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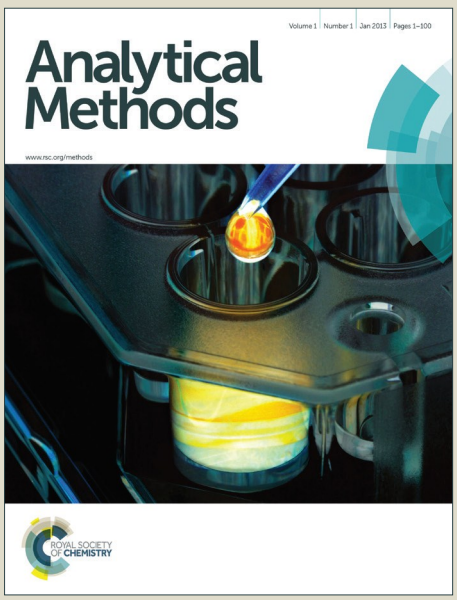

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ARTICLE

Sample preparation for cadmium quantification in sunflower (*Heliánthus ánnuus*) seeds using anodic stripping voltammetry

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Performance of acid extraction, dry ashing, and microwave digestion was evaluated for voltammetric cadmium quantification in oil crops with sunflower (*Heliánthus ánnuus*) seeds as an example. Standard additions, reference material analysis, and comparing to graphite furnace atomic absorption spectrometry were employed for validation. The main aim of the study was to address the difficulties of oil crops mineralization such as high fat content, material inhomogeneity, and complications in maintaining sample representativeness. 6 brands of fried sunflower seeds (Krasnodar region, Russia) were studied. Acid extraction with nitric acid and hydrogen peroxide provided low digestion efficacy, while dry ashing provided efficient sample decomposition, however, resulted in considerable time frame extension. Microwave-assisted digestion provided the best performance with a dynamic range of 0.05-0.75 µg/g, detection limit of 0.02 µg/g, spike recoveries of 95-100%, and within-run relative standard deviation of 5-7%, when using anodic striping voltammetry for Cd quantification.

Introduction

Sunflower (*Heliánthus ánnuus*) seeds are used for producing vegetable sunflower oil, for making deserts (sunflower halva) and they are also quite intensively consumed as a popular 'snack-seed' in Russian Federation and other countries.[1] Sunflower plant is known as Cd bioaccumulator.[2-5] Nevertheless, Cd pollution of sunflower seeds was not studied properly.[6]

In the current study, Cd content of sunflower seeds was investigated. Predominantly, for the determination of trace amounts of heavy metals atomic spectrometry[7-12] and electrochemical methods[13-17] are employed, whereas cereal or legume crops, *e.g.* rice, wheat or soybean, are the most frequently analyzed foodstuffs.[18, 19] Those samples are characterized by relatively low oil content, less than 1.5% for rice, 2.5% for wheat, and about 20% for soybeans.[18] Thus, reliable sample preparation approaches and valid assessment of trace elements using conventional techniques are implemented easier than for oil crops. Sunflower seeds contain a significant amount of oil up to 52%,[20, 21] complicating sample handling already at the stage of grinding and aliquot-taking. Additionally, sunflower seeds contain organic compounds able to form complexes with trace elements[4, 22] further complicating implementation of analytical techniques sensitive towards chemical speciation of the analyte including electrochemical methods.

The most common way of matrix interferences elimination, employed in trace element determinations, is total sample decomposition.[23-26] Nowadays, microwave-assisted digestion is becoming the most widely used technique for sample mineralization. However, in this technique the initial sample weight taken for mineralization is usually lower compared to conventional 'open vessel' approaches.[26] This states a problem of inadequate sample representativeness. Besides, in case of oil crops oil emission may accompany sample grinding[27, 28] with consecutive particle sticking worsening the representativeness even further. On the other hand, 'open vessel' digestion techniques provides better initial sample representativeness,[25, 29] yet their implementation may lead to substantial losses of the analyte in the form of volatile compounds or sample contamination.[23] The aim of the present study was to evaluate different approaches to sample preparation of oil-rich foodstuffs prior to voltammetric Cd quantification with sunflower seeds as an example.

