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Optimization of Extraction Parameters for Fat-Soluble Vitamins and Major Element Analysis in *Polygonum cognatum Meissn* Plant (Madımak)

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Abstract: The concentrations of fat-soluble vitamins (A and E) and various metal ions (Ca, Cd, Cr, Co, Cu, Fe, Mg, Mn, Pb, Zn) have been determined in *Polygonum cognatum Meissn* plant samples collected in Sivas, Turkey. Analysis of vitamins were carried out by HPLC after Soxhlet extraction and metal ions were determined by FAAS after microwave digestion, respectively. The study is focused on two main purposes: optimization of sample preparing parameters prior to determination and chemical analysis of a common edible plant in Anatolia. There was not enough information about this plant in point of vitamins and various metal species. Consequently, useful and detailed data were introduced to literature for a well-known and so consumed edible plant.

Keywords: *Polygonum cognatum meissn* (Madımak); vitamin A; vitamin E; metal analysis; HPLC; FAAS.

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INTRODUCTION

The analysis of micronutrients in food samples is of great interest both regarding nutrition and commercial aspects. This kind of analysis is so important in order to know the contents of foods and environmental samples in view of their toxicity and essential properties (1, 2). Trace elements play an important role in the formation of the active chemical constituents present in medicinal plants. However, most of the research work on medicinal plants pertain to constituents such as essential oils, vitamins, glycosides, and other organic compounds, while little attention has been paid to the elemental composition of the plants (3, 4).

Vitamins A and E are essential food ingredients that are usually supplied with the diet (5). Normally, the human organism cannot synthesize vitamins, and for this reason, they must be included in the human diet. The determination of fat-soluble vitamins A and E plays an important role in nutritional and biochemical studies, and analytical methods available and capable of determining these vitamins in different samples are imperative (6). The possible protective effect of vitamin E against environmental and drug toxicity has also attracted the attention of many investigators (7). The dietary reference intake of vitamin E for an adult person is 15 $mg.day^{-1}$ (8). Measurements of these vitamins in real samples like drugs, biological liquids, and foods are an important research area in order to check their intakes.

Due to their similar chemical properties, vitamins A and E are generally determined using an identical sample workup procedure. Typically, this includes a saponification step, extraction of the vitamins into an organic solvent, and separation by normal or reversed-phase HPLC coupled to UV or fluorescence detection (9, 10). Many analytical methods including colorimetry, thin-layer chromatography, gas chromatography, and high-performance liquid chromatography (HPLC) have been used for the analysis of fat soluble vitamins. HPLC is preferred for vitamin E quantification due to simplicity, giving more precision, and more specific than other methods (11). The most critical and time consuming step in the vitamin analysis is the extraction of the vitamin from real samples. So, newly developed methods are proposed together a useful sample preparation step.

The essential metal species play an important role in many biological systems due to their functions in metabolic activities. So, there are a lot of studies based on trace metals in food samples (12-14). The following of trace metals in food samples helps to understanding of their possible toxic effects. In addition, some of these metals are necessary for vital functions and they are taken by diet via food additives. Heavy metals may enter the food chain as a result of their intake by edible plants. So, determination of heavy metals in plant samples is a very important analytical problem. Some of these metals have significantly toxic and have hazardous effects on human health (15).

In food samples, a pre-treatment and separation procedure is often necessary to isolate the components of interest from sample matrices, to purify and concentrate the analyte (16). In order to release vitamins, various extraction methods such as soxhlet extraction (17), cloud point extraction (18), ultrasound assisted extraction (19), and accelerated solvent extraction (20) have been applied depending on the nature of the sample. The success of applied pre-treatment procedure affects directly the reliability of analysis results.

Polygonum Cognatum Meissn (Figure 1) is a wild edible plant called "madımak" in Turkish. This edible plant is perennial of slender woody stock. It grows on roadsides, slopes, and cliffs at altitudes of 720–3000 m (21-23). The young shoots with leaves are collected in the spring. In Turkish folk medicine, it has been used for various purposes, such as its diuretic effect and for the treatment of diabetes mellitus (24). According to Ozbucak *et al.* (25), the amount of protein in Madımak is higher that similar plants which are consumed as foods. Some wild plant species used as vegetables in the black sea region of Turkey were investigated by Civelek and Balkaya. The results of this study was shown that mineral and nutrient contents of *Polygonum cognatum Meissn* was comparable levels respect to other vegetables (26).

