the food and dietary supplement industry in bringing to market safe and high-quality phytoestrogen-containing products is important for the well-being of an increasing number of post-menopausal women consuming these products. On the other hand, a serious safety issue associated with food and herbal supplements is heavy metal contamination. Direct-injection mass spectrometric (DI-MS) technology has evolved as a powerful tool for screening of active ingredients in food, herbal and dietary supplement samples. In this work the developed DI-MS method provided simple and fast multi-target screening of phytoestrogens in food, raw materials and dietary supplement products without prior analyte separation by chromatography. Phytoestrogen marker compounds were identified in two of four herbal raw material samples and eight of twenty dietary supplement products. As for the heavy metal content determination, graphite furnace–atomic absorption spectroscopy (GF-AAS) was utilized. The average daily intake of heavy metals is well below the recommended tolerable daily intakes if the products are used according to the manufacturer's instructions, with the exception of one sample.

Keywords

phytoestrogen; metal impurity; functional food; herbal raw material; dietary supplement; regulation

Phytoestrogens are a diverse group of plant-derived compounds that mimic mammalian estrogens and therefore show potential benefits for human health. Depending on their chemical structure, phytoestrogens can be divided into flavonoid and non-flavonoid sub-groups. The four major phytoestrogen classes are isoflavonoids, stilbenes, coumestans and lignans. Isoflavones are the main representatives of the flavonoid phytoestrogens, while lignans and coumestans are main non-flavonoid phytoestrogens. These polyphenolic compounds have been extensively studied for a long time regarding protective effects against several complex diseases and reduction of post-menopausal symptoms.

Phytoestrogens are compounds found in a wide variety of plants such as soya, flax, hop, red clover and chaste tree. The most common sources of human exposure to phytoestrogens are foodstuffs, most notably soya and flax, and numerous dietary supplements, widely marketed as a natural alternative estrogen-replacement therapy [1].

Soya is an integral part of the traditional Asian diet with the daily intake of soya protein estimated at 20–30 g (containing 15–50 mg isoflavone phytoestrogens), while non-Asian diet contains less than 1 g of soya protein per day [2]. Recently, flaxseed has emerged as an attractive nutritional food because of its exceptionally high content of phytoestrogens. The content of lignan phytoestrogens is 5–15 $g \cdot kg^{-1}$ of whole flaxseed [3].

As consumption of dietary supplements is widely spread and on the rise, the nutritional and bioactive properties of phytoestrogen dietary supplements prepared from various sources are being intensively studied. The use of phytoestrogen-containing formulated products is extensive and noticeably increasing as botanical monoprepa-

Tajana Buhač, Daniela Amidžić Klarić, Biljana Nigović, Edvin Brusač, Mario-Livio Jeličić, Ana Mornar, Department of Pharmaceutical Analysis, Faculty of Pharmacy and Biochemistry, University of Zagreb, Ante Kovačića 1, 10000 Zagreb, Croatia.
Ilija Klarić, Department of Health Ecology, Public Health Brčko DC, R. Dž. Čauševića 1, 76000 Brčko DC, Bosnia and Herzegovina.

Correspondence author:
Ana Mornar, e-mail: amornar@pharma.hr

rations, single-constituent botanical products and multibotanical dietary supplements, due to synergistic efficacy, for relief of symptoms related to menopause. To produce beneficial and safe products, manufacturers of dietary supplements must consider several factors, including identity of the product's active ingredients and contaminant control. In terms of identity, many plants can be used to make herbal dietary supplements and even plants that look similar and are closely related may have very different effects. Several techniques for determination of active ingredients in the abovementioned samples have been already used [4]. Liquid chromatography coupled to a diode array detector and/or a mass spectrometric detector (LC/DAD/MS) has been widely used for analysis of these complex samples. This technique proved to be an effective and reliable approach for determination of even trace amounts of active ingredients [5–9]. However, it is questionable whether LC/DAD/MS is the simplest and fastest technique for routine analysis.

Recently, direct-injection mass spectrometric (DI-MS) technology has evolved as a powerful tool for multi-target screening of active ingredients in herbal and dietary supplement samples [10, 11]. The main strengths of the technique include the capability of supplying reliable results for a wide range of analytes in a short time, suitability for routine high-throughput analysis, as well as economic and environmental acceptability. From a toxicological point of view, food and herbal dietary supplements are a significant potential source of contamination with heavy metals, such as cadmium (Cd), arsenic (As), lead (Pb) and mercury (Hg). The contamination levels are related to contamination of agricultural soils, irrigation waters, atmosphere and crop inputs. Furthermore, processing equipment, excipients and product containers may also contaminate the products with various metals.

The extensive use of phytoestrogen-containing food and dietary supplements highlights the need for appropriate research and for data to support quality and safety assessment of these products. Protocols for evaluation of phytoestrogen-containing food and dietary supplements must consider many different factors, including the expected