Materials and Methods

Instrumentation

Voltammetric analyzer AKV-07MK (Akvilon, Moscow, Russia) with Polar® software was used throughout this work. Previously introduced electrochemical method[17] was modified for Cd measurement in sunflower seeds. The method provides linearity range of 0.3-6.5 µg/L with Cd limit of detection of 0.14 µg/L. Mercury modified carbositall electrode ARK-1 (Akvilon)

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Electronic Supplementary Information (ESI) available: Table S1 contains the data on microwave-assisted digestion program optimization. Fig. S1 contains voltammograms for supporting solutions (*a*) 6M HCl and 3.5 M NaCl; (*b*) 1M HNO_3 and 0.01M $Hg(NO_3)_2$; (*c*) acetate buffer (pH = 5.6); (*d*) 0.5M HCl; (*e*) 1M HCl and 0.01M $Hg(NO_3)_2$; (*f*) 1M HCl, 0.01M $Hg(NO_3)_2$ and 3.5M KCl.. See DOI: 10.1039/x0xx00000x

was used as a working sensor. Glassy carbon crucible was employed as an auxiliary electrode, whereas silver chloride electrode EVL-1M4 (Akvilon) was used as reference sensor. Voltammogram registration parameters were optimized according to the analyzer's manufacturer guidelines. These parameters are presented in Table 1.

Additional measurements were performed by graphite furnace atomic absorption spectrometry (GFAAS) using spectrometer MGA-915MD (Lumex, St. Petersburg, Russia), equipped with Massman-type integrated L'vov platform graphite furnace (Schunk Kohlenstofftechnik, Wettenberg, Germany) and hollow cathode lamp (228.9 nm, Cortec, Moscow, Russia). Measurements were performed in accordance with the optimized furnace program (Table 2). For GFAAS method, linearity range was 0.1-3.5 µg/L with Cd detection limit of 0.03 µg/L.

Muffle furnace LF-25/11-G1 (LOIP, St. Petersburg, Russia) was employed for dry ashing. Master MDS-10 system (Sineo, Shanghai, China) was used for microwave-assisted digestion. A source of ultrapure water was Milli-Q® Advantage A10 (Merck Millipore, Molsheim, France).

Standards and chemicals

Stock Cd standard solution was GSO 6690-93, 1.0 g/dm^3 (Monitoring, Moscow, Russia), which was serially diluted with Milli-Q® water or supporting electrolyte to produce spiking or calibration solutions, respectively. For selectivity studies stock solution of lead (Pb) GSO 7012-93, 1.0 g/dm^3 (Water Research and Control Center, St. Petersburg, Russia) was used. Reference material of soybean meal CCA № 10-160-2009 (Ormet, Ekaterinburg, Russia) was analyzed for validation purposes.

All used chemicals were at least of analytical grade. Concentrated nitric acid, HNO_3, Suprapure® 65% (Merck, Darmstadt, Germany) and 40% hydrogen peroxide, H_2O_2, (Nevareaktiv, St. Petersburg, Russia) were used for sample digestion. As supporting electrolytes the following solutions were tested: acetate buffer (pH = 5.6, NevaReaktiv); 0.5 M hydrochloric acid, HCl (NevaReaktiv); 1 M HNO_3 and 0.01M mercury nitrate, $Hg(NO_3)_2$ (Vekton, St. Petersburg, Russia); 1 M HCl and 0.01 M $Hg(NO_3)_2$; 1 M HCl, 0.01M $Hg(NO_3)_2$, and 3.5 M potassium chloride, KCl (NevaReaktiv).

