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Analytical Methods

Non-chromatographic speciation of inorganic arsenic in mushrooms by hydride generation atomic fluorescence spectrometry

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ABSTRACT

A non-chromatographic speciation method has been developed for the determination of inorganic arsenic in cultivated and wild mushroom samples from different origins. The ultrasound-assisted extraction of the toxic arsenic species As (III) and As (V) was performed for 10 min with 1 mol l^{-1} H_3PO_4 and 0.1% (m/v) Triton X-100. After phase separation the residue was washed with 0.1% (w/v) EDTA and centrifuged. As (III) and As (V) were determined by hydride generation atomic fluorescence spectrometry. Speciation was made using proportional equations corresponding to two different measurement conditions, (i) directly feeding sample extracts diluted with HCl and (ii) after reduction with KI and ascorbic acid for 30 min. The limits of detection of the method were 6.3 and 5.0 ng g^{-1} for As (III) and As (V), respectively. Recovery percentages varied between 91% and 108% for As (III) and from 90% to 109% for As (V) indicating that As species interconversion was avoided. As (III) concentrations from 264 to 81 μg g^{-1} and As (V) concentrations from 246 to 59 μg g^{-1} were found in Spanish cultivated mushrooms. For Chinese wild mushrooms As (III) varied between 624 and 117 μg g^{-1} and As (V) from 380 to less than 5 μg g^{-1}. The accuracy of the method was checked by the determination of total As (from the sum of As (III) and As (V)) in a tomato leaves reference sample, with good agreement with the certified value.

1. Introduction

Mushrooms are edible products with high protein and mineral content but low carbohydrate and fat values. Mushrooms provide relatively high concentrations of essential elements, but are able to accumulate elements such as As, Pb, Hg, Cs, Se, Cd, which could represent a serious risk to consumer health (Benbrahim, Denaix, Thomas, Balet, & Carnus, 2006; Rácz & Oldal, 2000). Therefore, intensive research has been carried out to detect and explain the presence and distribution of many heavy metals in edible mushrooms (Julshamn et al., 2007; Ouzouni & Riganakos, 2007; Smitht, Koch, & Reimer, 2007).

Different chemical forms of arsenic have different toxicity, thus, inorganic arsenic is generally toxic in both its arsenite and arsenate forms, but its non-toxic in some highly methylated forms, such as arsenobetaine and arsenocholine. Arsenic has been classified by the International Agency for Research on Cancer (IRAC) as a carcinogen to humans (Tsuda et al., 1992). There is a major interest in the speciation of As in foods and many papers have been published on the speciation of As in mushrooms. Table 1 summarises papers previously published on the speciation of arsenic in mushrooms using as the source the Science Citation Index (SCI) database of the Institute for Scientific Information (ISI, Philadelphia, PA, USA) from 1900 to 2008. As it can be seen, arsenite, arsenate, arsenobetaine, arsenocholine, monomethylarsonic acid and dimethylarsinic acid have been determined by using efficient separation techniques, such as high performance liquid chromatography (HPLC), size exclusion chromatography (SEC) (Wuilloud, Kannamkumarath, & Caruso, 2004) and gas chromatography (GC), coupled to atomic or ionic specific detectors, atomic fluorescence spectrometry (AFS) (Slejkovec, Elteren, & Byrne, 1998) or inductively coupled plasma mass spectrometry (ICP-MS) (Larsen, Hansen, & Gossler, 1998; Londesborough, Mattusch, & Wennrich, 1999; Smitht et al., 2007; Soeroes et al., 2005). However, the aforementioned procedures are tedious and require expensive instrumentation and, to our knowledge, there is no precedent on non-chromatographic speciation of As in this matrix. So, the main objective of this work was the development of a simple and fast analytical procedure for speciation of the most toxic chemical forms of As (As (III) and As (V)) in mushroom samples.

The methodology selected to do the present work is based on the different behaviour of As species in a sensitive detection system like AFS and, based on this fact, a proportional equations system can be obtained for experimental data found for the same sample under two different measurement conditions which could provide a different response for each one of the two considered species. So, fluorescence intensities I_A and I_B were obtained under

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Table 1
Methods proposed in the literature for arsenic speciation in mushrooms.