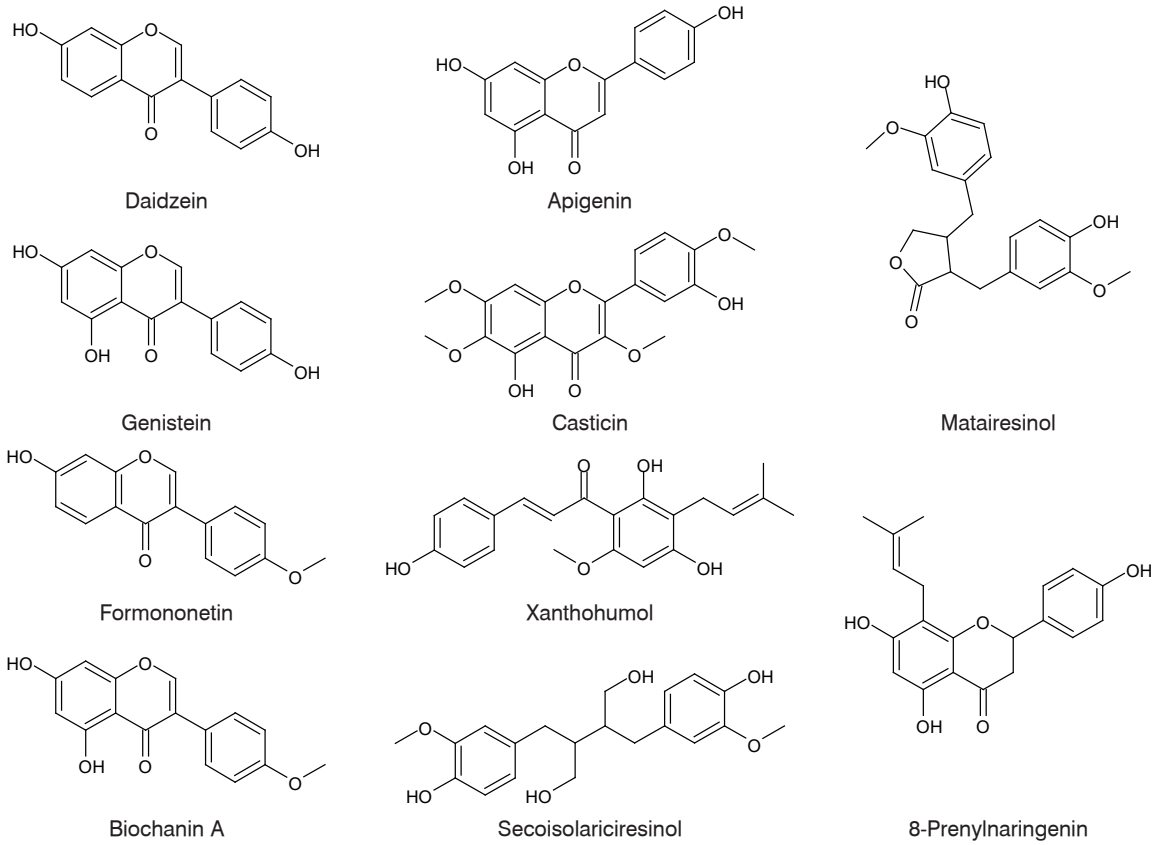

Fig. 1. Chemical structures of the studied phytoestrogens.

content of the analyte in the sample, analysis time, availability of equipment, environmental friendliness and cost of analysis.

For all the reasons above, this study aimed to identify ten marker compounds (Fig. 1) in various phytoestrogen-containing food, herbal raw material and dietary supplements as formulated products using DI-MS technique. From the regulatory point of view, another goal of this study was to evaluate heavy metal content by graphite furnace atomic absorption spectrometry (GFAAS) technique and to compare results to maximum allowed values.

MATERIALS AND METHODS

Chemicals and reagents

Phytoestrogen standards of biochanin A, daidzein, formononetin, genistein, matairesinol, 8-prenylnaringenin and secoisolariciresinol were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Apigenin, casticin and xanthohumol were obtained from Extrasynthese (Genay, France). Electrospray tuning standard solution mixture for calibration of mass spectrometer (MS) was obtained from Agilent Technologies (Santa Clara, California, USA). GFAAS mixed standard and the matrix modifiers were obtained from Perkin Elmer (Waltham, Massachusetts, USA). Nitric acid (TraceSelect Ultra, for ultratrace analysis, 65–71 %) and hydrogen peroxide solution (≥ 30%, TraceSelect Ultra, for ultratrace analysis), used as a reagent and cleaning agent, were obtained from Fluka (Buchs, Switzerland). Acetonitrile, ethanol and methanol (all HPLC grade) were from Merck (Darmstadt, Germany). Hydrochloric, sulphuric and boric acids were obtained from Kemika (Zagreb, Croatia). Kjeltabs Cu/3.5 (3.5 g K_2SO_4 and 0.4 g $CuSO_4 \cdot 5H_2O$), which was used as a catalyst, was obtained from Foss Tecator (Hillerød, Denmark). Ammonium sulphate, sodium hydroxide, bromocresol green and methyl red, as indicators, acetone and petroleum ether (boiling temperature 40–70 °C) were obtained from Kemika. All other reagents used in this work were of analytical reagent grade or better. Ultrapure water, prepared with a Milli-Q water purification system (Millipore, Billerica, Massachusetts, USA) with a resistivity of 18.2 MΩ cm (25 °C), was used in all experiments.

Samples

Four commercially available food samples of soybeans and flax (marked as F1–F4) from three different brands were obtained from a local "health food" store in Zagreb, Croatia. Five samples of herbal raw material (marked as RM1–RM5) were kindly donated by Specchiasol (Verona, Italy), while 20 phytoestrogen-containing formulated dietary supplement products (marked as DS1–DS20) manufactured by 15 companies from USA and European Union were obtained from a public pharmacy in Zagreb, Croatia. The goal of the sampling plan was to provide representative samples available and widely used in Croatia. Fifteen formulated dietary supplements were classified as botanical monopreparations, as they contained one of the investigated herbal extracts: soya (DS1–DS4), chasteberry (DS5–DS9), red clover (DS10 and DS11), hop (DS12–DS14) and flax (DS15). Five samples were multibotanical dietary supplements containing two or more bioactive ingredients: soya and chasteberry (DS16 and DS17), soya and red clover (DS18), soya and flax (DS19), and red clover and hop (DS20). The dietary supplements analysed in this work were in multiple dosage form including liquid extracts (2 samples), tablets (5 samples) and capsules (13 samples). Liquid products contained only ethanolic herbal extract while other products contained additional ingredients, such as vitamins and minerals. List of the products, together with manufacturers and descriptions, was presented previously [12].

