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Original Article

The effect of extraction techniques on the mineral content of black pepper fruit ethanolic extracts

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Abstract:
Black pepper is one of the most used spices around the world, with numerous biological activities. Precisely because of the frequent use of this spice, the aim of this research was to determine the elemental composition of black pepper fruit ethanol extracts (BPEEs). The presence of heavy metals in spices is of particular importance because it can lead to the accumulation of these elements in human organs, which can further cause various health problems. The preparation of samples was done by dilution of the initial extracts with distilled water to a concentration of 0.5 mg/cm^3. In the group of spice macro-elements, the concentration of potassium is the highest of all the tested elements (20.412-26.370 mg/g of dry extract). From the group of heavy metals, bismuth was the most present in the range of 0.134-0.156 mg/g of dry extract, while lead, cadmium, and mercury were detected in smaller amounts. The extracts that contained elevated levels of certain heavy metals should be consistently monitored. However, it is crucial to develop effective procedures for removing already-extracted heavy metals from BPEEs.

Key words:
black pepper extract, extraction techniques, macro-elements, micro-elements, heavy metals.

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Apstrakt:
Uticaj tehnika ekstrakcije na mineralni sadržaj etanolnih ekstrakata plodova crnog bibera
Crni biber je jedan od najčešće korišćenih začina širom sveta, sa brojnim biološkim aktivnostima. Upravo zbog česte upotrebe ovog začina, cilj ovog istraživanja bio je da se utvrdi elementarni sastav etanolnih ekstrakata ploda crnog bibera. Prisustvo teških metala u začinima je od posebnog značaja jer može dovesti do akumulacije ovih elemenata u ljudskim organima, što dalje može izazvati razne zdravstvene probleme. Priprema uzoraka je vršena razblaživanjem početnih ekstrakata destilovanom vodom do koncentracije od 0,5 mg/cm^3. U ekstraktima je iz grupe makroelemenata, koncentracija kalijuma bila najveća od svih ispitivanih elemenata (20,412-26,370 mg/g suvog ekstrakta). Iz grupe teških metala, bizmut je bio najzastupljeniji u opsegu koncentracija od 0,134-0,156 mg/g suvog ekstrakta, dok su olovo, kadmijum i živa detektovani u manjim količinama. Neophodno je kontinuirano pratiti ekstrakte koji sadrže neke teške metale u visokim koncentracijama. Međutim, ključno je razviti efikasne procedure za uklanjanje već ekstrahovanih teških metala iz etanolnih ekstrakata ploda crnog bibera.

Ključne reči:
ekstrakt crnog bibera, tehnike ekstrakcije, makroelementi, mikroelementi, teški metali

Introduction

Black pepper (*Piper nigrum* L.) is a very important species from the family Piperaceae, not only for its taste and aromatic properties but also for its potential health benefits. It is a perennial plant native to the tropic areas of India and Southeast Asia and is commercially cultivated in 26 countries around the world. In the Balkan countries, it is most often imported from Vietnam, Brazil, Indonesia, China, and Thailand, which are the major cultivating countries (Yogesh & Mokshapathy, 2013). As one of the most widespread spices in the world, it is traditionally used in cuisines around the world. In addition to its culinary applications, black pepper has been investigated for its medicinal and therapeutic properties (Nahak & Sahu, 2011; Nirwane & Bapat, 2012; Zarai et al., 2013; Tasleem et al., 2014; Prashant et al., 2017).

The chemical composition of black pepper is diverse, and this plant, in addition to the alkaloid piperine, also contains essential oil, phenolic compounds, fatty oil (saturated and unsaturated fatty acids), starch, proteins, vitamins (A, C, E, K, vitamin B complex, etc.), and minerals (Ca, K, Cu, Fe, Mg, Zn, etc.) (Meghwal & Goswami, 2012; Hossain et al., 2014). Overall, black pepper extracts contain a variety of minerals and vitamins that can contribute to a healthy diet. However, the exact mineral content may vary depending on the specific extract and the method of analysis used. Black pepper extracts are important because of the variety of active compounds that contribute to their numerous biological activities, such as antioxidant (Nahak & Sahu, 2011), antimicrobial (Zarai et al., 2013), anticancer (Prashant et al., 2017), hepatoprotective (Nirwane & Bapat, 2012), anti-inflammatory (Tasleem et al., 2014), etc. Given the widespread usage of black pepper fruit in the traditional Serbian food industry and cuisine, it is important to extensively analyze the medicinal potential of this plant.