Figure 1. An image taken in a local bazaar in Sivas.

This study aims to examine the chemical contents of *Polygonum cognatum Meissn* (Madımak) in terms of fat-soluble vitamins and various metals. As far as we know, this is the first study reporting the detail chemical analysis of this plant although it is overly consumed in the Anatolia. The soxhlet extraction method was optimized and used for sample preparation in vitamin analysis and the contents of vitamins A and E were determined by HPLC-DAD system. Microwave digestion system was also optimized to digest samples before metal analysis and the concentrations of metals were determined by flame atomic absorption spectrometer (FAAS).

EXPERIMENTAL

Instrumentation

The chromatographic system used is equipped with a pump model LC20-AD (Shimadzu), a thermostatic oven, CTO-10 AS (Shimadzu), auto sampler, SIL-20Ac (Shimadzu) and detectors: a DAD detector model SPD-M20A (Shimadzu). An LC solution software (Shimadzu) was used to transfer the data to the computer. An Inertsil C18 (250 mm×4,6×5 µm) column was used for chromatographic separation.

An atomic absorption spectrometer (Shimadzu AAS-6300), equipped with D_2-background correction, a hollow cathode lamp and an air-acetylene flame atomizer, was used for all determinations. All metal determinations were carried out according to manufacturer proposals.

SE (Soxhlet extraction apparatus, VWR Scientific Products, catalog no. 27611-049) was performed as described in EPA Method 3540. A microwave digestion system (CEM Mars X6, USA) was used to dissolve and prepare the samples to analysis. The pH measurements were carried out with a pH meter (Selecta 2001 pH-meter).

Chemicals

All chemicals were of chromatographic and analytical reagent grade. α-tocopherol (Vitamin E) and Retinol (Vitamin A) were purchased from Sigma (St. Louis, Steinheim, Germany), and n-hexane and isopropyl alcohol were from Merck (Darmstadt, Germany). Stock metal solutions were prepared by using their nitrate salts bought from Merck. Ultra-pure water with a resistivity of 18.2 MΩ cm was used in all experiments provided by ELGA (Flex III, UK) water purification system. The stock standard solutions of vitamins were prepared in ethanol, and the standards used were prepared by dilution of the appropriate volume of the stock in colored bottles and stored at –20 ^{0}C. Stock standard solutions of metal salts were prepared in 0.01 M HNO_3.

Sample Preparation

Madımak samples were collected from the flora of Sivas district on May, 2014. The experiments were carried out with two samples obtained from two different sub-districts and symbolized as M1 and M2. Edible parts of plant were cut and dried in laboratory conditions. Dried samples were ground by using a blender and kept in a refrigerator until analysis.

Sample preparation is one of the most critical steps of chemical analysis, which determines the quality and the credibility of the analytical results obtained, and it is estimated that it accounts for about 60 % of all errors during the whole analytical process (27). Manipulation is time consuming, expensive, and is the main source of errors and, therefore, it should be kept at a minimum if possible. Nevertheless, the relative amount of target species in the sample be analyzed can be very low, and it sometimes requires pre-concentration or separation procedures. Samples were prepared by using two different way for vitamin and metal analysis. Soxhlet

extraction was preferred in order to extract vitamins A and E from Madımak plant. Microwave digestion method was applied to samples before metal analysis.

Soxhlet Extraction

Conventional Soxhlet extraction method was applied to samples prior to the HPLC analysis. A 20 g sample portion of the dried plant was refluxed with 400 mL of organic solvent for 8 h at the boiling temperature of ethanol by using Soxhlet apparatus. Five parallel extraction were carried for repetition analysis. The extracts were filtered and concentrated by using a rotary evaporator until dryness. Approximately, 1 g of concentrated sample was obtained from each extraction system.