Ten tablets were weighed and the average weight of one tablet was determined. Afterwards, all tablets were finely ground and used in further investigations. Likewise, the content of 10 capsules was pooled and the average weight of one capsule was calculated.

Analysis of phytoestrogen compounds

Preparation of stock and working solutions

Optimization of DI-MS method was performed using standard solutions of each phytoestrogen in acetonitrile (0.1 $mg \cdot ml^{-1}$).

Extraction procedures

Approximately 1 g of each homogenized sample, accurately weighed, was transferred to a 15-ml centrifuge tube. The samples were extracted with 10.0 ml of ethanol during 15 min at room temperature using an ultrasonic bath. Finally, the solution was decanted by centrifugation at 3000 $\times g$ for 10 min at 25 °C using Z326K centrifuge (Hermle, Wehingen, Germany) and the supernatant was filtered through a Chromafil membrane filter (0.45 μm pore size; Carl Roth, Karlsruhe, Germany). Two samples of commercial dietary supplements that were in the form of

ethanolic tinctures were not subjected to the extraction procedures. The extraction procedure of food samples was carried out after removal of the lipid fraction from soya and flax.

Identification of phytoestrogens

The analytical system consisted of a mass selective detector (MSD) ion trap mass spectrometer (Agilent Technologies) with an electrospray ionization (ESI) probe. An external syringe pump (KD Scientific, Holliston, Massachusetts, USA) was utilized to transport the sample and wash the transfer line between injections. To evaluate the performance of DI-MS in this configuration, electrospray tuning standard solution mixture was injected into the instrument each day prior to analysis. Optimization of DI-MS method was performed by infusing standard solutions of phytoestrogen at a rate of 5 ml·min^{-1}. At the same flow rate, sample solutions were injected into the system. Carryover was analysed in each sample's spectra, none being observed using the washing solvent (mixture of acetonitrile and water, 80 : 20 v/v) in a volume of 1 ml. The ESI source was operated in positive ionization mode. Optimum source parameters were source temperature 325 °C, ion spray voltage 3.5 kV, nitrogen flow rate 10 l·min^{-1} and pressure 138 kPa. Continuous mass spectra were obtained by scanning from 100 to 500 *m/z*. A number of 10 000 ions was trapped in the analyser and the accumulation time was set at 200 ms. High-purity helium as collision gas was used for fragmentation and tandem mass spectrometry (MS^n) studies of standard solutions were carried out at the collision energy kept at 30 %. Tab. 1 shows the mass spectrometry parameters for multiple reaction monitoring (MRM) transitions. Acquisition of sample solutions was performed in MRM mode, and MSD Trap Control v.5.2 (Agilent Technologies) was used for data acquisition and processing.

Tab. 1. Mass spectrometry parameters for multiple reaction monitoring transitions.

Analyte	Transition	Fragment ions
Apigenin	271→225	153, 225, 243
Biochanin A	285→270	153, 229, 253, 270
Casticin	375→360	315, 345, 360
Daidzein	255→227	137, 181, 199, 227, 237
Formononetin	269→254	137, 213, 237, 254
Genistein	271→243	153, 197, 215, 243, 253
Matairesinol	359→341	137, 341
Secoisolariciresinol	345→327	137, 327
Xanthohumol	355→299	179, 235, 299
8-Prenylnaringenin	341→285	165, 221, 285

Determination of heavy metals

Microwave digestion

Approximately 0.2–0.6 g of each sample was weighed into polytetrafluoroethylene (PTFE) vessels and dissolved in 9.0 ml of nitric acid (65–71% HNO_3, TraceSelect, Fluka) and 1.0 ml of hydrogen peroxide solution (30% H_2O_2, TraceSelect, Fluka). Digestion was carried out in a microwave oven (Ethos UP systems, Milestone; Sorisole, Italy) and the operating program included two steps. In the first step, the temperature was increased to 200 °C during 15 min (power 1800 W), while in the next step the temperature was held constant at 200 °C (15 min, 1800 W). Three replicated digestions were conducted for each investigated sample and three blanks were prepared in an identical way without the sample. Furthermore, the standard solutions of each metal were digested three times by the above described procedure.

GFAAS analysis

The contents of heavy metals (Ag, As, Ba, Cd, Co, Cr, Cu, Mo, Ni, Pb, and Sn) in samples were determined by GFAAS using Perkin-Elmer AAnalyst 100 atomic absorption spectrometer equipped with HGA-800 graphite furnace and a deuterium background corrector (Perkin Elmer). Light sources for atomic absorption were Perkin Elmer single element hollow cathode lamps and the slit width was 0.7 nm for all measurements. Argon was used as an inert gas and the peak area signals were recorded. The determination of metals was done after microwave digestion procedures, and the sample volume was 20.0 μl. The main analytical parameters for the determination of metals by GFAAS are summarized in Tab. 2.

Mercury content

The content of Hg in the studied samples was analysed by using AMA-254 with HS cuvette (Altec, Dvůr Králové nad Labem, Czech Republic) under the following conditions: wavelength 253.6 nm, drying time 60 s, decomposition time 150 s, and cuvette clear time 45 s. Briefly, each sample (100 ± 3 mg) was weighed into a vessel, then placed in a furnace and thermally decomposed. The capability of the proposed method to be used in a routine laboratory was evaluated by the detection limit determination for individual metals. The limit of detection (*LOD*) and limit of quantitation (*LOQ*) were determined from the series of 10 blank analytical repetitions and were

calculated based on 3 and 10 standard deviations (*SD*) divided by the slope, respectively.