Analytical method	Sample pre-treatment	References
	Extraction	
HPLC–ICP-MS	Methanol/water 9:1	Kuehnelt et al. (1997)
	Methanol/water 9:1	Larsen et al. (1998)
	Methanol/water 9:1	Slejkovec et al. (1998)
	Methanol/water 1:1	Koch et al. (1999)
	Water	Londesborough et al. (1999)
	–	Slekovec et al. (1999)
	Methanol/water 1:1	Koch et al. (2000)
	Water and orthophosphoric acid	Soeroes et al. (2005)
	Methanol/water 1:1	Smitht et al. (2007)
HPLC–AFS	Methanol/water 9:1	Slejkovec et al. (1998)
HPLC–GFAAS	–	Slekovec et al. (1999)
HPLC–FAAS	–	Slekovec et al. (1999)
PT–GC–AFS	Methanol/water 9:1	Slejkovec et al. (1998)
SEC–UV–ICP-MS	NaOH and HCl	Wuilloud et al. (2004)

Note: HPLC, high performance liquid chromatography, ICP-MS, inductively coupled plasma mass spectrometry; AFS, atomic fluorescence spectrometry; GFAAS, graphite furnace atomic absorption spectrometry; FAAS, flame atomic absorption spectrometry, PT–GC–AFS, purge and trap-gas chromatography–atomic fluorescence spectrometry; SEC, size exclusion chromatography separation.
MMA, monomethylarsonic acid; DMA, dimethylarsenic acid; AB, arsenobetaine; AC, arsenocholine; TMAO, trimethylarsine oxide.

two selected conditions and they could be related to As (III) and As (V) concentrations, as follows:

$$I_A = a_A + b_{AIII}C_{III} + b_{AV}C_V$$
$$I_B = a_B + b_{BIII}C_{III} + b_{BV}C_V$$

In which I_A and I_B are the fluorescence signals obtained before and after KI reduction, a_A and a_B are the average of the intercept values of the regression lines for As (III) and As (V) in the absence of KI and the presence of KI respectively, b_B and b_A are the slopes obtained for calibration plots of As in the absence (A) and presence (B) of KI, with the same meaning for b_{AV} and b_{BV} concerning As (V).

The use of KI allows the reduction of arsenic species. The addition of KI, does not change the signal found for As (III). In contrast, after reduction of the standard solutions the sensitivity attainable for As (V) is clearly enhanced. So, it is possible to determine the concentration of species, As (III) C_{III} and As (V) C_v in the same sample, from just two fluorescent measurements made under different conditions and by solving the aforementioned simultaneous equations.

On the other hand, As speciation in solid samples, like mushrooms, constitutes a challenge since the different As species must be first quantitatively extracted from the matrix, while preserving the integrity of the overall equilibrium and avoiding species interconversion. In the past, several strategies to extract As species from solid samples have been developed based on microwave-assisted extraction (Bohari et al., 2002), accelerated solvent extraction (Vela, Heitkemper, & Stewart, 2001) and sonication (Cava-Montesinos, Nilles, Cervera, & de la Guardia, 2005; Matos-Reyes, Cervera, Campos, & de la Guardia, 2007, 2008). Therefore, in this study the use of a simple sonication extraction of inorganic As followed

