Application of ICP-MS in Various Fields of Research

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Introduction

Since the first inductively coupled plasma mass spectrometers (ICP-MS) have had been available in 1980s, inductively coupled plasma mass spectrometry has found its own place in scientific research of elements in various fields. Transport processes of toxic and essential trace elements from soil to plant and finally to humans is one of the most exciting areas of research. By the outstanding sensitivity of ICP-MS instruments – that means very low (sub-ppb [ppb=μg/L]) detection limits – and multielemental capacity, despite of its high capital and running costs, inorganic mass spectrometry has became a unique and unreplaceable analytical technique.

It is well known that the knowledge of the total concentration of an element is not sufficient to estimate its effect, but the distribution of its forms (species) is also needed information. Thus besides of the concentration screening of elements the speciation analysis of trace elements is also a developing area owing to ICP-MS, since coupling of an HPLC or sometimes a capillary electrophoresis with an ICP-MS is a good possibility to study ions in different oxidation states or determine various organo-metallic compounds. In this field different forms of chromium, mercury, tin, arsenic as potentially toxic and selenium as in an essential compounds have been studied thoroughly during the past decades.

Arsenic speciation is soil – Introduction

Arsenic has been gained a great interest because of the varying toxicity of its chemical forms such as arsenite ($As^{III}O_3^{3-}$), arsenate ($As^{V}O_4^{3-}$) and organoarsenic derivatives (methylated-oxoacids, arsenobetaine, arsenocholine etc.) [1]. Whilst inorganic arsenic compounds are basically presented in the environment (surface- and ground water, soil) organic compounds are produced in organisms due to the detoxification of arsenic owing to their lower (monomethyl-arsonic acid) or negligible (arsenobetaine) toxicity [2].

To understand the effect on crops and pathways of arsenic uptake from soil to plant and hence to a human body, a research field was set up at Nagyhörcsök, Hungary, where different levels of arsenic doses were applied on soil-plant system in 1991. One of our aims was – beside to determine the arsenic uptake by different plants – to study arsenic behavior in soil. Firstly it should be cleared that the soil samples which were collected some years ago are able to give appropriate basis for studying inorganic arsenic redox state without alteration in soil.

For this purpose an in lab experiment was made, where soil samples was treated with arsenite and arsenate respectively. Several extraction media and mechanic and ultrasonic agitation were tested to achieve the best recoveries of added arsenic quantity. After a year of storage of the model soils speciation analysis were done by HPLC ICP-MS coupling.

Experimental

For dilution and preparing solutions MilliQ water was used (18 MΩ cm, MilliPore Corporation, USA). In the experiment 200 g soils were mixed and homogenized with appropriate amount of arsenic-trioxide (As(III)) (Sigma-Aldrich) or potassium-dihydrogene-arsenate (As(V) (Sigma-Aldrich) to result an arsenic content of 30 mg kg^{-1}. Actual arsenic content and homogeneity of loading were verified successfully after wet digestion and determination by ICP-MS.

Two extraction modes were tested: a simple horizontal shaking for 1 hour and ultrasonic treatment in an ultrasonic bath for 3x15 minutes. Seven extraction media were applied: de-ionized water, 0.25, 0.30, 0.50 and 1.00 M ortophosphoric acid solutions, 0.25 M H_3PO_3, 0.05 M hydroxyl-amine solution and 0.50 M H_3PO_4 and 0.05 M ascorbic acid solution. Hydroxyl-amine and ascorbic acid were applied as potential preventive agents of arsenite → arsenate conversion during extraction.

Soil and extraction liquid ratios were 1:12.5 in all cases. In the case of ultrasonic treatment between the three consecutive steps 15 minutes centrifugation was applied for phase separation and the extract was decanted and new extraction solvent was added; finally the decanted extracts were combined.

After extraction the concentration of the extracted arsenic was measured by Thermo Scientific XSeries I ICP-QMS applying CCT (7% H_2 in He).