Statistical methods

All determinations were conducted in triplicate. The variables with normal distribution were described by the arithmetic mean and standard deviation, and those not showing normal distribution were represented by median and interquartile range. The Pearson product-moment correlation coefficient and Spearman rank correlation coefficient were determined to examine potential relationship between the contents of different compounds. *P* of 0.05 was a limit of statistical significance and *P* of 0.01 was a limit of high statistical significance. For each metal, incidence was expressed as percentage of samples in which the metal was found. The statistical package Statistica ver. 12.1 from StatSoft (Tulsa, Oklahoma, USA) was used for data analysis.

RESULTS AND DISCUSSION

Identification of phytoestrogens

The phytoestrogen-containing food, herbal raw materials and dietary supplements were analysed for content of ten phytoestrogens belonging to different classes. DI-MS technique was used upon optimization of a multi-target method for identification of marker compounds in phytoestrogen rich food, raw materials and dietary supplements prepared from various plant raw materials, namely, soya, red clover, chastetree, hop and flax. Selection of marker compounds was based on their distinctive role in quality control of these products as well as claims given on labels of products [12]. Therefore, the marker compounds with reported estrogenic activity were selected as follows: daidzein, genistein, formononetin, biochanin A (isoflavones), 8-prenylnaringenin, xanthohumol, (prenylflavonoids), apigenin (flavon), casticin (flavanol), matairesinol and secoisolariciresinol (lignans) (Fig. 1).

The external pump flow and the operating MS parameters of the ion source and trap were optimized to obtain the best performance of the mass spectrometer for identification of the selected ingredients. Parameters such as ionization mode, capillary voltage, nebulizer gas pressure, drying gas flow and temperature, ion accumulation time and number of ions trapped in the analyser were systematically evaluated until the highest signal intensity of all phytoestrogens was achieved. To select the most abundant parent and fragment ions as candidates for identification

Tab. 2. Analytical parameters for determination of metals by graphite furnace atomic absorption spectroscopy technique.

Metal	Wavelength [nm]	Current [mA]	Matrix modifier	Modifier volume [µl]	Calibration interval [µg·l^{-1}]	Calibration line	Correlation coefficient	*LOD** [µg·l^{-1}]	*LOD*** [µg·kg^{-1}]	*LOQ** [µg·l^{-1}]	*LOQ*** [µg·kg^{-1}]
Ag	328.1	10	0.015 mg Pd and 0.010 mg $Mg(NO_3)_2$	5	0–4	$y = 0.0319x + 0.0036$	0.9991	0.060	2.4	0.200	8.0
As	193.7	18	0.015 mg Pd and 0.010 mg $Mg(NO_3)_2$	5	0–20	$y = 0.0032x + 0.0005$	0.9995	0.750	30.0	2.500	100.0
Ba	553.6	30	0.005 mg Ca	5	0–40	$y = 0.0066x + 0.0010$	0.9984	0.600	24.0	2.000	80.0
Cd	228.8	8	0.015 mg Pd and 0.010 mg $Mg(NO_3)_2$	5	0.5–2.0	$y = 0.0612x + 0.0027$	0.9989	0.024	1.0	0.080	3.2
Co	240.7	15	0.05 mg $Mg(NO_3)_2$	5	0–20	$y = 0.0053x + 0.0020$	0.9993	0.300	12.0	1.000	40.0
Cr	357.9	25	0.05 mg $Mg(NO_3)_2$	5	0–20	$y = 0.0109x + 0.0017$	0.9997	0.300	12.0	1.000	40.0
Cu	324.8	15	–	–	0–20	$y = 0.0189x + 0.0030$	0.9998	0.300	12.0	1.000	40.0
Mo	313.3	35	–	–	0–20	$y = 0.0064x + 0.0001$	0.9996	0.300	12.0	1.000	40.0
Ni	232.0	40	0.05 mg $Mg(NO_3)_2$	5	0–40	$y = 0.0048x + 0.0043$	0.9994	0.600	24.0	2.000	80.0
Pb	283.3	12	0.20 mg $NH_4H_2PO_4$ and 0.010 mg $Mg(NO_3)_2$	5	0–40	$y = 0.0034x + 0.0038$	0.9985	0.600	24.0	2.000	80.0
Sn	286.3	20	0.015 mg Pd and 0.010 mg $Mg(NO_3)_2$	5	0–40	$y = 0.0012x + 0.0003$	0.9992	0.600	24.0	2.000	80.0

LOD – limit of detection, *LOQ* – limit of quantification, * – based on fortified method blanks (n = 20), ** – based on 0.250 g analytical portion (n = 20).

by MRM analysis of samples, the fragmentation pattern of each analyte was investigated.