Minerals, in their various forms, are essential elements that play an irreplaceable role in human nutrition and health. Their presence in food and extracts of plant origin can significantly affect the taste, nutritional value, and potential health effects of these products. Black pepper contains a wide range of minerals that contribute not only to its sensory characteristics but also to its health benefits. Several studies have investigated the mineral composition of black pepper pericarp and extracts (Nwofia et al., 2013; Delowar et al., 2014; Ameh et al., 2016; Olalere et al., 2019). The minerals that are typically more abundant in black pepper extracts and pericarp include potassium, calcium, and magnesium.

However, even though black pepper is an excellent source of nutrients, it can also contain accumulated heavy metals or harmful chemicals from the process of production or improper storage. Prolonged exposure to heavy metals can lead to detrimental effects on human health, such as fetal illnesses, premature labor, cognitive impairments in children, as well as fatigue, hypertension, and renal issues in adults (Inam et al., 2013). Heavy metals are described as those metals whose specific gravity is greater than or equal to 5 g/cm^3. The most common heavy metals are copper, nickel, chromium, lead, cadmium, mercury, and iron. However, some heavy metals, such as iron, nickel, and copper, are essential for survival in low concentrations (Olayiwola et al., 2017), and because of that, they are classified as micro-elements in this study. However, heavy metals such as lead, cadmium, and mercury are toxic to living organisms even in small quantities, causing anomalies in the metabolic functions of the organism, especially in larger amounts (Olayiwola et al., 2017).

The use of black pepper as a spice, which contains heavy metals, can lead to the accumulation of these elements in human organs and cause various health problems. For these reasons, interest in harmful metals present in food has increased in the last two decades. To date, comprehensive studies dealing with the mineral content of black pepper, especially in ethanolic extracts, are limited.

Although previously used methods for mineral identification were expensive or did not allow the simultaneous assessment of the micro-elements present in the samples, a precise method for multi-element analysis and isotope ratio determination, Inductively Coupled Plasma with Optical Emission Spectrometry (ICP-OES), has been established (Savić et al., 2019). This method enables the simultaneous analysis of a wide range of trace elements in the sample and was therefore used in this study.

The tendency of modern society to use natural raw materials and products has increased interest in research. Therefore, the aim of this study was to analyse the influence of extraction techniques (maceration-M, reflux extraction-RE, Soxhlet extraction-SE and ultrasonic extraction-UE) on the content of macro- (P, K, Ca, Mg, Na) and micro-elements (Fe, Mn, Cr, Cu, Li, Ni, Zn, Si) in black pepper ethanolic extracts (BPEEs) using the ICP-OES method. Also, the objective was to detect some heavy metals (Cd, Co, Pb, and Hg) in the obtained BPEEs.

Considering that black pepper fruit is often used in traditional Serbian cuisine, this study can be of great importance when it comes to comparing the effect of extraction technique on the mineral content of BPEEs, which are a potentially important source of nutrients for humans, natural raw materials for the development of dietary supplements, and can be used in the food and pharmaceutical industries.

Materials and Methods

Plant material

The commercial sample of black pepper fruit (*Piper nigrum* L.; country of origin: Vietnam; packed by Thessaloniki Gyros and spices cannon; Ugrinovački put, part 25-no 32, Zemun, Altina) was used in this investigation. The plant material was protected from light and refrigerated in its original package. Right before the analysis, the plant material was ground in an electric mill (laboratory electric mill „BRAUN

AROMATIC KSM2").

Reagents and chemicals

Ethanol 96% v/v (Reachem d.o.o.), Multistandard IV (multielement standard solution, Merck, concentration of 1000 ppm), standard solutions of Si, P, Hg and S (Reagecon, Ireland) and Argon 5.0 (99.999% purity) as the carrier gas were used for ICP-OES analysis. All other chemicals are of analytical reagent grade (p.a.).