Effect of storage was examined after a year storing of arsenic loaded model soils on room temperature and air-dry state (as the above samples from Nagyhörcsök Experimental Station). Extraction was performed by the extractant resulted the best efficiency. Quality and quantity of arsenic were determined by HPLC-ICP-MS coupling. Eluent was 1.5 mM ftalic acid (Fluka) and 1.5 mM TRIS (Tris-hydroxymethyl-aminomethane, Spektrum-3D, Hungary) with an isocratic flow of 1 ml min^{-1}, the pH of solution was 3.90.

Results and discussion

The best extraction efficiency on the soil samples treated with As (30 mg kg^{-1}) was achieved by 1.0 M phosphoric acid (*Figures 1, 2A, 2B*), however both 1.0 M phosphoric acid solution alone and a mixture of 1.0 M phosphoric acid and 0.1 M ascorbic acid gave the highest extraction efficiency for the control sample (6.53±0.60 mg kg^{-1} arsenic).

Better efficiency was always achieved on the sample treated with arsenate. In the view of mode of aggitation, ultrasonic treatment had given better efficiency (*TABLE 1.*). The last finding derives from that fresh extractant was used between the steps.

Because 1.00 M phosphoric acid gave the best extraction with sonication, for speciation analysis this type of sample preparation was applied. Arsenic forms determined in model soil samples are summarized in TABLE 2, total arsenic content is 36.5 mg kg^{-1}. Extraction efficiency is about 70% and 90% for arsenic(III) and arsenic(V) treated samples respectively.

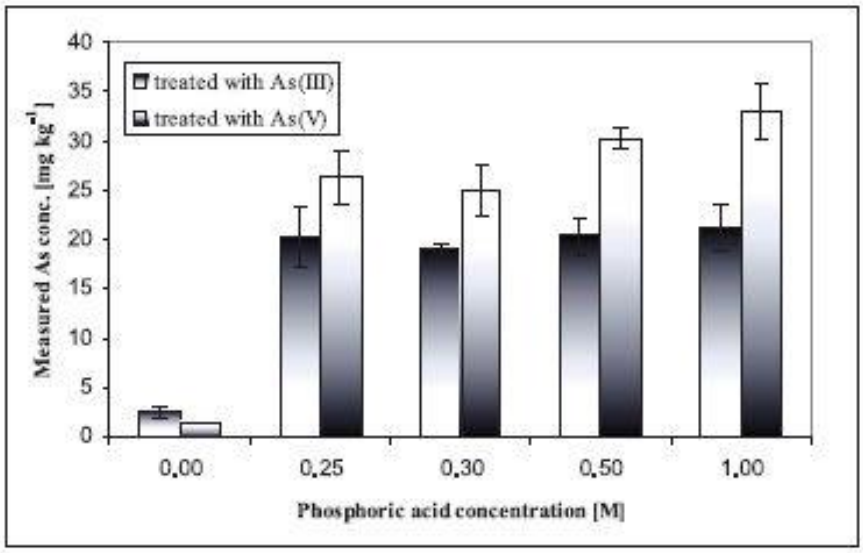

Fig. 1. Extracted arsenic regarding to phosphoric acid solution on treated soil - horizontal shaking, 1 h

