ICP-OES Determination of Some Trace Elements in Herbal Oils Using a Three-Phase Emulsion Method and Comparison with Conventional Methods

Dilek Bakircioglu^a, Nukte Topraksever^a, Selcuk Yurtsever^b, and Yasemin Bakircioglu Kurtulus^{a,*}

^a Trakya University, Faculty of Science, Department of Chemistry, Edirne, Turkey

^b Trakya University, Faculty of Science, Department of Biology, Edirne, Turkey

INTRODUCTION

Herbal oils have been used for hundreds of years all over the world because they contain naturally occurring antioxidants and essential elements, and are still used today for dietary and health benefits (1).

Nettle (*Urtica dioica L.*) is used as a folk medicine in many parts of Turkey due to its antioxidant characteristics for preventing and curing cancer (2). It is also used for diuretic, hypoglycemic, anti-inflammatory, anti-rheumatic and hypertensive conditions (3). In addition, it is an important source of vitamin C (2, 4).

Linseed (*Linum usitatissimum L.*), also known as flax seed, contains 40–50% oil and meal, 23–34% protein, 4% ash, 5% fiber and lignin precursor (5). It is a high source of ω -3 fatty acid, α -linolenic acid, short-chain polyunsaturated fatty acids, soluble and insoluble fibers, phytoestrogenic lignans, proteins and antioxidants. The α -linolenic acid (ALA) (52% of total fatty acids) decreases hypercholesterolemia, thrombosis, risk of cancer, mammary and prostate gland anti-inflammatory activity, meno-pausal symptoms and osteoporosis, cardiovascular diseases, and reduces platelet adhesiveness (6, 7). Recent studies have confirmed the pharmacological actions and probable use of linseed in the treatment of diseases such as atherosclerosis, cancer, (breast, colon, ovary, and prostate) and Alzheimer's disease (8). In gen-

ABSTRACT

The concentrations of cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn) were determined in five (Nettle, Linseed, Pumpkin seed, Sesame, and Chamomile) herbal oil samples, using inductively coupled plasma atomic emission spectrometry (ICP OES) after ultrasonic extraction (UE), wet digestion (WD), and the three-phase emulsion method (TPE). The concentrations for Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn were found to be 0.037–0.064, 0.014–0.085, 0.735–1.243, 2.105–19.487, 0.104–0.592, 0.105–1.582, 0.025–0.125, and 1.024–2.583 mg kg⁻¹, respectively. The highest concentrations in the herbal oils were Cr, Fe, Mn, and Zn, in linseed oil, Cd and Pb in sesame oil, Cu in nettle oil, and Ni in chamomile oil, respectively. The three sample preparation methods investigated were compared and it was found that the results of TPE and WD are comparable. The statistical results show that the TPE and WD methods usually give similar results.

eral, flax seed oil is used in food products, such as juices, milk and dairy, baked and dried foods, such as muffins, macaroni, etc (7). Linseed oil is also used in a wide range of industrial applications (plastics, paint, paper, textile, etc.) (9).

Pumpkin (*Cucurbita pepo L.*) seed is dark green in color, very rich in protein (28%) and fat (55%). It is used as a salad oil and contains rich-free fatty acids (10). It is a

source of phytoestrogens similar to other plant sources such as soy foods, flax seed, sunflower seed, sesame seed, etc. (11). Pumpkin seed oil contains a significant level of the special fatty acid of oleic acid and linoleic acid. The important pharmacological activities of pumpkin seed oil are in slowing the progression of hypertension, potential anti-inflammatory activity in arthritis, and also decreases the formation of stones in the bladder (12). It could be used as an alternative to the expensive olive oil.

Sesame (*Sesamum indicum L.*) seed is an annual plant, and widely used in human nutrition as oil and for food production (Halaweh, java beans, and salads) (13). It is one of the oldest oil seed crops known, domesticated well over 3000 years ago. Because it contains very high amounts of unsaturated fatty acids and lignan compounds, it has excellent oxidative stability (14–16). Its oil is rich in sesamin and sesamol and therefore more thermally stable than other vegetable oils (17). Because it has phytosterols as the predominant sterol, this compound increases oxygen solubility in the oil (18, 19). Sesame oil increases plasma gamma-tocopherol and vitamin E activity, which has potential health benefits against heart disease and cancer. The high amount of polyunsaturated fats helps in the reduction of cholesterol (20).