Preparation of extracts
Extraction techniques

Ground and homogenised plant material (black pepper fruit) was used for extraction in all applied extraction techniques. For maceration, 2 g of plant material, and the solvent ethanol 70% v/v in the ratio 1:10 m/v were used, and the extraction process was carried out for 120 minutes at room temperature. Reflux extraction followed a similar process but was carried out at boiling temperature. Ultrasonic extraction lasted 60 minutes at room temperature in a thermostated ultrasonic bath, and involved pouring 70% v/v ethanol over 2 g of plant material at a ratio of 1:10 m/v. Finally, Soxhlet extraction was performed under specific conditions using 25 g of plant material and 250 cm^3 of 70% v/v ethanol for a 240-min extraction. The obtained liquid extracts were separated by filtration using a „Buchner" funnel.

ICP-OES Analysis
Reagents and standard solutions

Distilled purified water from Fisher Chemical (HPLC grade) was used to dilute the extracts. Argon 5.0 (99.999% purity) was used as the carrier gas. In addition to the samples for analysis, calibration standards were prepared earlier. For the preparation of the calibration solution, Multistandard IV - multielementary standard solution (Merck) was used, which contains Ag, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, and Zn, as well as additional standard solutions of Si, P, Hg, and S (Reagecon, Ireland) in a concentration of 1000 ppm. The preparation of standard solutions for testing selected elements was performed by diluting Multistandard IV so that the concentrations of standards for making calibration diagrams were in the range of expected concentrations of tested elements.

Sample preparationy

The evaporated ethanolic extracts of black pepper fruit were diluted with distilled water to a concentration of 0.5 mg/cm^3. The samples were filtered through cellulose filters (0.45 µm), and after preparation subjected to ICP-OES analysis.

All measurements were performed on ICP-OES (Inductively Coupled Plasma - Optical Emission Spectrometry, ARCOS FHE12, SPECTRO, Germany) by the method of quantitative analysis. The operating conditions of the ICP-OES instrument and the parameters for determining the concentration of the selected elements are given in **Tab. 1**.

Table 1. Operating conditions for ICP-OES analysis

ICP-OES operating parameter	Parameter value
Plasma power (W)	1400
Gas flow (L/min)	
-Coolant	13
-Auxiliary	0.80
Nebulizer type	Cross flow
Nebulizer flow rate (L/min)	0.95
Pump speed (rpm)	30
Stabilization time (s)	0
Number of probes for each measuring	3
Plasma observation	Axial

Results and discussion

Mineral composition of BPEEs

The presence of 24 elements in the extracts was determined, and their content is given in **Tab. 2**.

Table 2. Content of macro- and micro-elements in BPEEs (mg/g d.e.)

	mg/g d.e.			
	1	**2**	**3**	**4**
Macro-elements (P, K, Ca, Mg, Na, S)				
P	11.696	7.796	11.738	5.876
K	26.370	22.790	24.850	20.412
Ca	n.i.	n.i.	n.i.	n.i.
Mg	0.650	0.534	0.744	0.246
Na	2.850	5.206	3.080	3.450
S	2.730	3.120	2.440	2.756
Micro-elements (Ag, Fe, Mn, Cr, Cu, Li, Ni, Zn, and Si)				
Ag	n.i.	n.i.	n.i.	n.i.

Fe	n.i.	n.i.	n.i.	n.i.
Mn	n.i.	n.i.	n.i.	n.i.
Cr	n.i.	n.i.	n.i.	n.i.
Cu	0.042	0.044	0.040	0.044
Li	0.008	0.008	0.008	0.008
Ni	n.i.	n.i.	n.i.	n.i.
Zn	0.102	0.182	0.110	0.208
Si	2.472	2.490	2.462	2.268
Elements with unknown benefits (B, Ba, In, Sr)				
B	n.i.	n.i.	0.098	n.i.
Ba	0.050	0.052	0.05	0.054
In	0.030	0.032	0.030	0.032
Sr	0.012	0.034	0.016	0.038
Heavy metals (Bi, Cd, Co, Pb, Hg)				
Bi	0.156	0.144	0.134	0.152
Cd	0.006	0.006	0.008	0.006
Co	n.i.	n.i.	n.i.	n.i.
Pb	n.i.	0.016	0.008	n.i.
Hg	0.072	0.092	0.100	0.090

*1-maceration; 2-reflux extraction; 3-Soxhlet extraction; 4-ultrasonic extraction; n.i.-not identified

The obtained results vary in the analysed samples depending on the extraction technique by which the extracts were obtained. Based on the presented results, it can be concluded that the presence of silver, boron, calcium, cobalt, chromium, iron, manganese, and nickel was not detected in any of the analysed samples, i.e. their content in the samples was below the detection limit (**Tab. 2**).