All isoflavones (daidzein, genistein, formononetin and biochanin A) produced the most abundant ion corresponding to the protonated molecule $[M+H]^+$ at *m/z* of 255, 271, 269 and 285. The $[M+H]^+$ ions afterwards generated complex electrospray ionization tandem mass (ESI-MS/MS) spectra. Isoflavones are characterized by a common 3-phenyl-chromen-4-one core structure and differ by substituents such as methoxyl and hydroxyl. Analysis of tandem mass (MS^2) spectral data of daidzein and genistein revealed the presence of the prominent ions at *m/z* of 237 for daidzein and *m/z* of 253 for genistein. These ions were assigned to a fragment ion $[M+H-H_2O]^+$ obtained by loss of 18 Da. On the other hand, MS^2 fragmentation of *O*-methylated isoflavones resulted in formation of the most prominent, stable product ions at *m/z* of 254 (formononetin) and 270 (biochanin A) assigned to a radical ion $[M+H-CH_3]^{+\bullet}$. $[M+H]^+$ ions of all isoflavonoids can also exhibit neutral loss of one or two CO groups, proposed to occur from the C-ring, resulting in fragment ions *m/z* of 227 and 199 for daidzein, *m/z* of 243 and 215 for genistein, *m/z* of 213 for formononetin and *m/z* of 229 for biochanin A. The fragment at *m/z* of 181 for daidzein and *m/z* of 197 for genistein correspond to additional loss of H_2O (18 Da) and formation of $[M+H-2CO-H_2O]^+$ fragment ions, while notable fragment ions at *m/z* of 237 (formononetin) and *m/z* of 253 (biochanin A) present a unique pattern in the mass spectrum of *O*-methylated isoflavones obtained by loss of CH_3OH from the B-ring. Taking into account the formation of these notable fragment ions at *m/z* of 237 (formononetin) and *m/z* of 253 (biochanin A), it is possible to assume that the loss from the B-ring is favoured for *O*-methylated isoflavones. The retro Diels-Alder reaction (RDA) diagnostic ions were found in MS^2 spectra of all investigated isoflavonoids (*m/z* of 137 for daidzein, *m/z* of 153 for genistein, *m/z* of 137 for formononetin and *m/z* of 153 for biochanin A). Still, RDA fragments of both *O*-methylated isoflavones had low relative abundance (less than 3 %; Fig. 2A).

MS spectrum of apigenin produced the most abundant ion corresponding to the protonated molecule $[M+H]^+$ at *m/z* of 271, while analysis of MS^2 spectral data revealed the presence of only three fragment ions: *m/z* of 243 ($[M+H-CO]^+$), *m/z* of 225 ($[M+H-CO-H_2O]^+$ – base peak) and *m/z* of 153 ($[M+H-C_8H_8O]^+$ – RDA diagnostic ion). On the other side, methoxylated flavonoid casticin showed the progressive loss of methyl groups with the prominent ions at *m/z* of 360, 345 and 315. These typical fragmentation patterns of isoflavones and flavones are in accordance with data previously reported by Madeira et al. [13]. Lignans secoisolariciresinol and matairesinol showed a characteristic fragmentation pathway by progressive loss of 18 Da (H_2O) from the positive $[M+H]^+$ ions at *m/z* of 345 and 359, respectively. Both dimers of phenylpropane, linked by β-β bonds, that differ only by an additional five-membered oxolane ring present in the structure of metairesinol, gave the characteristic ion at *m/z* of 137 after cleavage of the central linkage.

In positive ion detection mode, prenylated chalconoid xanthohumol gave a protonated molecule ion at *m/z* of 355. The most prominent, stable product ion at *m/z* of 299 $[M+H-C_4H_8]^+$ was formed after loss of 2-methylpropene from the prenyl group [14]. Fragmentation via RDA reaction gave a product ion at *m/z* of 235, while the third fragment ion at *m/z* of 179 was obtained by additional loss of the prenyl substituent from A ring of RDA-fragmented ions. The MS^2 spectrum of the prenylated flavonoid, 8-prenylnaringenin, showed the presence of three major fragment ions at *m/z* of 285, 221 and 165. Similar fragmentation patterns were reported by Yilmazer et al. [15] and Prokudina et al. [16].

After method optimization, the most stable and prominent ions were designated for MRM transitions (Tab. 1) and the usefulness of the method was evaluated by analysis of samples of food, herbal raw materials and dietary supplements. Using this selective mode very little matrix interference was observed. All fragment ions selected for MRM transitions obtained by analysis of standard solutions had signal intensities higher than 6×10^3 ion counts. To evaluate the reproducibility of the method, three injections of each sample type were carried out. Intensities of all ions were within the acceptance limit of ±5 %. Furthermore, other less abundant fragment ions were also monitored to aid in proper identification of the selected phytoestrogens.

Finally, the method was successfully applied to identification of phytoestrogens in various samples. None of the phytoestrogens was found in food samples, while three out of five herbal raw materials contained detectable levels of phytoestrogens. Daidzein and genistein were found in RM1 (soya extract) and RM3 (red clover extract). As expected, RM3 was found also to be rich in *O*-methylated isoflavones formononetin and biochanin A.

The formulated dietary supplements (DS1–DS15) were classified as botanical monopreparation, while 5 consisted of two or more

herbal extracts (DS16–DS20). Four samples (DS1–DS4) were labelled as containing soya and this was confirmed by analysis that revealed contents of soya isoflavones daidzein and genistein. The fragmentation consistency of both isoflavones was found in all samples since the fragment ions given in Tab. 1 were noticed in all investigated samples. The signal intensities of MRM transition 255→227 were in a range from 9.7×10^2 ion counts (DS1) to 1.6×10^4 ion counts (DS4) and signal intensities of MRM transition 271→243 were in a range from 2.3×10^2 ion counts (DS3) to 6.0×10^3 ion counts (DS4), indicating that sample DS4 had the highest content of soya phytoestrogens. Furthermore, soya ingredients were expected to be found in three samples containing soya and chasteberry (DS17), red clover (DS18) and flax (DS19). Still only in DS17, daidzein (signal intensity of MRM transition 255→227 was 1.1×10^3 ion counts) and genistein (signal intensity of MRM transition 271→243 signal intensity was 3.2×10^2 ion counts) were found. Five monobotanical chasteberry-based products were investigated (DS5-DS9) and none of marker compounds, apigenin and casticin, was detected in those samples. Detectable levels of isoflavones formononetin (signal intensities of MRM transition 269→254 were higher than 6.0×10^3 ion counts) and biochanin A (signal intensities of MRM transition 285→270 were higher than 3.7×10^3 ion counts) were found in one red clover monobotanical (DS10) and one multibotanical dietary supplement (DS20). Analysis of MS2 spectral data revealed the presence of all fragment ions given in Tab. 1 for red clover marker compound formononetin. Its MS^2 spectrum in sample DS10 is presented in Fig. 2B. Detectable levels of both prenyl flavonoids (8-prenylnaringenin and xanthohumol) were not found in any hop-based dietary supplements (DS12, DS13, DS14 and DS20).