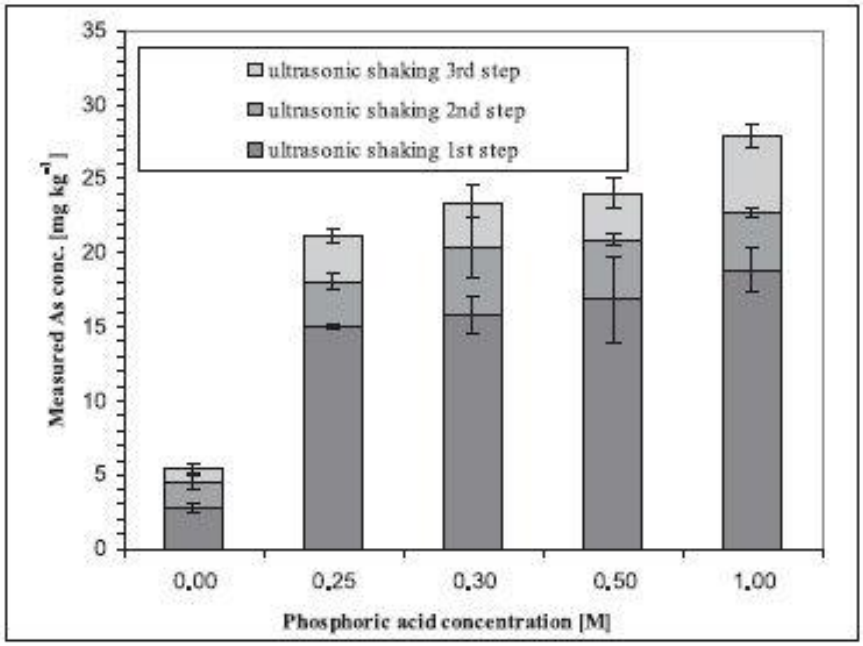

Fig. 2A. Extracted arsenic regarding to phosphoric acid solutions on treated soil - ultrasonic shaking, 3x15 min, soil treated with As(III)

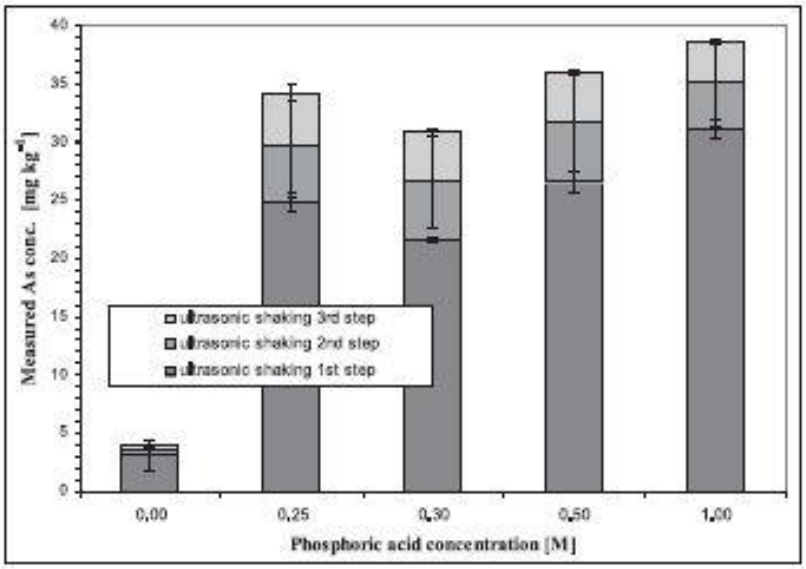

Fig. 2B. Extracted arsenic regarding to phosphoric acid solutions on treated soil - ultrasonic shaking, 3x15 min, soil treated with As(V)

TABLE 1. Amount of extracted arsenic with various modes and extractants

Shaking, 1 h			Sonication, 3 x 15 min		
solution	extr. As [%]		solution	extr. As [%]	
De-ionized water	6.1[a]	3.6[b]	De-ionized water	13.8[a]	10.3[b]
0.25 M H_3PO_4	51.3[a]	66.5[b]	0.25 M H_3PO_4	53.6[a]	86.6[b]
0.30 M H_3PO_4	48.2[a]	63.1[b]	0.30 M H_3PO_4	59.3[a]	78.0[b]
0.50 M H_3PO_4	51.3[a]	76.5[b]	0.50 M H_3PO_4	60.8[a]	91.1[b]
1.00 M H_3PO_4	53.5[a]	83.6[b]	1.00 M H_3PO_4	70.5[a]	97.8[b]
0.5 M H_3PO_4 + HA[c]	45.3[a]	70.6[b]	0.5 M H_3PO_4 + HA[c]	57.4[a]	93.8[b]
1.0 M H_3PO_4+ AA[d]	35.9[a]	70.5[b]	1.0 M H_3PO_4 + AA[d]	66.8[a]	96.9[b]