In the second step, the obtained extracts were re-dissolved by using various solvents such as acetonitrile, ethanol, methanol, n-hexane, and acetone. 100 mg of extract was weighed and 2.5 mL of solvent was added onto the extracts. Then, the samples were mixed vigorously by a vortex for 10 minutes. Finally, the samples were filtered by using 0.45 µm membrane and transferred to HPLC vials.

Microwave Digestion

The samples were digested by using a microwave digestion system prior to metal analysis. The microwave parameters were given in Table 1. After digestion, the samples were filtered through 0.45 µm filter paper and neutralized by a few drops of ammonia until its acidity was eliminated. Then, they were diluted to a 10 mL final volume with 0.1 M HNO_3 and the metal contents of samples were determined by using FAAS.

Table 1. Microwave parameters for digestion procedure

Amount of sample	0.50 g of plant sample	
Digestion reagent	+ 10 mL of HNO_3	
Steps	0-5 min.	100 ºC
	5-10 min.	150 ºC
	10-20 min.	180 ºC

Analysis of samples

Analysis of vitamin A and E by HPLC-DAD: For determination of vitamins A and E, a reverse-phase column (Inertsil C18 (250 mm×4.6×5 µm)) was used throughout the experiments. The mobile phase consisted of methanol/acetonitrile (95:5) with a flow rate of 1.2 mL/min at isocratic mode. Injection volume was 20 µL for each analysis. A DAD detector was used for quantification of vitamins A and E at 324 nm and 292 nm, respectively. Retention times were 4.49 min for vitamin A and 11.20 min for vitamin E. The results were calculated with the help of the peak areas of standard solutions. Lab solution software provided by Shimadzu was used for evaluation of chromatograms.

Analysis of metals by FAAS: An atomic absorption spectrometer was used to determine metals in digested samples by microwave digestion system. All samples were filtered before analysis by a 0.45 μm filter. All determinations were performed in air/acetylene flame at conditions proposed by the manufacturer.

RESULTS AND DISCUSSIONS

Optimization of Soxhlet Extraction Parameters

Soxhlet extraction technique was employed for the extraction and separation of chemical constituents in Madımak. 20.00 g of plant sample was refluxed in 400 mL of solvent for 8 hours. Three different solvents (ethanol, methanol, and n-hexane) were used in order to choose the best extraction conditions. Five parallel extraction systems were used for repetitive analyses. The extracts were filtered and concentrated by using a rotary evaporator until dryness. After extraction, solvents were evaporated and raw extracts were obtained about 1 g for each samples. The concentrated extracts were re-solved (diluted) prior to HPLC analysis. The solvent selection in this step is also very critical for sensitive and reliable determinations. We tried five different solvents in this step namely ethanol, methanol, acetonitrile, n-hexane, and acetone. Analysis results were illustrated in Figure 2. Standard addition method was applied to samples in order to evaluate the recovery of extraction procedures. 20.00 μg mL^{-1} of vitamin was spiked into the samples and recovery values were calculated by considering the measured and added concentrations.

As seen in Figure 2, the best signals were obtained by ethanol. According to experimental results, ethanol is a good solvent both for main extraction and re-dissolution of the extract. So, subsequent extractions were carried out by using ethanol.

A series of experiments were also made to choose ideal extraction time. The extraction of 20.00 g of the sample was carried out by using ethanol throughout 3, 6, 8, and 12 hours. According to the experimental results, there is not an important change in the amount of extractable vitamins after 8 hours. So, the samples were refluxed for 8 h in the next studies.