Chamomile (*Matricaria recutita L.*) is a member of the Asteraceae family and belongs to a major group of aromatic-medicinal plants. Its flowers contain the essential oil. It also contains several medical substances such as sesquiterpenes, flavonoids, and coumarins (21).

* Corresponding author.
E-mail: ybakircioglu@trakya.edu.tr
Tel: +90 284 2359592

Because it has anti-inflammatory and spasmolytic effects, it is used to treat some diseases (22). It is also widely used in different types of industrial products such as cleaning materials, cosmetics, medicine, etc. Some researchers report that the essential oil extracted from chamomile has anti-microbial activity, which prevents the generation of some species of bacteria, fungi, and viruses (23). Chamomile oil also exhibits antioxidant activities (24).

The quality criteria of herbal oils and their products is increasing day by day because they contain naturally occurring antioxidants and essential elements (25). The presence of trace elements in herbal oils may catalyze the decomposition of hydroperoxides to free radicals and secondary oxidation products. This can increase the carcinogenic effect by interacting with other food constituents (26) and influence the oxidative stability of oil quality (27). Trace element contamination of herbal oils is a major concern and is due to environmental pollution from industrial and traffic emissions and agricultural activities, use of fertilizers, fungicides, and insecticides.

In this study, the concentrations of some trace elements such as Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn in various herbal oil samples (Nettle, Linseed, Pumpkin seed, Sesame seed, and Chamomile) were deter-

mined by inductively coupled plasma optical emission spectrometry (ICP-OES) after ultrasonic extraction (UE), wet digestion (WD), and the three-phase emulsion method (TPE). Additionally, sample preparation methods are discussed.

EXPERIMENTAL

Instrumentation

Element concentrations were determined using a Varian Vista-MPX CCD simultaneous inductively coupled optical emission spectrometer (ICP-OES) (Varian Inc., Victoria, Australia). The operating conditions of the ICP-OES are listed in Table I.

An ultrasonic bath (Bandelin Electronic GmbH, Germany) was used for ultrasonic extraction experiments in the preparation of the herbal oil samples.

A thermostatic bath (A Nüve ST 402, Ankara, Turkey) provided the means to maintain samples at an experimental temperature.

A temperature-controlled water bath (WiseBath WB-22, Daihan Scientific Co., Ltd, South Korea) was employed to perform the three-phase emulsion method.

Standards and Reagents

A multi-element stock solution containing 100 g L⁻¹ of cadmium, chromium, copper, iron, manganese, nickel, lead, and zinc (Merck, Darmstadt, Germany) was prepared daily in 5% HNO₃.

Analytical reagent grade hydrochloric acid (37%), nitric acid (65%), and hydrogen peroxide (35%) used in the extraction and digestion experiments were bought from Merck (Darmstadt, Germany).

The commercial Triton[®] X-114 (Sigma-Aldrich, USA) was used as received. Acidic Triton[®] X-114 (7% Triton X-114/10% HNO₃) for use in the three-phase emulsion method was prepared by dissolving 7.0 g of Triton X-114 in 100 mL of 10% (v/v) HNO₃.

Deionized water (18 MΩ·cm resistivity, ELGA Elgastat Maxima, ultra-pure water purification system) was used for preparation of the reagents and standard solutions.

Samples Used for Analysis

A total of 25 herbal oil samples (five of chamomile oil, linseed oil, nettle oil, pumpkin oil, and sesame oil samples) were purchased from different local supermarkets in Turkey. The oil samples were packed in polyethylene containers and kept in a refrigerator (about 4 °C) until the time of analysis.

Sample Preparation for Herbal Oil Samples

Three different sample digestion methods were investigated: ultrasonic extraction (UE), wet digestion (WD), and three-phase emulsion method (TPE).