Samples

In the current investigation, 6 samples of fried sunflower seeds from 6 major brands originating from Krasnodar region of Russian Federation were analyzed. According to manufacturers, all seeds under study were harvested in 2013. Samples included into the study were randomly selected amongst several batches of packages for each studied brand. All samples were purchased on the local market. For all packages expiration dates, integrity of hermetic packs, and batch numbers were checked prior to the inclusion into the study. Samples were pre-dried (2 h, 130 °C) and grounded using a household blender or a vibration mill GSM 06, (Siebtechnik, Duisburg, Germany). The first portion of crushed material was discarded to avoid contamination.[30] After that,

Table 1

Voltammograms registration parameters

Measurement parameter	Value
Scanning mode	Positive
Electrode cleaning potential	0.0 V
Cleaning time	180 s
Scanning speed	0.05 V/s
Electrolysis potential	– 1.3 V
Detection potential	– 0.7 V
Scanning amplitude	1.0 V
Accumulation time	60 s
Cycle number	2

Table 2

Furnace program for atomic absorption spectrometry measurements

Stage	Temperature, °C	Time, s	Shear Ar flow, L/min
Drying	100	45	0.2
Pyrolysis	450	20	0.2
Atomization	1460	2.5	0.0
Cleaning	1800	2	0.8
Pause	-	90	0.8

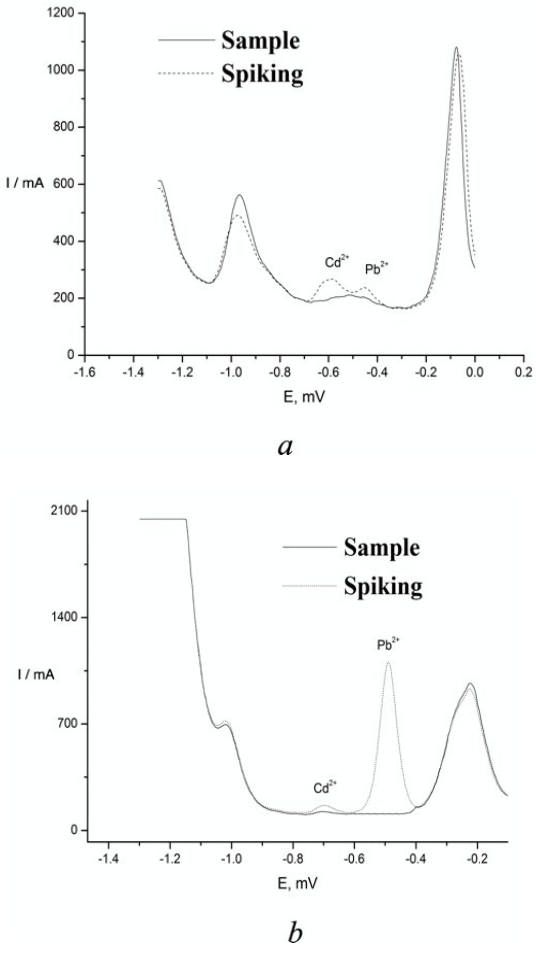

Fig. 1. Voltammograms for sunflower seeds samples and these with spikings of Cd and Pb; supporting electrolytes were 1M HCl and 0.01 M $Hg(NO_3)_2$ (*a*) and 1M HCl, 0.01M $Hg(NO_3)_2$, 3.5M KCl (*b*)

samples were submitted to digestion. For sample comminution studies a set of nylon sieves (Ecochim, St. Petersburg, Russia) was used. Blank solutions were prepared using the same procedures, reagents and materials as for the samples under study. All presented results correspond to dry weight and are blank subtracted.

Statistics

Standard parametric statistics (Student, Fisher, Cochrane) were employed for data evaluation. Throughout the paper, results are presented as mean ± confidence interval for the confidence level β = 0.05. Standard Excel® 2007 software (Microsoft, Redmond, WA, USA) was used for calculations.

RESULTS AND DISCUSSION

Supporting electrolyte selection

Several solutions were tested to select the optimal supporting electrolyte. Characteristic peaks of Cd were obtained only for supporting solutions containing 1M HCl and 0.01M $Hg(NO_3)_2$ or 1M HCl, 0.01M $Hg(NO_3)_2$ and 3.5M KCl. Contrary to the data of *e.g.*[13-16] for the supporting electrolytes containing: 6M HCl and 3.5 M NaCl; 1M HNO_3 and 0.01M $Hg(NO_3)_2$; acetate buffer (pH = 5.6); or 0.5M HCl, reliable analytical signals of Cd were not observed. On the other hand, according to the results of *e.g.* Rusinek *et al*.[31], 'bare' carbon electrode without mercury modification shows quite poor sensitivity for Cd. For more details, please, see ESI (Fig. S1). To confirm peak identification sequential spikings of Cd and Pb corresponding to 0.25 μg/g were made. Results are presented in Fig. 1 (*a*, *b*).