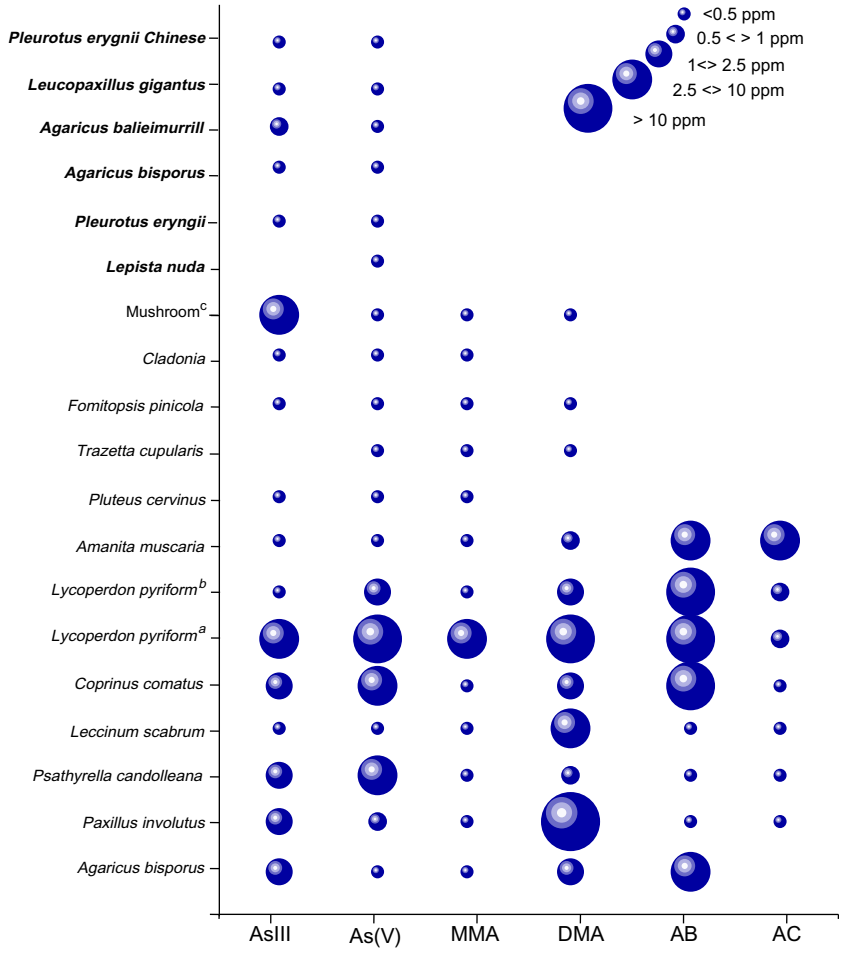

Fig. 1. Concentration ranges of arsenic species determined in mushrooms from published studies (Koch et al., 1999, 2000; Kuehnelt et al., 1997; Slejkovec et al., 1998; Slekovec et al., 1999; Smitht et al., 2007; Soeroes et al., 2005). *Note:* [a] Mushroom grown in the south of Yellowknife, [b] Mushroom located in the north of Yellowknife, [c] scientific name was not provided by the authors. Bold letters belongs to mushrooms analysed.

by fluorescence measurements by HG-AFS of extracts made before and after KI reduction has been used in order to obtain measurements corresponding to As (III) and As (V) species with different sensitivities.

2. Experimental

2.1. Apparatus and reagents

A PSA Millenium Excalibur 10055 continuous flow hydride generation spectrometer from PS Analytical (Orpington, UK), equipped with a high intensity source BDHCL (boosted discharge hollow cathode lamp) superlamp from Photron (Victoria, Australia), for excitation of As and a multireflectance filter, to achieve isolation of analyte emission lines, was employed for HG-AFS determination of As.

An ultrasound water bath from Selecta (Barcelona, Spain), of 350 ml volume with 50 W power and 50 kHz frequency, was employed for sample sonication in order to improve the extraction process. A Gelec Electronic centrifuge (Buenos Aries, Argentina) was used for extracts separations and a Telstar Cryodos lyophiliser (Barcelona, Spain) was used for fresh mushrooms sample lyophilisation.

All reagents used were of analytical grade and all solutions were prepared in nanopure water, with a minimum resistivity of 18.2 M Ω cm^{-1}, obtained from a Milli-Q Millipore system (Bedford, MA, USA). For the extraction, 37% HCl from Merck (Deventer, Holland), 85% H_3PO_4 obtained from BDH Chemicals (Poole, UK), a 0.1% (w/v) solution of the disodium salt of ethylenediaminotetraacetic acid and Triton X-100 both purchased from Panreac (Barcelona, Spain) and Antifoam A from Fluka (Buchs, Switzerland) were employed.