Method A: Conventional Wet Digestion (WD)

The herbal oil samples (0.5 g each) were treated with an acid digestion method proposed by Ansari et al. (28). About 10 mL of a daily prepared mixture of concen-

TABLE I
Instrumental Operating Conditions of ICP-OES

RF power	1.00 kW
Plasma flow	15.0 L min ⁻¹
Auxiliary flow	1.5 L min ⁻¹
Nebulizer flow	0.75 L min ⁻¹
Pump rate	15 rpm
Replicates	3
Replicate read time	10 s
Elements monitored (nm)	Cd (214.4), Cr (267.7), Cu (327.4), Fe (238.2), Mn (257.6), Ni (231.6), Pb (220.3), Zn (213.9)

trated $\text{HNO}_3\text{-H}_2\text{O}_2$ (2:1, v/v) was added and kept standing for 10 minutes at room temperature. Afterwards, they were heated to 80 °C on a hot plate until digestion of the sample was completed. The digested sample was evaporated, cooled to room temperature, and 5 mL 0.2 M HNO_3 was added and filtered through a blue filter paper, then made up to 10-mL volume with ultra-pure water before ICP-OES analysis. The blanks were prepared in a similar manner without sample.

Method B: Ultrasound Extraction (UE)

Herbal oil samples in the amount of 0.1 g were placed into 50-mL polypropylene centrifuge conical tubes and 10 mL of 1% HNO_3 and 0.2% HCl (1:1) solutions were added to every tube. The solution was agitated for 30 seconds employing a vortex mixer. Every tube was put into the ultrasonic water bath and sonicated at 170 W for 5 minutes. After sonication, the supernatant solution was separated

into two parts by centrifugation for 5 minutes at 4000 rpm. Determination of the elements was carried out in the aqueous phase. Blanks (without sample) were analyzed for all methods (29).

Method C: Three-Phase Emulsion (TPE)

The use of a three-phase emulsion step (TPE) for sample preparation was based on a procedure published in previous literature (30, 31). In brief, primary emulsion (water-in-oil) was prepared using 2 mL of the Triton® X-114 (acidic) solution. Then, 2 mL of herbal oil was added dropwise into the acidic surfactant phase while stirring with a vortex. Emulsification time was fixed at 5 minutes and the temperature kept at 25 °C during homogenization for all experiments. Then the tube was placed into a water bath at 80 °C to allow the emulsion breakdown and to separate into three phases: (a) surfactant-rich lower phase (s), (b) oil-rich upper phase (o), and (c) acidic aqueous middle phase (m) which contains

the extracted elements (see Figure 1). The middle phase is equal to a total volume of 2 mL. The separated middle phase (about 1.5 mL) was transferred into a new tube using a micro-pipette. This step of the TPE method is probably the most important. If phase separation is not performed properly, unclear results will be obtained. The studied element concentration in 1.5 mL, which is higher than recommended by the instrument manufacturer, can be reduced by increasing the dilution volume.

Calibration Procedure and Statistical Analysis

To conduct quantitative analysis by ICP-OES, calibration was performed daily according to the program options of the apparatus. The acidity and concentration of the standards were kept the same as the sample solutions and were prepared in 1% HNO_3 from the stock solution. The range of calibration curves (5 points) for the studied elements over the 0–4.0 mg L^{-1} concentration range were obtained by

Fig. 1. Emulsion before (1a, 2a, 3a, 4a, 5a) and after breaking by heating at 80 °C (1b, 2b, 3b, 4b, 5b). (o) Herbal oil-rich phase; (m) acidic aqueous middle phase; (s) surfactant-rich lower phase.

ICP-OES. The correlation coefficient of the determination was in the range of 0.9998-0.9999. The statistical significant differences between the concentrations of the trace elements in herbal oil using the various digestion methods were analyzed by ANOVA and determined using by the P values of <0.05 . The different results of the statistical evaluation were obtained with the TUKEY test, and the data are expressed as the mean $\pm$ standard deviation (32).

RESULTS AND DISCUSSION

Herbal oils are the oldest known traditional medicinal products. Their importance is still increasing because many people, particularly in the developing countries, rely on herbal materials for treatment of various ailments, but they are also used in the production of oils and foods. Thus, prevention and detection of contamination are very important criteria for health authorities, pharmaceutical companies, and the population. The study of toxic elements in Turkish herbal oils has to our knowledge never been reported in the literature and should also include the determination of microorganisms, microbial toxins, pesticides, radioactivity (33).

The preparation of herbal materials during their processing includes assessment of the conditions of the starting material, intermediate product or finished product, sampling, packaging, storage, and transport. During these cycles, undesired trace element contamination can occur due to plant types, metal-containing fertilizers, and pesticides (34, 35).

In addition, Fe, Cu, Ni and Mn enhance autoxidation and thus have a significant effect of light on the oil products. On the other hand, Cr, Cd, and Pb are very toxic and impact important metabolic functions in humans and animals (36).