Notably, only for supporting solution '*b*' (1M HCl, 0.01 M $Hg(NO_3)_2$, and 3.5M KCl) adequate resolution of Cd and lead peaks was observed, while electrolyte '*a*' did not provide reliable selectivity.

'Open vessel' mineralization

Although microwave-assisted decomposition is largely introduced for digestion of different matrices, conventional 'open vessel' approaches such as wet digestion and dry ashing are still being used in many laboratories[32]. Some recent studies recommended dry ashing for food elemental analysis as reliable sample preparation technique if compared to acid extraction.[33-35] Additionally, this is legally approved sample preparation technique for food analysis in Russian Federation.[29] At the first stage of the study, the performance of acid extraction with HNO_3 / H_2O_2 and dry ashing was evaluated as sample digestion techniques prior to Cd quantification in sunflower seeds by anodic striping voltammetry (ASV).

In case of acid extraction, each sample of 6 studied brands (*ca*. 1 g) was placed into a glass cup and kept with concentrated HNO_3 (40 mL) for at least 30 minutes. After that cup content was quantitatively transferred to a round bottom flask. Concentrated H_2O_2 (5 mL) was added and the reaction mixture was kept boiling under reflux for 4 hours. Afterwards, digestates were cooled, filtered through a double defatted paper filter (d = 80 g/m^2, total oil and resin content ≤ 0.04%, ash content < 0.1 mg per filter), quantitatively transferred to volumetric flasks (250 mL), diluted with supporting electrolyte and submitted to Cd determination.

Table 3

Determination of Cd in sunflower seeds after acid extraction and dry ashing using ASV and GFAAS (mean ± confidence interval, n = 3, P = 0.95)

	Mass fraction of Cd, μg/g			
Sample	Acid extraction		Dry ashing	
	ASV	GFAAS	ASV	GFAAS
1	0.10 ± 0.06	0.18 ± 0.07	0.25 ± 0.06	0.22 ± 0.06
2	0.07 ± 0.04	0.16 ± 0.08	0.23 ± 0.05	0.22 ± 0.05
3	0.09 ± 0.06	0.16 ± 0.07	0.24 ± 0.03	0.23 ± 0.04
4	0.05 ± 0.01	0.13 ± 0.05	0.17 ± 0.04	0.16 ± 0.04
5	0.10 ± 0.06	0.17 ± 0.05	0.25 ± 0.03	0.24 ± 0.03
6	0.05 ± 0.04	0.11 ± 0.03	0.19 ± 0.02	0.19 ± 0.01

Ashing procedure was as follows: sample (*ca.* 2 g) was placed into a porcelain crucible and gently heated till full carbonization. Crucibles with carbonized specimens were placed into the muffle furnace with initial temperature of 200°C. The temperature was gradually raised to 450°C within 5 hours. Totally, sample was being incinerated during a working shift (8 hours). After that, the samples were cooled, the content of the crucibles was wetted with a few drops of HNO_3, acid was evaporated and the residue was cooled again. The crucibles were placed into the muffle furnace and heated (450 ^{0}C) for another 30 min. Criterion of mineralization fulfillment was pale pink color of incineration residue and absence of any dark inclusions or particles. The resulting incineration ash was dissolved in HNO_3, quantitatively transferred to a volumetric flask (25 mL) and diluted with supporting electrolyte. Table 3 represents the results of Cd determination in 6 digestates of sunflower seeds after acid extraction and dry ashing using ASV and GFAAS as a reference method. For dry ashing spike recoveries (0.25 μg/g Cd) were in the range 89-95% for ASV and 92-96% for GFAAS. Acid extraction provided worse performance with the spike recoveries of 70-84% and 79-88% for ASV and GFAAS, respectively.