Of the macro-elements whose content was determined in four extracts (P, K, Ca, Mg, Na, and S), the highest content of potassium (20.412-26.370 mg/g of dry extract) was detected in all samples, and the most of it was in the M extract. These results agree with the studies of Özcan & Akbulut (2008), Al-Jasass & Al-Jasser (2012), Lee et al. (2020), where potassium was the most present of all macro-elements with values of 663-2377 mg/100 g. The study by Lee et al. (2020) showed that the pericarp of black pepper contains a much higher level of potassium compared to white beans (463.1 mg/100 g), one of the best sources of K. Potassium is a highly important electrolyte for maintaining the acid-base balance in the body as well as the osmotic pressure. It has a similar role in the body as sodium, calcium, and magnesium, and is important for the nerve impulse transmission, the proper functioning of the intestines and muscles, and heart contractions. Also, potassium participates in carbohydrate metabolism and membrane transport (Petrović et al., 2015). Calcium and magnesium are essential elements that help in bone formation, heart strengthening function, muscle relaxation, memory function, and glucose metabolism. Although the samples in the mentioned works contained a high level of calcium, it was not detected in our research. The Mg level in BPEEs was detected in the range of 0.246-0.744 mg/g of dry extract, with the highest content of it in SE extract. The ME and SE extracts were better sources of Mg compared to tuna (64 mg/100 g) and brown rice (44 mg/100 g). The recorded concentrations of Mg in BPEEs from this study were similar to concentrations in black pepper pericarp from the study of Al-Jasass & Al-Jasser (2012). When it comes to sodium, it is the most common cation that participates in water metabolism, muscle contraction, and allows the transfer of carbon dioxide to the lungs. The sodium content of BPEEs was in the range of 2.850-5.206 mg/g of dry extract (the highest value - RE extract), which is higher than the sodium content of black pepper pericarp according to previous studies (Özcan & Akbulut, 2007; Al-Jasass & Al-Jasser, 2012; Ameh et al., 2016; Lee et al., 2020). Sulphur is the third most abundant mineral in our body after calcium and phosphorus, with no recommended intake. It is essential for the biosynthesis of chondroitin in bone and cartilage, heparin, and insulin. Also, sulphur is an important component of the enzyme glutathione peroxidase (Nimni et al., 2007). Current data is insufficient to establish a tolerable upper intake level (Institute of Medicine, 2006). In the BPEEs, the content of sulphur was detected in the range of 2.440-3.120 mg/g of dry extract (the highest content was detected in RE extract).

The presence of lithium, zinc, copper, and silicon was detected in the micro-elements in all BPEEs samples. The study by Cicero et al. (2022) showed the presence of chromium, iron, and manganese which were not detected in the present investigation, as well as potentially dangerous minerals such as cadmium, lead, mercury, and aluminum (Cicero et al., 2022). The higher quantities of zinc were detected in current research (0.102 to 0.208 mg/g d.e.) than in the studies of Özcan & Akbulut (2008) (0.93 mg/100 g), Al-Jasass & Al-Jasser (2012) (0.9 mg/100 g), and Lee et al. (2020) (1.02 mg/100 g). Zinc is a micro-element that is required for the effective functioning of the brain and biomembrane, as well as the treatment of mental diseases. It is also required for protein and carbohydrate metabolism, prostate function, DNA synthesis, and is important in the development and function of reproductive organs (Savić et al., 2019). Copper, vitamin C, and zinc play an important role in preserving skin elasticity and providing essential support for the structural integrity of the skin. Copper deficiency can lead to conditions such as anemia, hair depigmentation, and bone deformities. The World Health Organisation (WHO) recommends a daily intake of 1 mg of copper (WHO, 1989). A diet lacking essential elements like zinc (Zn), iron (Fe), and copper (Cu) can potentially lead to the accumulation of toxic metals, including cadmium (Cd) or lead (Pb) (WHO, 1996). In the current study, the concentration of copper in the BPEEs samples ranged from 0.040 to 0.044 mg/g (as indicated in **Tab. 2**). It's important to note that the WHO has set a maximum permissible limit for copper in edible plants at 3.00 mg/kg, and based on this recommendation, all analysed samples (BPEEs) in this study have copper above the permissible limit (FAO/WHO, 1984).