In this study, five different herbal oils have been subjected to trace element analysis using ICP-OES after UE, WD, and TPE sample preparation (see Tables IIa and IIb). The average Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn concentrations were found to be 0.050-0.054, 0.018-0.080, 0.826-1.155, 2.604-18.548, 0.162-0.508, 0.163-1.323, 0.033-0.148 and 1.164-2.403 mg kg⁻¹ in herbal oil samples, respectively. For the investigated trace elements, the level of iron was found highest at 18.5, 16.5, and 14.3 mg kg⁻¹ in the linseed, chamomile, and sesame oil samples, respectively, while the concentration of Cr was the lowest at 0.018 mg kg⁻¹ in pumpkin and chamomile. In general, Fe contamination arises from environmental contamination due to mineralization of crops, processing equipment, storage material, and application of agricultural sources (36).

Because of the biological role of Cr, which is related to the carbohydrate, lipid and protein metabolism, it is an essential element for living organisms. Apart from its biological role, the hexavalent form is toxic if taken in excess (37). The concentration of Cr was about 0.020 mg kg⁻¹, in sesame oil at 0.09 mg kg⁻¹ and linseed oil at 0.080 mg kg⁻¹.

Iron has the following important functions in the human body: (a) as a component of several proteins and enzymes for a healthy immune system; (b) required for the transport of oxygen from tissues to the body for metabolism; (c) acts as pro-oxidant and antioxidant; and (d) decreases the incidence of anemia. Iron deficiency affects growth, results in high menstrual loss, and problems during pregnancy (38). This study shows that the oils of chamomile, sesame, and linseed contain higher amounts of Fe (14.265 to 18.548 mg kg⁻¹) than all other herbal oils analyzed (2.604 to 6.153 mg kg⁻¹).

Copper, Fe, and Mn are directly involved in redox reactions, because they have unpaired electrons which participate in a redox reaction by transferring and accepting electrons or changing the oxidation state. They are essential components of biological systems, which bind to large molecules such as proteins to form metallo-proteins, and small molecules (phosphates, phytates, polyphenols, and other chelating compounds). Many of the metals in metalloproteins have structural and storage functions, but at the same time they are part of the enzymatic systems. In biological systems, the functions of Fe, Mn, Cu, and Zn are to protect human health. Deficiency or inadequate intake of these metals can lead to unwanted pathological problems. It was found that the potential contamination of Cu and Fe comes from the oil processing equipment, while Pb and Cu contamination can be present in oils due to environmental contamination (36).

The concentration of Mn was in the range of 0.16 to 0.51 mg kg⁻¹. Pumpkin seed had the lowest Mn level and linseed the highest. A sesame seed herbal oil sample differs also from the other oils with regards to its Pb concentration (0.15 mg kg⁻¹). Copper was found in the range of 0.83 to 0.92 mg kg⁻¹ in all herbal oil samples studied, except for the linseed (1.02 mg kg⁻¹) and the nettle oil samples (1.15 mg kg⁻¹).

The Pb and Cu levels in herbal products were lower than the European pharmacopoeia limits of Pb at 5 mg kg⁻¹ and Cu at 150 mg kg⁻¹ (39). Most of the sesame seed oil results are parallel to the results of Juranovic Cindric (35). However, in the present study the Fe, Zn, and Ni levels in pumpkin seed oils are lower. Cindric and coworkers (35) found the trace element levels of sesame seed oil at 14.8 mg kg⁻¹ for

TABLE IIa
The Contents of Cd, Cr, Cu, and Fe (mg kg⁻¹) in Five Different Herbal Oils Determined
Using Three Different Sample Preparation Methods (Mean±SD; n=5)