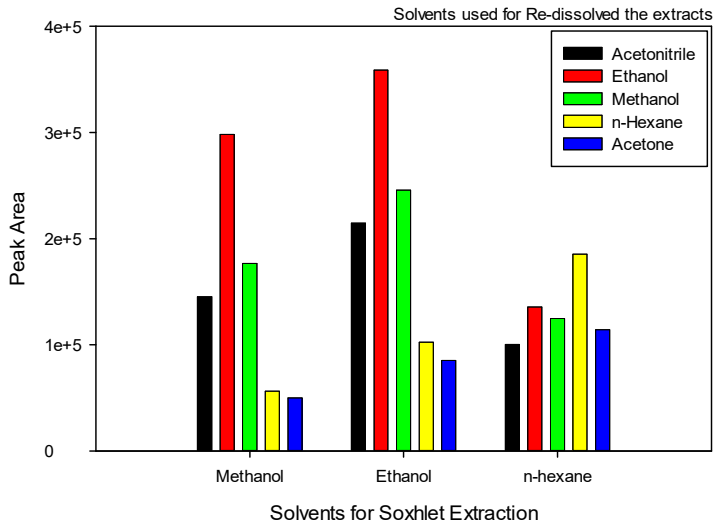

Figure 2. The effects of various solvents on extraction

Amounts of Vitamin A and E in the Plant Samples

After the conditions were optimized, samples were prepared and their vitamins A and E contents were determined by HPLC-DAD system. Chromatograms obtained from the standard and the sample were shown in Figure 3. As can be seen from the figure, the peak of vitamin A was not observed in the samples. Analysis results were presented in Table 2.

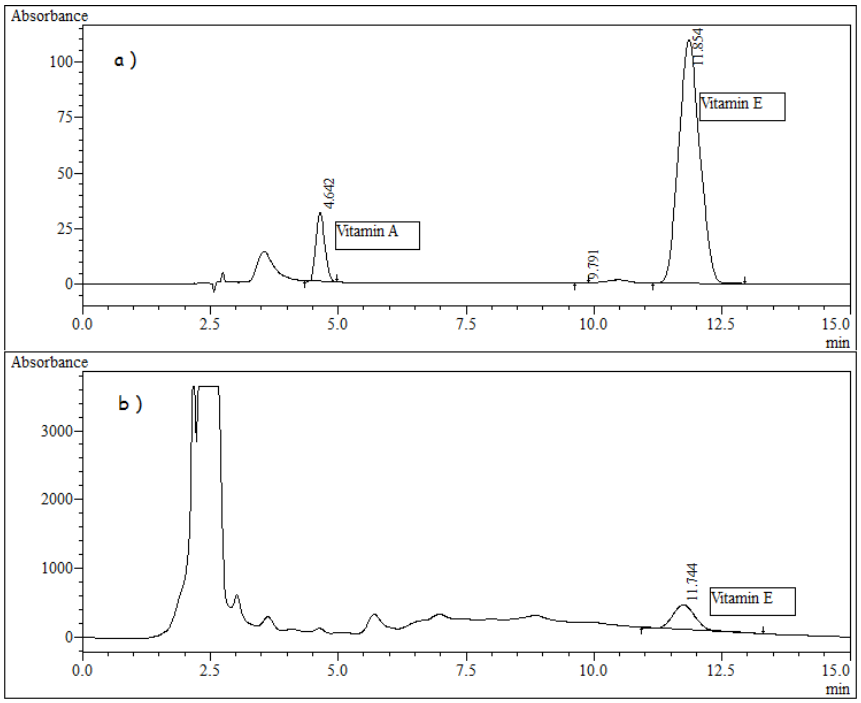

Figure 3. Chromatograms for standard solution (a) and sample (b)

Table 2. The contents of vitamins A and E in Madımak (N:5)

Vitamins		**M1**			**M2**		
	Added	**Found mg kg^{-1}**	**% RSD**	**% R**	**Found mg kg^{-1}**	**% RSD**	**% R**
	0	Not detected	-	-	Not detected	-	-
A	20.00	21.26±1.42	6.67	106.30	20.65±1.39	6.73	103.25
	40.00	40.92±2.02	4.94	102.30	39.88±2.22	5.57	99.70
	0	63.01±4.55	7.22	-	59.18±5.14	8.69	-
E	20.00	84.20±5.88	6.98	101.43	78.66±5.55	7.06	99.30
	40.00	101.99±6.24	6.12	99.00	97.87±6.12	6.25	98.67

Optimization of Metal Analysis Procedure

The preparation of samples were carried out by microwave digestion system explained in the previous section. For this purpose, the explained procedure in Table 1 was applied to the samples. The concentrations of metal ions were determined by using a flame atomic absorption spectrometer.