Our results showed that detectable levels of lignans, flax marker compounds, were not found in food or herbal raw materials. Still, a detectable level of secoisolariciresinol (signal intensity of MRM transition 345→327 was 3.0×10^2 ion counts) was found in one flax-based dietary supplement (DS17) indicating that this lignan is the most abundant phytoestrogen in flax-based products. Unfortunately, analysis of MS^2 spectral data of secoisolariciresinol in sample DS17 revealed fragmentation inconsistency as one of fragment ions (*m*/*z* of 137) was not found. As expected, it was found that intensities of fragment ions had higher variabilities at lower contents in highly complex matrices, and the extent of variability depended on physico-chemical properties of the analytes [17].

The results obtained by DI-MS assay are comparable with our previously published LC-MS/MS data on phytoestrogen contents in phytoestrogen-containing samples from food to dietary supplements [12]. Application of the method demonstrated that multitarget screening DI-MS method is indeed a simple and fast technique particularly suited for routine analysis. It is well suited for selection of herbal raw materials appropriate for further formulation to dietary supplement products. On the other hand, it is not appropriate for identification of low levels of phytoestrogens, since contents of phytoestrogens lower than 100 $mg \cdot kg^{-1}$ were detectable only using LC-MS/MS technique. Due to the complexity of samples, especially multibotanical ones, ion suppression of low abundance ions may occur, as well as fragmentation inconsistency.

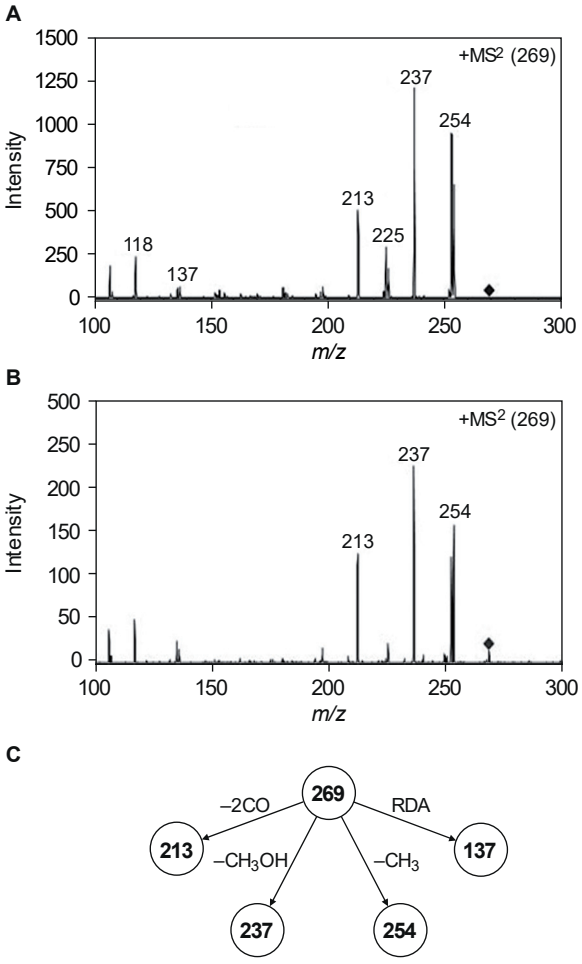

Fig. 2. Tandem mass spectral data of formononetin.

A – tandem mass spectrum in standard solution, B – tandem mass spectrum in sample DS10, C – fragmentation pathway. RDA – retro Diels-Alder reaction.

Tab. 3. Heavy metal content in oral phytoestrogen-containing food, herbal raw materials and dietary supplement samples.

Metal	Incidence [%]	Number of quantified samples	Mean values [mg·kg^{-1}]	Median [mg·kg^{-1}]	Range of quantified values [mg·kg^{-1}]	Interquartile [mg·kg^{-1}]	*RSD** [%]	Amount of metal per RDs [μg]		Batch-to-batch variability *RSD*** [%]
								Food samples (n = 4)	Dietary supplements (n = 20)	
Ag	0	0			< *LOQ*		–	–	–	–
As	0	0			< *LOQ*		–	–	–	–
Ba	100	29	17.208	6.256	0.563–89.655	3.400–9.840	0.3–9.2	156.28–770.71	6.26–231.72	0.2–19.4
Cd	24	7	0.052	0.021	0.008–0.257	0.009–0.064	0.3–5.8	0.34–8.30	0.02–1.23	0.7–9.2
Co	100	29	0.555	0.243	0.041–3.747	0.129–0.570	0.1–26.5	48.24–124.79	0.12–9.36	0.1–39.2
Cr	100	29	4.878	3.337	0.521–16.940	1.233–7.893	0.2–4.5	315.59–739.90	2.47–57.21	0.1–12.0
Cu	100	29	11.950	4.110	0.440–95.151	1.707–10.901	0.1–3.6	388.56–1503.55	1.76–381.19	0.2–13.6
Hg	100	29	4.01[a]	3.51[a]	0.16–15.81[a]	1.00–5.49[a]	0.1–7.9	0.25–0.66	< 0.070	0.3–67.1
Mo	100	29	0.971	0.504	0.080–7.825	0.135–1.289	0.3–7.6	36.58–90.95	0.27–31.30	0.6–115.0
Ni	100	29	1.689	1.536	0.425–4.854	1.004–2.223	0.3–4.9	132.40–436.90	1.09–35.46	0.2–10.4
Pb	41	12	0.568	0.502	0.170–1.445	0.433–0.650	0.2–3.3	–	0.32–7.43	0.9–43.5
Sn	0	0			< *LOQ*		–	–	–	–