	Sesame	Linseed	Nettle	Pumpkin	Chamomile
Cd					
WET	0.053±0.007	0.057±0.006 (a)	0.058±0.009 (a)	0.055±0.005 (a)	0.062±0.010 (a)
UE	0.048±0.005	0.035±0.009 (b)	0.034±0.006 (b)	0.033±0.004 (b)	0.028±0.002 (b)
TPE	0.054±0.009	0.050±0.010 (ab)	0.053±0.004 (a)	0.051±0.003 (a)	0.052±0.004 (a)
P	N.S.	***	***	***	***
Cr					
WET	0.089±0.007 (a)	0.055±0.007 (a)	0.038±0.04 (a)	0.022±0.003	0.109±0.019 (a)
UE	0.077±0.014 (ab)	0.037±0.014 (b)	0.053±0.008 (b)	0.018±0.003	0.022±0.002 (b)
TPE	0.069±0.003 (b)	0.080±0.003 (c)	0.022±0.005 (c)	0.018±0.001	0.018±0.004 (b)
P	*	***	***	N.S.	***
Cu					
WET	0.954±0.094 (a)	0.792±0.123 (a)	1.011±0.181 (a)	0.712±0.025 (a)	1.220±0.114 (a)
UE	0.786±0.035 (b)	1.108±0.050 (b)	0.526±0.043 (b)	0.605±0.013 (b)	0.871±0.042 (b)
TPE	0.908±0.013 (a)	1.019±0.08 (b)	1.155±0.077 (a)	0.826±0.078 (c)	0.920±0.87 (b)
P	**	***	***	***	***
Fe					
WET	17.155±0.690 (a)	27.684±0.738 (a)	6.938±0.435 (a)	3.454±0.471 (a)	28.505±0.663 (a)
UE	8.759±0.862 (b)	11.017±1.252 (b)	3.211±0.482 (b)	1.203±0.049 (b)	10.160±0.455 (b)
TPE	14.265±0.447 (c)	18.548±0.673 (c)	6.153±0.923 (a)	2.604±0.393 (c)	16.553±0.762 (c)
P	***	***	***	***	***

SD: standard deviation.

N.S.: not significant at 0.05 levels.

*, **, ***: indicate significance levels beyond the 0.05, 0.01, and 0.001 levels.

(a),(b),(c): indicate whether compared means are same or not.

Fe, 3.1 mg kg⁻¹ for Zn, 0.56 mg kg⁻¹ for Cu, and 0.30 mg kg⁻¹ for Ni. These values were close to the results of this study. The elements Mn, Cr, and Pb cannot be compared because the concentrations found by Cindric et al. are below the detection limit. No data have been published with regard to trace elements in edible herbal oils.

The metals Cd, Ni, and Pb are known to cause a decrease of some essential nutrients in the cells, and result in diseases such as decrease in immunological defenses, intrauterine growth retardation, psycho-social dysfunctions, and gastrointestinal cancer. Cadmium, which is a highly toxic element, can cause damage even in small

amounts to the arteries, kidneys, and the liver. These metals may enter the food chain from soils consisting of sludge, composts, or phosphate-containing fertilizers. For this reason, these products must be used carefully such as cadmium-containing fertilizers (37). The concentration of Cd in the analyzed oil samples was about 0.050 mg kg⁻¹ which is lower than the daily recommended intake (DRI) value listed by FAO/WHO (40).

The state of Zn²⁺ prevents participation in redox reactions. Nevertheless, it acts as an antioxidant by replacing metals that are active in catalyzing free radical reactions, such as Fe (39). The chamomile, pumpkin seed, and linseed oil sam-

ples contain higher amounts of Zn (2.0 to 2.4 mg kg⁻¹) compared to the other oils (1.16 to 1.35 mg kg⁻¹). Ni was found in the range of 0.163 to 0.402 mg kg⁻¹ in all herbal oil samples, except the chamomile oil (1.323 mg kg⁻¹).

The Joint FAO/WHO Expert Committee on Food Additives, Environmental Protection Agency (EPA), and the US National Council (NRC) have set a limit for metal intake based on body weight. For an adult, the daily recommended intake (DRI) values of Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn were reported as 0.07 mg, 1.5 mg, 0.9 mg, 48 mg, 9.8 mg, 1.4 mg, 0.25 mg, and 15 mg, respectively (40-44). The results of this study show

TABLE IIb
The Contents of Mn, Ni, Pb, and Zn (mg kg⁻¹) in Five Different Herbal Oils
Determined Using Three Different Sample Preparation Methods (Mean±SD; n=5)