Acid extraction of sunflower seeds before voltammetric Cd determination seems to be unsuitable sample preparation technique, judging from acquired results (Table 3). Cd signal underestimation was related, possibly, to low efficacy of organic matrix decomposition. On the other hand, dry ashing was applicable for the determination of Cd by ASV providing complete decomposition of the organic matrix. Nevertheless, since dry ashing is too time-consuming and lacks robustness for serial analyses,[26] it could hardly be recommended for contemporary food elemental analysis rather than as reference decomposition approach for validation purposes.

Microwave digestion

In order to select optimal conditions of sample decomposition in the microwave oven power was altered in the range 400-1000 W, and decomposition time was varied in the range 18-30 min. Digestion efficacy was controlled visually (absence of

particles, turbidity or opalescence) and by assessing spike recoveries (corresponding to 0.25 μg/g Cd). Reagent quantities for initial optimization were in accordance with digesting system guidelines (5 mL HNO_3 and 1 mL H_2O_2). The best performance with spike recovery of 96% was observed for the power of 800W with 30 min microwave exposure. For more detail on this stage of optimization, please, see ESI (Table S1).

Reagent combinations (HNO_3 and H_2O_2) were also optimized. In the preliminary experiments incomplete sample decomposition was observed when total reagent volume was below 3 mL. Reagent optimization was performed as follows: sample (*ca.* 0.2 g) was put into the decomposition vessel and variable volumes of HNO_3 and H_2O_2 (0.5-5.0 and 1.0-3.0 mL for HNO_3 and H_2O_2, respectively) were added. Performance was evaluated through visual examination, assessment of relative standard deviation (RSD) and spike recovery (0.25 μg/g Cd). The most reliable results were acquired for the volume ratio of HNO_3 to H_2O_2 of 1 : 2. That was quite unexpected as normally excess of HNO_3 over H_2O_2 is used. To the best of our knowledge, in all original papers concerning oil or cereal grain Cd determination, including microwave-assisted digestion, nearly exclusively HNO_3 excess over H_2O_2 was used, see *e.g.*,[30, 36-39] whereas Kucukkolbasi *et al.*[40] used equal volumes of HNO_3 and H_2O_2 for vegetable oils decomposition. However, our own results showed that in case of sunflower seeds using of H_2O_2 excess provided better performance, possibly, due to better oil-rich organic matrix decomposition.

Sample comminution influence

Sample comminution techniques contribute greatly to decomposition kinetics, increasing contact surface[41] as well as cause additional problems such as sample contamination.[42] When using microwave digestion, one should not disregard that a sample fraction taken for further pre-treatment is minor compared to 'open vessel' techniques, so for inhomogeneous samples like sunflower seeds severe problem of unrepresentativeness may arise. Thus, the effect of sample comminution on the measured Cd was investigated.

In the preliminary studies, a vibration mill was tried for sample crushing in order to produce ground sample with better particle size homogeneity. Although the vibration mill provided more regular particle size than household blender, copious oil extraction during sample comminution resulted in worse performance (RSD and spike recoveries), possibly, due to substantial loss of Cd or sample contamination. So further experiments were carried out using comminution with a blender. To study sample comminution effect on Cd quantification, pre-dried samples were crushed and sifted using a set of nylon sieves (pore diameters ranging 0.25-1.0 mm) to produce fractions of different particle size. After that, samples were submitted to microwave digestion according to the previously optimized procedure. Results of ASV determination of Cd are presented in Fig. 2.

A tendency of higher measured Cd concentration for smaller particle was observed, possibly, due to more efficient decomposition of fine particles. On the other hand, for smaller particles increased RSDs were acquired. In turn, this may be related to lower size homogeneity, oil emissions, and sequential particle sticking at the stage of grinding and sampling prior to digestion. Notably, significant difference in measured Cd mass fraction was found only for the size fraction of ≥ 1.0 mm, so samples should be grounded to the state below this limit and particle size homogeneity should be provided to ensure result precision.