RSD – relative standard deviation range, RDs – recommended daily servings, * – obtained by analysis of each sample in triplicate, ** – batch-to-batch variability is given for dietary supplement products, a – value expressed in micrograms per kilogram.

Mineral and heavy metal contents

The present study was also designed to evaluate metal contaminants in the samples. The metal content found in all food samples were compared to the maximum levels (*ML*s) established by European Food Safety Authority (EFSA) [18–23] and World Health Organization (WHO) for these elements [24]. Since this kind of dietary supplement formulation is used continuously for an extended time and *ML*s of several analysed metals in dietary supplements are not defined, International Conference on Harmonisation Q3D (ICH Q3D) guidelines were used in the assessment and compared to permissible daily exposure (*PDE*) [25]. According to this guideline [25], metal contaminants in drug products are classified into four classes and 12 analysed metals were evaluated as follows: class 1 (As, Cd, Hg, and Pb), class 2A (Co and Ni), class 2B (Ag) and class 3 (Ba, Cr, Cu, Mo, and Sn).

From the results presented in Tab. 3, it can be observed that contents of seven metals (Ba, Co, Cr, Cu, Hg, Mo, and Ni) were determined in all analysed samples. On the other hand, Ag, As, and Sn were not detected using the graphite technique in any of the samples. It must be pointed out that the *LOQ* value for these metals was significantly below their *ML*s and *PDE*, which indicates satisfactory sensitivity of the GFAAS method.

Arsenic, cadmium, lead, and mercury (class 1) are naturally occurring elements and severe environmental contaminants. These heavy metals are considered systemic toxicants, even in small doses of exposure, inducing multiple organ injury and failure. Arsenic, as a ubiquitous metalloid, can be found in the inorganic and organic form in nature. It is considered that organic forms are relatively non-toxic, except for those which are synthesized and developed as pesticide ingredients. Inorganic As (III) is more toxic than As (V) form, although both forms of this heavy metal are potentially harmful to human health. Literature survey reveals that human exposure to arsenic can occur via different routes, while food and drinking water intake are the principal routes of exposure to arsenic [18].

The lead content in 12 samples ranged from 0.170 mg·kg^{-1} to 1.445 mg·kg^{-1}, while

it was below *LOQ* in the remaining 17 samples. After intake of contaminated food and dietary supplements, lead is rapidly absorbed from the gastrointestinal tract and has adverse effects on the blood, cardiovascular, central nervous, immune, muscular, renal and reproductive systems [19, 26]. Consequently, the prescribed limit values for Pb are extremely low, maximum permitted concentration (*MPC*) for all solid food is 6 $mg \cdot kg^{-1}$, *PDE* is 5 $\mu g \cdot d^{-1}$ [25, 27]. The obtained results indicate that a high total amount of this heavy metal per recommended daily serving (7.43 $\mu g \cdot d^{-1}$) was found in the capsule sample DS15.

Regarding the cadmium content, it was determined in only 7 samples in a narrow range (0.009–0.257 $mg \cdot kg^{-1}$). This heavy metal is an environmental contaminant, both through natural occurrence and from industrial and agricultural sources. As shown in Tab. 3, the median and mean values of all samples indicated low cadmium contents in all samples. Thus, Cd content of soya food samples (F1 and F2) was below the defined cadmium *ML*s for soybeans (0.2 $mg \cdot kg^{-1}$ wet weight) [20]. It was observed that the flax sample (F3) had the highest content of this element (0.257 $mg \cdot kg^{-1}$). The cadmium *ML*s are 1.0 $mg \cdot kg^{-1}$ for dietary supplements [20] and Cd content for all the investigated samples was below this value. The maximum value was found in a multibotanical dietary supplement tablet formulation DS17 (0.245 $mg \cdot kg^{-1}$). Total amount of cadmium per recommended daily serving for investigated food samples (< 8.30 μg) and dietary supplements (< 1.23 μg) was lower than the prescribed limit values for this heavy metal (*PDE* 5 $\mu g \cdot d^{-1}$; tolerable weekly intake (*TWI*) 2.5 $\mu g \cdot kg^{-1}$ body weight (bw)).