	Sesame	Linseed	Nettle	Pumpkin	Chamomile
Mn					
WET	0.339±0.013 (a)	0.555±0.064 (a)	0.193±0.043 (a)	0.161±0.024 (a)	0.378±0.850 (a)
UE	0.042±0.012 (b)	0.053±0.008 (b)	0.011±0.002 (b)	0.004±0.0004 (b)	0.006±0.007 (b)
TPE	0.342±0.036 (a)	0.508±0.070 (a)	0.179±0.042 (a)	0.162±0.038 (a)	0.366±0.020 (a)
P	***	***	***	***	***
Ni					
WET	0.541±0.061 (a)	2.427±0.133 (a)	0.338±0.064 (a)	1.186±0.081 (a)	1.333±0.128 (a)
UE	0.305±0.071 (b)	1.090±0.078 (b)	0.649±0.048 (b)	0.556±0.042 (b)	0.384±0.027 (b)
TPE	0.402±0.056 (b)	0.256±0.047 (c)	0.163±0.051 (c)	0.172±0.022 (c)	1.323±0.173 (a)
P	***	***	***	***	***
Pb					
WET	0.149±0.009 (a)	0.062±0.029 (ab)	0.041±0.017 (a)	0.093±0.006 (a)	0.116±0.015 (a)
UE	0.046±0.009 (b)	0.037±0.010 (a)	0.024±0.007 (b)	0.039±0.006 (b)	0.080±0.010 (b)
TPE	0.148±0.256 (a)	0.088±0.008 (b)	0.033±0.006 (b)	0.050±0.005 (c)	0.038±0.004 (c)
P	***	***	N.S.	***	***
Zn					
WET	1.407±0.113 (a)	5.905±0.303 (a)	1.075±0.142 (a)	2.045±0.133	2.043±0.111(a)
UE	2.277±0.087 (b)	3.935±0.385 (b)	1.537±0.094 (b)	2.329±0.111	3.111±0.68 (b)
TPE	1.354±0.101 (a)	2.403±0.196 (c)	1.164±0.116 (a)	1.996±0.566	2.254±0.157 (c)
P	***	***	***	N.S.	***

SD: standard deviation.

N.S.: not significant at 0.05 levels.

*, **, ***: indicate significance levels beyond the 0.05, 0.01, and 0.001 levels.

(a),(b),(c): indicate whether compared means are same or not.

that the mean values of the elements determined were in accordance with the dietary recommended intake (DRI) limits given by FAO/WHO, EPA, and NRC.

The mean values of herbal oils obtained from the five different samples (five replicates for each treatment) using the three different methods are comparable indeed. As can be seen in Tables IIa and IIb, 37 of the 40 ANOVA tests are significant statistically and only three tests reveal no sign of difference. The three methods showed significant statistical different results in a total of 15 cases. The pair-wise comparisons using the posterior TUKEY test clearly reveal that the UE method is comparable to the

two other methods of WD and TPE. In most of the treatments, the UE method resulted in lower mean values than the WD and TPE methods. Although the mean value of the WD method was higher than of TPE and UE in most cases, and 14 of the pair-wise comparisons for WD and TPE showed no sign of difference. Accordingly, it can be presumed that the TPE method is preferred over the WD and UE methods. The mean values obtained from TPE were significantly the same as the conventionally used wet digestion methods.

CONCLUSION

This study presents the results in the determination of elements in

nettle seed, linseed, pumpkin seed, sesame seed, and chamomile oil samples by inductively coupled plasma optical emission spectrometry (ICP-OES) after ultrasonic extraction (UE), wet digestion (WD), and the three-phase emulsion (TPE) method. The highest concentration of iron was found in the linseed, chamomile, and sesame oil samples, whereas the chromium concentration was minimum in pumpkin seed and chamomile oil samples. Contamination of herbal materials with metals can be attributed to environmental pollution (i.e., factories, contaminated water, pesticides), soil composition and fertilizers, and the external conditions during the growing seasons, harvesting, and the manufacturing

process. The most important advantages of this TPE method for the quantitative analysis are the simultaneous determination of many trace elements by ICP-OES, determination in a wide concentration range, simple and fast sample preparation, excellent and effective, less time-consuming, no use of large amounts of concentrated reagents and no contamination. It is an alternative method to the WD method with much lower equipment cost than the conventional WD and UE methods.

The results with the proposed TPE method were comparable to those obtained after the sample preparation by WD digestion and UE method. The 36 out of 40 ANOVA tests are statistically significant for the three methods. Moreover, the pair-wise comparisons (Turkey) show that 17 tests are not different from WD and TPE and usually give similar results. On the other hand, TPE and UE usually give different results, because the 29 statistical tests were different. Therefore, the TPE method is recommended as an alternative approach to the conventional sample pretreatment method of wet digestion.

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