A 50% (w/v) KI solution and a 10% (w/v) ascorbic acid solution from Sharlau (Barcelona, Spain), were employed to reduce As (V) after extraction of As species. Sodium tetrahydroborate, from Fluka, dissolved in 0.1 mol l^{-1} NaOH, from Scharlau, was used to generate the corresponding As hydrides prior to the AFS measurements. This solution was prepared daily and filtered before use.

Stock solution of As (V) (1000 mg l^{-1}) dissolved in 5% HNO_3, from Merck, and an As (III) solution (1000 mg l^{-1}) dissolved in 5% HNO_3, from Scharlau, were employed for calibration.

Argon C-45 (purity higher than 99.995%) and synthetic air, both supplied by Carburos Metálicos (Barcelona, Spain), were employed as carrier gas and to dry the formed hydride respectively.

Determination of total arsenic was carried out using a Perkin Elmer Model Optima 5300 DV spectrometer (Norwalk, CT, USA) ICP–OES, equipped with an AS 93-plus autosampler and a Meinhard nebulizer. Argon C-45 (purity higher than 99.995%) supplied by Carburos Metálicos was employed as plasmogen and carrier gas.

For sample digestion HNO_3 69% (w/v) from J.T. Baker (Deventer, The Netherlands) and H_2O_2 35% reagent grade from Scharlau were employed.

An Ethos SEL microwave laboratory system, from Millestone (Sorisole, Italy), equipped with an optical fibre sensor for automatic temperature control, an automatic gas detector and 10 high pressure vessels of 100 ml inner volume, operating at a maximum exit power of 1000 W, was employed for microwave-assisted digestion of samples.

2.2. Samples

Two different cultivated types of Spanish mushrooms (*Pleurotus eryngii* and *Agaricus bisporus*), obtained fresh in the local market, and four wild Chinese mushrooms (*Agaricus balieimurrill, Leucopaxillus giganteus, P. eryngii, Lepista nuda*), obtained dried in a specialised Chinese market were analysed in our laboratory.

The certified reference material NIST 1573a tomato leaves were obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

2.3. Non-chromatographic speciation procedure

It is widely recognised that moisture can contribute to the extent of sample heterogeneity. In order to avoid this heterogeneity, food samples need to be dried prior to blending, storage, or analysis since the moisture will affect the final results. Additionally, the lyophilisation procedure also facilitates the storage and sampling steps. Thus, fresh Spanish mushrooms were lyophilised for a minimum of 48 h at a chamber pressure of 0.05 mbar. The dried samples were crumbled and pulverised with a mill until to obtain a homogeneous mixture. The resulting fine powder was stored in a dessicator prior to analysis.

The extraction step was based on the method reported by Matos-Reyes et al. (2008). One gram (±0.0001) of powdered sample was weighed inside a 50 ml polyethylene tube and 10 ml of H_3PO_4, containing 0.1% (v/v) Triton X-100, were added. To minimise foam formation 0.5 ml of Antifoam A were added before sonication. The mixture was sonicated for 10 min and the extracts separated by centrifugation at 3500 rpm for 15 min. The remaining solids were washed with 10 ml of 0.1% (w/v) EDTA solution and centrifuged again for additional 15 min. The washing solution was mixed with that previously separated and the mixture used for further analysis.

Three millilitres of the final extract were mixed with 8.75 ml concentrated HCl and analysed by HG-AFS. For the reduction of As (V), 3 ml of the extract was mixed with 0.6 ml of a solution of a mixture of 50% (w/v) KI and 10% (w/v) ascorbic acid, and let to react for 30 min before measurement by HG-AFS using in both cases the experimental conditions established before (Cava-Montesinos, Cervera, Pastor, & de la Guardia, 2003) shown in Table 2.

2.4. Acid digestion for total As determination procedure

For the determination of total arsenic content by using ICP–OES, a microwave-assisted digestion of samples was carried out using an adapted procedure from that of Koch et al. (1999), according to the microwave laboratory system manufacturer recommendations and our own experience. To avoid cross-contamination, teflon digestion vessels were previously cleaned in a bath of 10% (v/v) nitric solution for 48 h.