Optimized digestion procedure

Optimized procedure of sunflower seeds microwave mineralization was as follows: sunflower seed sample, pre-dried and crushed with the household blender (*ca*. 0.2 g), was placed into a vessel for microwave digestion. 1 mL of concentrated HNO_3 and 2 mL of concentrated H_2O_2 were added and microwave decomposition was performed for 30 min using autoclave pressure of 2.0 MPa and power of 800 W. After microwave digestion, samples were quantitatively transferred to volumetric flasks (25 mL), diluted with supporting electrolyte and submitted to Cd quantification (according to the parameters in Table 1).

Validation and application of the method

The proposed method was validated using reference material of crushed soybean meal. Acquired Cd mass fraction of 0.16 ± 0.02 μg/g was in concordance with the target value of 0.15 ± 0.02 μg/g (recovery of 107%), which confirmed the accuracy of the method. The developed method was used for the analysis of the real sunflower seeds samples originating from the Krasnodar region. Alternatively, Cd in these samples was measured by GFAAS. Results for both methods are presented in Table 4.

The acquired results for GFAAS and ASV were in concordance with each other, which also indicated the validity of the method. Analysis of the data in Table 4 shows that at the optimum decomposition conditions the method spike recovery for ASV ranged from 95 to 100% and for GFAAS from 98 to 100%, which excels the results obtained for dry ashing (89-95%and 92-96% for ASV and GFAAS, respectively). Noteworthy, the measured Cd mass fractions in all the samples under study were found to be decreased if compared to the data of Rojas-Cifuentes *et al.*[43]. Lee *et al.*[5] reported considerable Cd adsorption from contaminated soils resulting in elevated Cd content in all parts of sunflower plant including the seeds. For soils containing 1.5 μg/kg Cd, seeds Cd content of 3.60 μg/g was reported, whereas for extremely contaminated soil with 30 μg/kg Cd the value[5] reached 39.6 μg/g. Much lower Cd content was reported for vegetable oils including those produced of sunflower. For instance, Mendil *et al.* [39] reported Cd level of 3.76 ± 0.40 μg/kg in sunflower oil produced in Turkey, whereas the level below 3.6 μg/kg was found in virgin olive oil [44]. Interestingly, according to results published by Acar[36] and Pehlivan *et al.*,[45] Cd content of sunflower oil originating from Turkey was in the range 0.8-4.5 μg/kg (for different brands) and 43 ± 6 μg/kg, respectively, which is still much lower compared to our data for the seeds. On the other hand, for crude unrefined sunflower oils from Pakistan much higher Cd level of up to 6.18 ± 0.88 μg/g was reported [37]. More recent data[40] showed high Cd removal

Table 4

Sunflower seeds Cd content of 6 different brands assessed by ASV and GFAAS (mean ± confidence interval, $P = 0.95$, $n_{ASV} = n_{GFAAS} = 5$). Spikings of 0.25 µg/g Cd were added to each sample to assess recoveries

Specimen	Mass fraction of Cd, µg/g				Spike Recovery, %	
	Sample		Sample + spike			
	ASV	GFAAS	ASV	GFAAS	ASV	GFAAS
1	0.21 ± 0.03	0.30 ± 0.03	0.45 ± 0.04	0.55 ± 0.02	97.8	100.0
2	0.22 ± 0.06	0.25 ± 0.04	0.45 ± 0.08	0.49 ± 0.06	95.7	98.0
3	0.18 ± 0.04	0.19 ± 0.03	0.43 ± 0.06	0.43 ± 0.05	100.0	97.7
4	0.14 ± 0.03	0.15 ± 0.03	0.38 ± 0.07	0.40 ± 0.06	97.4	100.0
5	0.20 ± 0.05	0.23 ± 0.04	0.45 ± 0.05	0.47 ± 0.05	100.0	97.9
6	0.18 ± 0.06	0.23 ± 0.05	0.41 ± 0.08	0.47 ± 0.06	95.3	97.9

efficacy from crude sunflower oil during refinement, being more than 99% (Cd level for crude oil was 1.51± 0.18 µg/g,after refinement decreased to 0.50 ± 0.38 µg/kg). Anyway, it should be noted that Cd control in oil crops still remains somehow disregarded. Most published works relate to the element analysis of cereal grain – rice, wheat *etc.*[46-49]