[a] soil treated with 30 mg kg^{-1} arsenic trioxide (As^{III})
[b] soil treated with 30 mg kg^{-1} potassium-dihydrogene-arsenate (As^{V})
[c] HA - 0.1 M hyroxylamine
[d] AA - 0.1 M ascorbic acid

TABLE 2. Speciation in model soils after 1 year of storage

	As(IIII)	As(V)	As(III)+As(V)	total As	organo-As
Soil A[a] [mg kg^{-1}]	18.5	4.39	22.9	24.8	1.93
%	74.5	17.7	92.2	100 %	7.8
Soil B[b] [mg kg^{-1}]	4.21	28.2	32.4	36.1	3.77
%	11.7	77.9	89.6	100 %	10.4

[a] soil treated with 30 mg kg^{-1} arsenic trioxide (As^{III})
[b] soil treated with 30 mg kg^{-1} potassium-dihydrogene-arsenate (As^{V})

Conclusions

The speciation of trivalent and pentavalent arsenic treated soils showed that during the storage of these soil samples at room temperature a certain amount of both arsenic species have been transformed. Moreover organic arseno-compounds could be formed. Approximatly 25% of arsenic(III) was converted to arsenic(V) and organo-arsenic compounds, and 22% of arsenic(V) was converted to arsenic(III) and organo-arsenic compounds.

This means the soil samples collected from the above long term experiment of Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Nagyhörcsök can not be used for speciation analysis except they were collected recently.

Selenium speciation in plant – Introduction

Trace mineral selenium is an essential nutrient of fundamental importance to human biology, therefore it is very important to be in our daily diet. Although it is known that selenium content of foods show big variety, the most widely consumed food have low selenium content. The main reason is that it has been shown, that selenium species have different bioavailability, utilization, accumulation and toxicity, but the main difference was manifested in the anti-cancer effect of selenium [3]. For example, a number of studies have confirmed that our body utilizes the organic Se species (selenomethionine, selenocysteine, Se-methylselenocysteine) easier and organic Se species are proved to be more effective in cancer prevention compared to inorganic forms of selenium [4].

As various selenium compounds have different effect on a human body, a selenium-enriched wheat sprouts were prepared applying selenite and selenate salts and we wanted the following question to be answered: in which selenium chemical forms are stored in wheat sprouts? Moreover selenium speciation analysis of wheat sprouts fortified with selenium can provide important informations related to the following issue: can selenium-enriched sprouts ensure the selenium requirement of a human body whose diet is deficient in selenium?

Experimental

Wheat seeds (*Triticum aestivum*) were used for germination studies. Total selenium content of sprout samples was measured after microwave assisted closed vessel wet digestion by Thermo Scientific XSeries II ICP-QMS applying CCT (7% H_2 in He).

Selenium was used in form of sodium selenite ($Na_2SeO_3.5\ H_2O$) (Fluka, Buchs, Switzerland) and sodium selenate (Na_2SeO_4) (Sigma-Aldrich, Steinheim, Germany), the concentration of the solutions used for germination was 10 mg dm^{-3}. Control treatment meant germination in ultrapure water.

For speciation purposes different extraction solvents were tested (0.1 M and 0.2 M HCl or 10 mM citric acid buffer). Speciation analysis was done on Hamilton PRP-X100 anion-exchange column, with an isocratic elution by 10 mM citric acid buffer, pH set to 4.6.

Analysis of selenium species of sprout sample in the extracts was performed by an attached system (a high performance liquid chromatograph (HPLC) with an anion exchange column and an inductively coupled plasma mass spectrometer (ICP-MS)). There was also the following question to find the appropriate answer: which extraction solvent results the best extraction efficiency?

Results and discussion

During germination a remarkable accumulation of selenium was experienced, both for selenite and selenate with near equal quantities (TABLE 3.)