A portion of 0.5 g (±0.1 mg) of powdered and homogenised sample was treated with 5 ml of concentrated HNO_3 and the mixture was sonicated in an ultrasound water bath for 30 min in order to obtain an homogeneous dispersion. After that, 4 ml of H_2O and

Table 2
Instrumental conditions employed for the determination of As by HG-AFS.

Parameter	Value
Resonance wavelength (nm)	197.3
Primary current (mA)	27.5
Boost current (mA)	35
Sample volume (ml)	2
Delay time (s)	15
Analyse time (s)	30
Memory time (s)	15
HCl (mol l^{-1})	3.5
$NaBH_4$ % (w/v)	0.7
Ar flow rate (ml min^{-1})	330
Air flow rate (l min^{-1})	2.5
Sample carrier flow rate (ml min^{-1})	9
$NaBH_4$ flow rate (ml min^{-1})	4.5

Table 3
Operating parameters employed for ICP–OES determination of total arsenic.

Parameter in curry samples	Value
Plasma flow	15 l min^{-1}
Auxiliary flow	2.0 l min^{-1}
Nebulizer flow	0.8 l min^{-1}
Power	1300 W
Sample uptake rate	1.3 ml min^{-1}
View	Axial
Background correction	2-Point
Number of replicates	3
Nebulizer	Meinhard

1 ml of H_2O_2 were added and the mixture was placed in the ultrasound water bath for 30 min. The resulting solution was transferred to a teflon reactor and once the vessel was capped, it was placed inside the microwave oven and the following program was run: step 1, 10 min to reach 110 °C; step 2, 10 min to reach 200 °C; step 3, 10 min at 200 °C; step 4, cooling down. After cooling to ambient temperature, the reactor was opened and sonicated again to eliminate nitrous vapours. The resultant solution was directly analysed by ICP–OES using the experimental conditions summarised in Table 3.

3. Results and discussion

3.1. Strategy for the speciation analysis of As (III) and As (V)

As it has been aforementioned, the basis of the non-chromatographic speciation of As through HG-AFS is based on the measurement of standards and samples at two different experimental conditions which allows us to establish a set of two independent proportional equations for the analysis of each sample, the intensity obtained in each case being proportional to the concentration of As (III) and As (V):

$$I_{(A)} = 16 + 339.96[\text{As III}] + 138.46[\text{As V}]$$
$$I_{(B)} = (-6) + 342.76[\text{As III}] + 331.2[\text{As V}]$$

The two different conditions A and B correspond to direct measurements of diluted extracts (A) and measurements after reduction with KI and ascorbic acid of the extract (B), as has been described in Section 2. Table 4 shows the calibration lines obtained for As (III) and As (V) both directly and after adding KI. It can be concluded that KI does not changes statistically the signal of As (III) and provides for As (V) a sensitivity value comparable for that found for As (III). On the other hand the intercept values are very low.

3.2. Analytical characteristics

The limit of detection (LOD) was calculated as the concentration corresponding to signals equal to three-times the standard deviation of 10 replicates of a blank solution. Additionally, LODs were calculated in the original samples (ng g^{-1}), taking into consideration the amount of sample and the final dilution employed in the recommended procedure. The detection limits of the method were around 6.5 ng g^{-1} for As (III) and As (V).

Table 4
Calibration lines obtained for As (III) and As (V) standards by HG-AFS directly and after reduction with KI.

Element	Calibration lines	R^2 ($n = 6$)	LOD (ng g^{-1})
As (III)	$Y = 339.96x + 16$	0.9996	6.5
AS (III) red	$Y = 342.76x + 5$	0.9998	6.5
As (V)	$Y = 138.46x + 16$	0.9996	5
As (V) red	$Y = 331.20x - 11$	0.9983	6.5

Table 5
Recovery of spiked concentrations of As (III) and As (V) added to mushroom samples before their analysis by the proposed HG-AFS speciation method.