Of the analysed micro-elements, the highest silicon (2.268-2.490 mg/g of dry extract) content was detected in all samples, and iron, manganese, chromium, and nickel were not identified in extracts. Silicon is an essential element for growth of crops, but also has a role in human health. The bioavailability of silicon in the human diet, for example, strengthens bones and improves the immune response, as well as the health of neurons and connective tissue (Farooq & Dietz, 2015). The literature data doesn't provide any specific information on the silicon content of black pepper.

Environmental pollution with heavy metals and long-term cumulative health effects are among the leading health problems worldwide. In this study, the presence of heavy metals was detected in BPEEs. Cobalt was not identified in extracts. Of the all analysed heavy metals, the highest bismuth (0.134-0.156 mg/g of dry extract) content was detected in all samples. There is not much information about the direct toxic effects of this element (Sano et al., 2005), but the toxicity of bismuth and its compounds occurs mainly when used as a therapeutic agent during prolonged administration, which mainly leads to kidney failure or mental disorders (Michalke et al., 2008). Bioaccumulation of lead (Pb) in the human body interferes with the functioning of mitochondria, which affects breathing and causes swelling of the brain, paralysis, and eventual death. Lead was detected only in RE and SE extracts with values that exceed the maximum allowable levels set for this metal by the WHO—10 mg/kg (WHO, 2007). Studies by Savić et al. and Al-Eed et al. showed lower values than this study and no presence of lead, respectively (Al-Eed et al., 2002; Savić et al., 2019). The presented results also correspond with the analyses of spice samples conducted in other European countries, for example in Serbia, where the lead concentration in black pepper samples varied in the range of 0.280 mg/kg to 0.42 mg/kg. However, it should be emphasised that black pepper is not grown in these countries, but is exclusively imported (Blagojević et al., 2015). The cadmium was detected in all BPEEs in the concentration range of 0.006-0.008 mg/g d.e., which exceeded the limit value set by the World Health Organisation (WHO) at the level of 0.3 mg/kg, i.e. 0.0003 mg/g (WHO, 2007).

Based on the mineral content of BPEEs and also taking into account the content of heavy metals in the samples, the best extract is the one obtained by maceration. Why is it so? This extract contains the highest amount of potassium, which is the most abundant of the macro-elements, but also the lowest amount of heavy metals compared to the other extracts. However, M extract cannot be considered an ideal choice, but in the future, greater attention should be directed to quality control of imported raw materials on the soil of Serbia and other Balkan countries. The differences in the obtained results, and those reported in previous studies may be due to environmental factors that prevail in production areas, cultivars used to produce seeds, and due to the different methods used to prepare samples (Fatoki, 2000). Also, some techniques of remediation is ex-situ soil washing using chelating agents or purification of already isolated extracts (Tandy et al., 2004; Shyam Sunder et al., 2020).

Conclusion

The use of the ICP-OES method provides a simple and efficient procedure for determining the nutritional profile of spices and spice extracts. The obtained results showed that the analysed extracts of black pepper (BPEEs) are a good source of potassium, phosphorus, sodium, and sulphur and therefore represent an important source of nutrients for humans. From the group of macro-elements, the most common element was potassium (20.412-26.370 mg/g of dry extract), while the lowest content was recorded for magnesium (0.246-0.744 mg/g of dry extract). The analysed BPEEs showed the presence of heavy metals, among which the highest content of bismuth was detected (0.134-0.156 mg/g of dry extract), lower quantities of lead, cadmium, and mercury, while the presence of cobalt was not detected. The human need for spices is very small on a daily basis, so there should be no danger of using them in the diet or food industry in that quantity. Also, if isolated extracts were to be used as a source of minerals in another industry, it is important to analyse and find a way to eliminate harmful extracted minerals. In the future, it is important that extracts containing some toxic metals at a relatively high level, should be under continuous monitoring. However, it is essential to find appropriate and efficient methods for the removal of already extracted heavy metals from BPEEs.

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