Firstly, the concentration of nitric acid used in digestion procedure was optimized to avoid the high acidic medium. According to manufacturer's proposals, digestion procedure was applied by using 0.50 g of sample and 8.0 mL HNO_3 as solvent at different concentrations. The extraction of spiked model solutions were repeated with various concentration of HNO_3 in the range of 5-16 mol L^{-1}. As seen in Figure 4, there is not an important change on amounts of extractable metals after 10 mol L^{-1} concentration. In addition, it needs as little as possible acidic conditions in the digestion in order to avoid high ionic strength. If the solution is more acidic, it will be more difficult to neutralize the solution prior to determination. So, 10 mol L^{-1} of HNO_3 was selected in the digestion procedure. As seen in Figure 4, 10 mol L^{-1} HNO_3 is enough for a quantitative digestion. So, determination of metal species were carried out by using these optimal parameters.

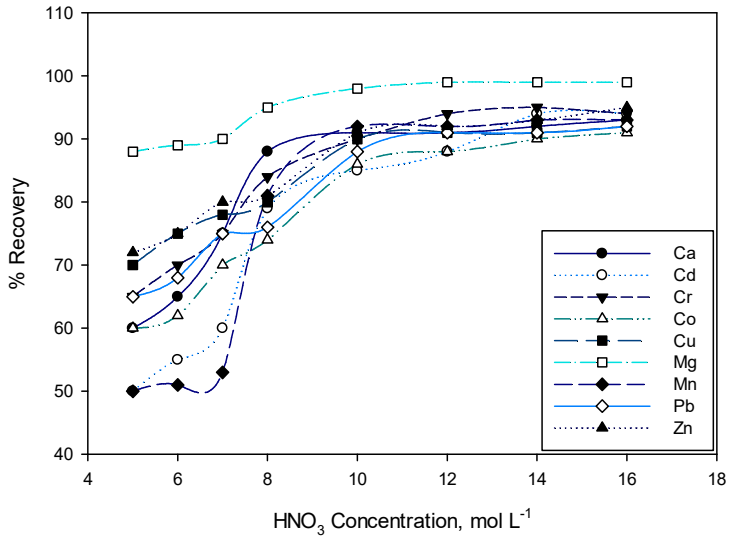

Figure 4. Optimization of HNO_3 concentration

Amounts of Various Metals in the Plants Samples

The plant samples were prepared by using the optimized digestion method as explained in previous section and their analysis were performed by flame atomic absorption spectrometer (FAAS). The obtained results were presented in Table 3.

CONCLUSIONS

In this study, the detailed chemical analysis of *Polygonum cognatum Meissn* (Madımak) has been performed for the first time. There are a few studies in literature dealing with some properties of Madımak such as anti-microbial activity, antioxidant activity (20) but they are inadequate to display its nutrient properties. According to vitamin analysis results, the plant includes vitamin E at important amounts while does not include vitamin A. Both direct analysis and spiked analyses were shown that the amount of vitamin A was under the limit of detection for our detection method. When analysis results and recommended values were considered together, it can be said that Madımak contributes to intake essential elements and vitamin E by daily diet.

Table 3. Results of metal analysis in Madımak Plant (N:5)