TABLE 3. Total selenium concentration (mg kg^{-1}) of wheat sprouts in case of control, 10 mg dm^{-3} selenite and 10 mg dm^{-3} selenate treatments (n=3)

Treatments	Se concentration in wheat
control	0.089±0.014
selenite	12.4±0.3
selenate	13.1±0.3

The concentrations of organic and inorganic selenium species in selenium-enriched wheat sprouts are summarized in TABLES 4-7.

TABLE 4. Concentration (µg kg^{-1}) of inorganic selenium species in selenite-enriched wheat sprouts when 0.1 M and 0.2 M HCl or 10 mM citric acid as extraction solvent was applied (n=3)

Extraction	inorganic Se species		Total inorganic
solvents	selenite	selenate	Se content
0.1 M HCl	2.37±0.21	2.60±0.30	4.97
0.2 M HCl	16.2±3.1	2.22±0.82	18.4
citric acid	2.85±1.08	4.44±1.34	7.29

TABLE 5. Concentration (µg kg^{-1}) of organic selenium species in selenite-enriched wheat sprouts when 0.1 M and 0.2 M HCl or 10 mM citric acid as extraction solvent was applied (n=3); 5. organic selenium species=SeMet)

Extraction	organic Se species					Total organic
solvents	1.	2.	3.	4.	5.	Se content
0.1 M HCl	52.6±2.2	40.8±4.7	602±62	3.79±0.15	18.1±3.2	717
0.2 M HCl	41.6±7.4	43.0±9.3	466±26	13.4±2.78	3.3±0.4	567
citric acid	38.7±3.3	160±7	524±2	145±4	364±22	1232

TABLE 6. Concentration (μg kg^{-1}) of inorganic selenium species in selenate-enriched wheat sprouts when 0.1 M and 0.2 M HCl or 10 mM citric acid as extraction solvent was applied (n=3)

Extraction	inorganic Se species		Total inorganic
solvents	selenite	selenate	Se content
0.1 M HCl	126±33	4601±774	4727
0.2 M HCl	147±14	2728±6	2875
citric acid	218±7	159±66	377

TABLE 7. Concentration (μg kg^{-1}) of organic selenium species in selenate-enriched wheat sprouts when 0.1 M and 0.2 M HCl or 10 mM citric acid as extraction solvent was applied (n=3); 5. organic selenium species=SeMet)

Extraction	organic Se species					Total organic
solvents	1.	2.	3.	4.	5.	Se content
0.1 M HCl	90.3±16.4	58.7±22.9	66.6±25.9	4.5±3.6	68.8±13.5	290
0.2 M HCl	48.6±1.2	48.9±3.8	29.0±3.2	5.76±0.56	76.5±25.9	209
citric acid	62.7±3.8	243±11	79.4±10.9	215±16	541±57	1141

Conclusions

From the results it may be concluded that selenium was successfully converted to organo-selenium species when selenite was applied, whilst applying selenate most of the selenium have been taken up by sprouts remained in original form and minor part had been converted to organo-selenium compounds.

Therefore it can be concluded that it is more appropriate to apply selenium as selenite to produce selenium-enriched wheat sprouts in order to achieve higher organic Se-content.

In addition our research has also shown that regarding to the effectiveness there is significant difference between the applied extraction solvents. Whilst applying hydrochloric acid as extraction solvent prone to be successfully in detection of inorganic species, citric acid was facilitated mainly the release of organic Se-species.

From the preliminary date it seems that in order to achieve high extraction efficiency both strongly acidic (0.2 M HCl) and mild organic acidic media (10 mM citric acid at pH 4.6) should be applied sequentially.

Although it also has to be mentioned that extractions must be optimized in the future, because low efficiencies had been experienced regarding to the total selenium content of sprouts.

Determination of iodine in human milk – Introduction

Essentiality of iodine is well known. Insufficient iodine intake may cause serious health problems as goitre, mental retardation and cretinism as well. Fortification of table

salt with iodine is an effective strategy against iodine deficiency disorders in many countries, but it is very important, that infants should have an adequate iodine intake both during fetal development and later during lactation. In the latter case milk is the only source which determines the level of iodine intake [5].