Mercury is a toxic heavy metal (*MPC* for all food in solid form 500 $\mu g \cdot d^{-1}$; *PDE* 30 $\mu g \cdot d^{-1}$, limit for total daily consumption 16 $\mu g \cdot d^{-1}$) with no known biological function in humans [21, 25, 27]. The organometallic form of this metal is more toxic than the inorganic form since it is more readily absorbed after ingestion. Besides this, the toxicity of mercury is also due to its accumulation in biological tissues (bioaccumulation) and high exposure to this dangerous contaminant of the environment may cause neurological disorders, including seizures and even death. For the reasons stated above, mercury content was determined by the proposed method. It should be noted that sensitivity is one of the advantages of this method, as evidenced by the extremely low values of *LOD* (18 $ng \cdot kg^{-1}$) and *LOQ* (54 $ng \cdot kg^{-1}$). The determined total mercury content in all samples covered a very narrow range between 0.16 $\mu g \cdot kg^{-1}$ and 15.81 $\mu g \cdot kg^{-1}$. Furthermore, a statistically significant difference was not found between the content of Hg in food (5.88 $\mu g \cdot kg^{-1}$), herbal raw materials (3.95 $\mu g \cdot kg^{-1}$) and dietary supplement (3.67 $\mu g \cdot kg^{-1}$) samples.

Class 2 elements are generally considered as route-dependent human toxicants (Ag, Co and Ni). Cobalt is a mineral naturally occurring in various forms. The only physiological role of this trace element in humans is that of a component of vitamin B12. The average daily intake of this essential trace element from food is estimated to be 5–40 $\mu g \cdot d^{-1}$ [28]. It is interesting to note that the cobalt content in the studied soybean food samples (mean 1.296 $mg \cdot kg^{-1}$) was higher than in flax samples (mean 0.407 $mg \cdot kg^{-1}$). Furthermore, the cobalt content in dietary supplements was below 1 $mg \cdot kg^{-1}$ except for two samples (DS6; 3.747 $mg \cdot kg^{-1}$ and DS9; 2.301 $mg \cdot kg^{-1}$).

On the other hand, nickel has not been shown to be essential for humans. The current chronic dietary exposure to this metal is of concern for the general population. The tolerable daily intake (*TDI*) for Ni is 2.8 $\mu g \cdot kg^{-1}$ bw [22]. Soybeans and oilseeds were also mentioned as food groups containing the highest levels of nickel. The investigated soya samples had higher Ni levels (mean 4.082 $mg \cdot kg^{-1}$) than flax samples (mean 1.892 $mg \cdot kg^{-1}$). The obtained results were still below the values required by EFSA to be reported (5.2 $mg \cdot kg^{-1}$ for soybeans and 5.1 $mg \cdot kg^{-1}$ for soya products) [22]. The herbal raw material containing soya (RM1) had higher Ni content of 4.039 $mg \cdot kg^{-1}$ than the other samples. It was also found that high contents of this mineral had also soya-based dietary supplements DS2, DS16 and DS18.

The elements in class 3 (Ba, Cr, Cu, Mo and Sn) have relatively low toxicities at oral administration with high *PDE* values (generally 500 $\mu g \cdot d^{-1}$) [25]. Chromium, as a trivalent ion, is considered an essential trace element in humans present in various foods. On the other hand, hexavalent chromium, being carcinogenic to humans, is usually a consequence of anthropogenic contamination. Chromium dietary exposure across all age groups is well below *TDI* (0.3 $mg \cdot kg^{-1} \cdot d^{-1}$ bw) and, therefore, does not raise concerns for public health [23]. The investigated soya-containing food samples had higher Cr contents (mean 8.06 $mg \cdot kg^{-1}$) than flax-containing samples (mean 4.21 $mg \cdot kg^{-1}$). Among the investigated herbal raw materials, sample RM5 (flax) had the highest Cr content (3.87 $mg \cdot kg^{-1}$). Four dietary supplements (DS9, DS11, DS16 and DS20) had the total chromium contents greater than 10 $mg \cdot kg^{-1}$.

It is noteworthy that values obtained for the

Ba and Cu contents in the analysed samples had quite a wide range. The values obtained for mean (17.208 mg·kg^{-1}) and median (6.256 mg·kg^{-1}) Ba content of all analysed samples were statistically significantly different. The interquartile range was narrower and 7 samples had values higher than 25 mg·kg^{-1}.

Four samples (DS9, DS10, DS19 and DS20) had copper content greater than 25 mg·kg^{-1}, still the amounts taken following manufacturers' recommendations would lead to intake lower than the tolerable upper intake level recommended by EFSA (5 mg·d^{-1}) [29].

In contrast, the determined molybdenum contents were in a very narrow range. The investigated botanical monopreparations DS9 (7.825 mg·kg^{-1}) and DS2 (3.535 mg·kg^{-1}) had the highest levels of this ultratrace element, while the age-dependent tolerable upper intake levels for Mo are in the range from 0.1 mg·d^{-1} to 0.6 mg·d^{-1} [29].

Batch-to-batch variability

The batch-to-batch quality consistency of selected products was evaluated using two different batches of each dietary supplement and it was interesting to note variable batch-to-batch uniformity among various brands. Relative standard deviation (*RSD*) values were between 0.12 % (for Cd) and 115.0 % (for Mo), and high variability of two different batches of dietary supplements products was found for Co and Mo ($RSD \leq 39.2$ % and ≤ 115.0 %, respectively). The obtained results, presented in Tab. 3, showed that mercury was present at extremely low levels in the analysed dietary supplement samples, so the high *RSD* value (67.1 %) does not represent a large batch-to-batch variability (individual values were 1.940 μg·kg^{-1} and 5.438 μg·kg^{-1}).

CONCLUSIONS

The DI-MS method provided sufficient sensitivity and selectivity for rapid identification of phytoestrogens in complex samples without prior analyte separation by chromatography and with a total sample preparation and analysis time of less than 30 min. The obtained results indicate that the metal content in the investigated phytoestrogen-containing food, herbal raw materials and dietary supplement samples was below the international established limits for these heavy metals, except for Pb content in one capsule sample DS15. The results obtained indicate necessity for continuous monitoring of metal content in these type of samples.

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