Elements		**M1**			**M2**		
	Added	**Found mg kg^{-1}**	**% RSD**	**% R**	**Found mg kg^{-1}**	**% RSD**	**% R**
	0	65.190±2.553	3.9	-	44.500±4.473	8.4	-
Ca	20.000	81.255±3.895	4.8	95.39	63.545±4.985	7.8	98.5
	40.000	96.847±5.587	5.8	92.07	88.384±5.354	6.1	104.6
	0	0.045±0.003	6.7	-	0.105±0.006	6.1	-
Cd	0.020	0.063±0.004	6.4	96.92	0.128±0.010	7.8	102.4
	0.040	0.090±0.005	5.6	105.88	0.144±0.014	9.7	99.3
	0	0.080±0.002	1.9	-	0.079±0.007	8.8	-
Cr	0.020	0.096±0.004	4.1	96.0	0.103±0.008	7.8	104.0
	0.040	0.115±0.009	7.8	95.8	0.125±0.010	8.0	105.0
	0	0.091±0.008	8.8	-	0.124±0.011	8.4	-
Co	0.020	0.115±0.010	8.7	103.6	0.140±0.012	8.6	97.2
	0.040	0.136±0.012	8.8	103.8	0.161±0.013	8.1	98.2
	0	0.399±0.031	7.8	-	0.351±0.012	3.4	-
Cu	0.100	0.487±0.041	8.4	97.5	0.463±0.021	4.5	102.7
	0.200	0.586±0.043	7.3	97.8	0.569±0.025	4.4	103.3
	0	6.774±0.445	6.6	-	7.646±0.237	3.1	-
Fe	2.000	8.650±0.551	6.4	98.6	9.815±0.443	4.5	101.8
	4.000	10.698±0.612	5.7	99.3	11.843±0.563	4.8	101.7
	0	66.522±2.550	3.8	-	66.983±1.483	2.2	-
Mg	20.000	88.147±3.654	4.1	101.8	87.548±3.654	4.2	100.6
	40.000	109.224±5.552	5.1	102.6	110.278±4.654	4.2	103.1
	0	4.535±0.123	2.7	-	4.289±0.095	2.2	-
Mn	2.000	6.866±0.254	3.7	105.1	6.120±0.107	1.7	97.3
	4.000	8.442±0.360	4.3	98.9	6.250±0.101	1.6	99.4
	0	0.046±0.004	7.8	-	0.047±0.004	8.5	-
Pb	0.020	0.065±0.006	9.2	98.4	0.070±0.006	8.6	104.5
	0.040	0.083±0.007	8.4	96.7	0.085±0.007	8.2	97.7
	0	10.364±0.433	4.2	-	9.139±0.230	2.5	-
Zn	2.000	12.875±0.687	5.3	104.1	11.312±0.645	5.7	101.6
	4.000	14.215±0.702	4.9	98.9	13.444±0.727	5.4	102.3

Results of metal analysis can be evaluated according to their toxicity and essential functions. The amounts of Cd, Cr, Co, and Pb in the analyzed samples were lower than legal limits according to FDA. As seen in Table 3, the amounts of these metals are under 0.15 mg kg^{-1}. So, it can be said that the studied plant samples does not include common toxic metal components according

to our determination conditions. Cu concentration in the samples were determined as 0.351 and 0.399 mg kg^{-1} in samples. We think that herbicides or insecticides may cause this results. Because, this kind of chemicals are used on April-May period in the sampling region to destroy unwanted plants. As known, most of these chemicals may include copper. So, the amount of copper is higher than expected. One of important results of study is the content of Mn, Fe and Zn in Madımak. As known, Zn, Fe and Mn are essential elements for a healthy diet.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that there is not unethical situation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Türkçe Öz ve Anahtar Kelimeler

Polygonum Cognatum Meissn Bitkisinin (Madımak) Temel Element Analizi ve Yağda Çözünen Vitaminleri için Ekstraksiyon Parametrelerinin Uygunlaştırılması

Halil İbrahim Ulusoy, Hilal Acıdereli, Uğur Tutar

Öz: Yağda çözünen vitaminlerin (A ve E) ve çeşitli metal iyonlarının (Ca, Cd, Cr, Co, Cu, Fe, Mg, Mn, Pb, Zn) derişimi Sivas, Türkiye'de toplanan *Polygonum cognatum Meissn* bitki örneklerinde tespit edilmiştir. Vitamin analizleri Soxhlet ekstraksiyonunun ardından HPLC ile, metal iyonlarının varlığı ise mikrodalga çözünürleştirmesinin ardından FAAS ile tayin edilmiştir. Çalışma iki ana hedefe odaklanmıştır: Anadolu'da yetişen yaygın bir yenebilir bitki türünün kimyasal analizi ve tayinden önce örnek hazırlama parametrelerinin en uygun hale getirilmesi. Vitamin ve çeşitli metal türleri açısından bu bitki hakkında yeterli bilgi bulunmamaktadır. Sonuç olarak, iyi bilinen ve bolca tüketilen bir bitki için literature faydalı ve ayrıntılı bilgiler sunulmuştur.

Anahtar kelimeler: *Polygonum cognatum meissn* (Madımak); vitamin A; vitamin E; metal analizi; HPLC; FAAS.