ICP-MS is a good choice to determine iodine in different samples, such as human milk. In the analysis of iodine it is very important that in acidic medium iodide forms hydrogen-iodide, which is volatile so special care should be taken to prevent iodine losses during sample preparation. Thereby applying basic dissolution media, such as ammonia or tetramethylammonium-hydroxyide solution is a good way for these purposes [6].

Another specific problem in iodine determination by ICP-techniques, that iodine causes memory effect in spray chamber. To overcome this problem ammonia solution was successfully applied as a washing solvent.

For the measuring of iodine in human milk the following objectives were taken into consideration:

- to keep dilution as low as possible, thereby keep iodine levels above limit of detection
- to prevent any loss of iodine
- to get a homogeneous solution, that cannot cause clogging in sample introduction system consisted of standard Meinhard-type concentric nebulizer.

Experimental

For solubilization of human milk samples 0.001 M NaOH and KOH, concentrated ammonia (25 m/v %) solution and two surfactants (2 mM sodium-dodecyl-sulfate and 1 g L^{-1} Triton X-100) were tested.

Iodine levels were determined by Thermo Scientific XSeries II ICP-QMS applying CCT (7% H_2 in He).

Final sample preparation methodology was as follows: frozen human milk samples were let to thaw on room temperature. After fully thawing, precipitated fat and protein were homogenized by hand shaking until agglomerates were broken up. 1 ml of thawed, homogenized sample was taken into a quartz tube, then 5 ml of concentrated ammonia solution was added. Quartz tubes were sealed with teflon tape and placed to a teflon vessel. The solubilization was made in a microwave digestion system (Milestone Start D) on 80°C for 5 minutes. Each teflon vessel contained 10 ml of water (MilliQ) to assess heat transfer.

After solubilization samples were diluted up to 10 ml. For measurement a further dilution was applied to decrease organic matter content, thus 1 ml of prepared sample was analyzed after adding 0.1 ml Rh internal standard solution (final Rh concentration 100 μg L^{-1}) and dilution to a final volume 5 ml.

Results and discussion

During the experiments the most appropriate solubilizing agent was ammonia at a concentration of 25 (V/V)%. The overall dilution was approximately 50-fold, that ensures yet the iodine levels above limit of detection of iodine.

In human milk samples iodine concentrations varied between 50-650 μg L^{-1}. 85% of analyzed milk samples were in 100 and 300 μg L^{-1} of concentration range.

Among the analysis of standards and samples to prevent the sample introduction system against the memory effect NH_3 solution at a concentration of 0.5% was successfully applied as wash solution (*FIG. 3.*).

Conclusions

Owing to the broad applicability of ICP-MS and wide range of element capability we successfully applied the above elaborated analytical method in the determination of iodine content of human milk samples. Regarding to the special behavior of iodine an appropriate sample preparation should have developed.

Applying concentrated basic ammonia solution excessively assisted the solubilization of precipitated materials on 80°C in a human milk.