Data processing and analytical figures of merit

Limit of detection was evaluated using corresponding blank solutions. The microwave-assisted method provided a dynamic range of 0.05 to 0.75 µg/g Cd per sample (initial sample weight of 0.2 g) with detection limit of 0.02 µg/g. For dry ashing method the limit of detection was 0.01 µg/g with dynamic range of 0.03 to 0.25 µg/g owing to larger initial sample weight of *ca*. 2 g.

For microwave decomposition average within-run precision (relative standard deviation, RSD) did not exceed 10% and usually was at the level of 5-7%. However, between-digestion RSDs were sometimes greater than 20%. That might be related partially to the influence of sample comminution (Fig. 2) but mainly to the low initial sample weight causing difficulties in maintaining of sample representativeness. Oil extraction during sample crushing may also contribute to high RSD values.

Although quite high RSDs were obtained for independent replicate digestions, no outliers amongst acquired data were found using Student *t*-test. Maximal calculated *t* values of 1.75 and 1.47 for ASV and GFAAS respectively did not exceed critical values of 1.87 ($n = 5$, $P = 0.95$). ASV and GFAAS were pairwise compared for each sample using Fisher's *F*-test ($P = 0.95$, $v_1 = v_2 = 4$). The dispersion of both methods was found to be statistically equal. Maximal calculated *F*-value of 1.96 was found for the sample No. 2, yet it still did not exceed $F_{crit.}$ of 6.39. Notably, the sample No. 2 was the most oil-rich one amongst all 6 studied sunflower seed specimens. High oil content, possibly, worsened the precision, especially for ASV. 'Within method' data for different samples were also compared using Cochrane *C*-test ($P = 0.95$, $n = 6$). In this case dispersion was also found to be equal for all the samples ($C_{ASV} = 0.26$ and $C_{GFAAS} = 0.27 < C_{crit.} = 0.5065$).

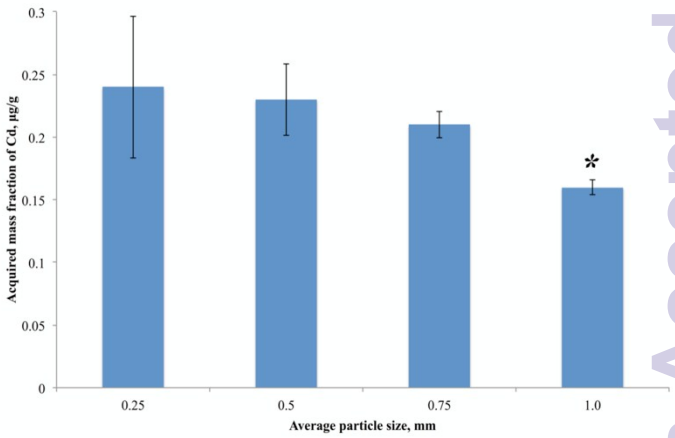

Fig. 2. Measured Cd content for different size-fractions of crushed sunflower seeds. Error bars represent confidence interval ($n = 5$, $P = 0.95$)

Conclusions

Using of conventional acid extraction with the mixture of concentrated HNO_3 and H_2O_2 was shown to be inappropriate for Cd quantification in sunflower seeds, when employing electrochemical detection approach. And even for GFAAS Cd detection acid extraction did not provide reliable results. On the other hand, dry ashing provided adequate sample decomposition for both techniques but it can hardly be called a 'technique of choice' owing to its laboriousness and time requirement. Microwave decomposition was quite expectedly found the most appropriate technique for the determination of Cd in sunflower seeds. Nevertheless, even using of microwave digestion does not eliminate all the obstacles as it may result in sample unrepresentativeness due to low initial sample weight and possible influence of sample comminution and particle size inhomogeneity.

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