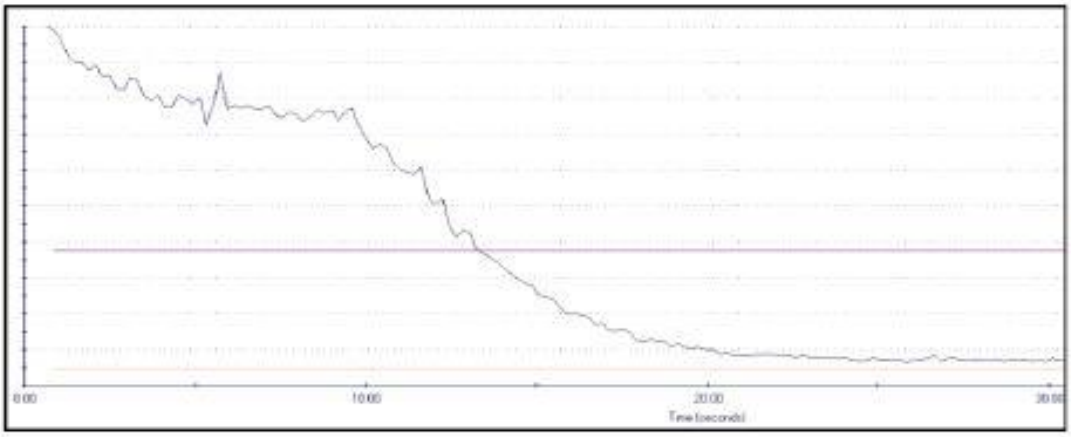

FIG. 3. Monitoring of washing out after sample containing 20 μg L^{-1} iodide.Signal intensity decreased to blank level after 25 seconds of washing on 100 rpm (4 ml min^{-1})

Determination of trace element content in wines by direct analysis – Introduction

The determination of minor, trace and ultra-trace element concentrations of wine samples provides data for geographical origin, authenticity, quality and food safety investigations. Data-analysis is requiring sophisticated statistical techniques such as cluster analysis, principal component analysis and related methods. Nevertheless not only

accurate and reliable results are consequential but a necessary number of wines should be analyzed for such investigations, which involves increase of analytical costs.

For the most instrumental methods it is needed to apply an appropriate sample preparation method to determine the concentrations of elements. The usually applied wet digestion procedures in wine analysis are time consuming particularly if a large number of wines should be analyzed, moreover due to the increase of operation steps and laboratory equipment used the probability of contamination also increases. The amount of inorganic acid and oxidizing agent utilized for digestion is also not a negligible point of view.

There are some papers reporting that in the case of wine – derived from its features – it is possible to omit digestion and wine can be introduced directly or after dilution by conventional nebulizers of an atomic absorption spectrometer (AAS) or an ICP instrument into the measuring system, hereby time and reagent can be saved, however during this sample preparation the organic matter of the wine remains after dilution causes interfering matrix effects [7].

Our aim was to develop a wine analytical method without any digestion sample preparation which is suitable for microelement determination of wine samples with an inductively coupled plasma mass spectrometer (ICP-MS) and to ensure reliable results for all kind of wine samples. For this purpose we compared different dilution degrees of wine aliquots and matrix-matched external calibrations. Because a certified wine reference material was not available in our laboratory the reference values were determined with standard addition method. The results were also compared with values determined in digested samples prepared by a nitric acid microwave wet digestion.

Experimental

For preliminary studies three house-hold made wine samples were used. Elemental composition was determined with a Thermo Scientific XSeries II ICP-QMS and by Thermo Scientific iCAP 6500 Duo ICP-OES as well for verification the results. ICP-OES measurement was applied only for digested samples.

Nitric acid wet digestion method was performed by a Milestone START D microwave digestion oven with the following temperature program: heating up to 180°C from room temperature 10 min. duration, hold on at 180°C for 10 min. For digestion distilled nitric acid was used (Milestone SubPur Distillation System): 8 ml cc. HNO_3 was added to 10 ml of wine; after digestion samples was diluted to 25 ml.

For direct analysis by ICP-MS different dilutions were tested: 100-fold, 50-fold, 10-fold and 5-fold. To increase precision and to correct matrix effects Rh, Te, Cs, Ta were used as internal standards in concentration of 100 $\mu g\ l^{-1}$. All measurements were made in triplicate.

Results and discussion

We have compared different dilutions in case of direct wine analysis. For the presented elements it can be concluded that Ba, Cu, Mn, Sr, and Zn could be determined

after a dilution as high as a hundred fold, but cadmium and lead concentrations could be determined just after a minimum ten-fold dilution.

The measured concentrations are in a good agreement with those determined after wet digestion by ICP-OES, except lead, which measured concentrations were higher in all cases than determined by ICP-MS. This last phenomenon could be aroused by lead contamination during the sample preparation (*TABLE 8A and 8B*).

Conclusions

With a simple dilution the time consuming sample preparation could be left out and beyond that the possibility of contamination decreased as well. The matrix-effects originated from the organic matters in the analyzed samples could be corrected by internal standardization.

From the result we concluded that a ten-fold dilution is enough to moderate matrix-effects of the organic matrix and for trace elements it is the maximum dilution factor to keep concentrations above instrumental detection limit. Furthermore it has been shown that to get reliable results matrix-matching of the calibration standards in case of external calibration is obligatory. Because wines can have different characteristics of organic constituents e.g. acids, sugar – manly for sweet wines – and alcoholic content, it is ultimate that not only the matrix-matching have to be used, but internal standardization is required to correct the changes of matrix-effects derived from the varying composition of individual wine samples in a single run.

Acknowledgements

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TABLE 8A. Comparison of measured concentrations [μg l^{-1}] in three wine samples

	dilution factor	Ba	Cd	Pb	Sr
SAMPLE_1					
ICP-OES	df=2.5	40.2 ± 2.4	< 5	18.7 ± 8.5	190 ± 6
ICP-MS	df=100	39.8 ± 2.0	< 1	< 10	204 ± 9
	df=50	41.8 ± 2.9	< 1	6.31 ± 0.47	240 ± 12
	df=10	35.5 ± 0.4	0.277 ± 0.022	4.73 ± 0.04	196 ± 4
	df=5	43.3 ± 1.0	0.235 ± 0.015	5.19 ± 0.08	207 ± 5
SAMPLE_2					
ICP-OES	df=2.5	77.9 ± 2.1	< 5	33.9 ± 2.7	253 ± 5
ICP-MS	df=100	91.1 ± 16.9	< 1	27.2 ± 0.5	255 ± 1
	df=50	85.0 ± 3.1	< 1	25.2 ± 0.2	279 ± 3
	df=10	82.2 ± 3.0	0.537 ± 0.060	22.4 ± 0.6	284 ± 8
	df=5	82.3 ± 0.8	0.429 ± 0.035	20.6 ± 0.4	286 ± 6
SAMPLE_3					
ICP-OES	df=2.5	102 ± 3	< 5	29.7 ± 2.5	599 ± 28
ICP-MS	df=100	111 ± 12	< 1	< 10	619 ± 13
	df=50	106 ± 6	< 1	< 10	637 ± 80
	df=10	98.1 ± 3	0.784 ± 0.085	5.53 ± 0.49	545 ± 14
	df=5	95.8 ± 2	0.766 ± 0.076	4.65 ± 0.18	536 ± 14

TABLE 8B. Comparison of measured concentrations [μg l^{-1}] in three wine samples

	dilution factor	Cu	Mn	Sr	Zn
SAMPLE_1					
ICP-OES	df=2.5	120 ± 1	1180 ± 37	190 ± 6	179 ± 20
ICP-MS	df=100	119 ± 9	1056 ± 47	204 ± 9	197 ± 20
	df=50	126 ± 5	1170 ± 74	240 ± 12	184 ± 11
	df=10	102 ± 2	1064 ± 17	196 ± 4	171 ± 3
	df=5	107 ± 3	1238 ± 19	207 ± 5	208 ± 2
SAMPLE_2					
ICP-OES	df=2.5	42.6 ± 2.0	982 ± 8	253 ± 5	501 ± 24
ICP-MS	df=100	59.2 ± 2.7	1003 ± 17	255 ± 1	604 ± 12
	df=50	46.4 ± 0.7	1052 ± 10	279 ± 3	605 ± 9
	df=10	38.7 ± 0.9	1023 ± 21	284 ± 8	554 ± 14
	df=5	37.4 ± 0.4	1008 ± 20	286 ± 6	510 ± 10
SAMPLE_3					
ICP-OES	df=2.5	67.2 ± 1.4	1129 ± 33	599 ± 28	451 ± 16
ICP-MS	df=100	70.9 ± 14	1280 ± 187	619 ± 13	475 ± 50
	df=50	63.3 ± 7.2	1114 ± 177	637 ± 80	430 ± 55
	df=10	61.2 ± 2.3	1025 ± 28	545 ± 14	483 ± 10
	df=5	67.6 ± 1.3	1079 ± 13	536 ± 14	446 ± 6

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