Mushroom sample	As (III) (ng g^{-1})	As (V) (ng g^{-1})	Recovery As III (%)	Recovery As V (%)
P.E	264 ± 29	242 ± 36	–	–
P.E + As (III)[a]	420 ± 13	218 ± 26	91 ± 3	
P.E + As (V)[b]	276 ± 3	494 ± 5		109 ± 10
P.E + As (III)+As (V)[c]	374 ± 5	332 ± 6	104 ± 8	90 ± 10
A.B	81 ± 14	59 ± 23	–	–
A.B + As (III)[a]	269 ± 7	68 ± 3	95 ± 3	
A.B + As (V)[b]	82 ± 4	272 ± 21		105 ± 8
A.B + As (III) + As (V)[c]	168 ± 4	144 ± 4	93 ± 5	91 ± 10
P.E[d]	117 ± 9	118 ± 3	–	–
P.E[d] + As (III)[a]	324 ± 7	119.8 ± 30.5	101 ± 4	
P.E[d] + As (V)[b]	113 ± 12	348 ± 12		108 ± 4
P.E[d]+ As (III) +As (V)[c]	237 ± 6	220 ± 10	108 ± 2	100 ± 5

Note: P.E: Pleurotus eryngyii and *A.B: Agaricus bisporus* correspond to Spanish mushrooms.
Spiked were made before sample sonication.
[a] Samples spiked with 200 ng g^{-1} As (III).
[b] Samples spiked with 200 ng g^{-1} As (V).
[c] Samples spiked with 100 ng g^{-1} As (III) and 100 ng g^{-1} As (V).
[d] *P.E: Pleurotus eryngii* corresponds to Chinese mushrooms.

To evaluate the accuracy of the whole procedure in terms of species integrity, recovery studies were carried out on three types of mushroom samples spiked prior to ultrasonic extractions at two concentration levels 100 and 200 ng g^{-1}. The experiments were performed in triplicate and results are presented in Table 5. As it can be seen, quantitative recovery values were obtained for the two considered species of As in spiked samples, evidencing that the extraction methodology is suitable for non-chromatographic speciation of As and preserves the integrity of the species equilibrium, avoiding the interconversion of these two redox forms of inorganic As.

The certified reference material (NIST-SRM 1573a) was analysed by using the proposed methodology in order to assess the accuracy of the whole procedure. The results obtained for the concentration of As (III) and As (V) in the reference material (tomato leaves) are reported in Table 6 and total As calculated from the aforementioned results was compared with the certified value. It can be seen that the sum of As (III) and As (V) compares well with the certified value for total As concentration.

3.3. Analysis of market samples

Table 7 summarises the As (III) and As (V) concentration data obtained for six different types of mushrooms. It is noticeable that As (III) and As (V) have been found in all the samples, except in one Chinese sample where the concentration of As (V) was lower than the detection limit of the procedure.

It should be mentioned that the total concentration of As in all samples analysed are under the maximum level allowed by the Spanish legislation, which restricts its concentration to 1 mg kg^{-1} (Food legislation surveys No. 6, 1993). In spite of the fact that the concentration of As (III) (the most toxic As species) was in all the cases equal or higher than that of As (V).

As compared with data reported previously in the literature (Koch, Wang, Reimer, & Cullen, 2000; Koch et al., 1999; Kuehnelt,

Table 6
Validation of the accuracy of HG-AFS non-chromatographic speciation of As (III) and As (V) using a certified reference material NIST 1573a (tomato leaves).

Sample	As (III) (ng g^{-1})	As (V) (ng g^{-1})	Total As (ng g^{-1})	Accuracy (%)[a]
1	70.18	41.22	111.4	98.7 ± 0.8
2	60.14	50.36	110.5	
3	57.48	52.15	109.6	

[a] Certified value 0.112 ± 0.004 mg Kg^{-1}.

Table 7
Determination of As (III) and As (V) in mushrooms by HG-AFS and total arsenic in mushrooms by ICP–OES. Values expressed in ng g^{-1}.

Mushroom samples	Type	HG-AFS			ICP–OES
		As (III)	As (V)	Total As	
Wild from China	*Agaricus balieimurrill*	624 ± 81	264 ± 98	888 ± 11	832 ± 85
	Leucopaxillus giganteus	417 ± 31	380 ± 45	797 ± 10	1400 ± 82
	Pleurotas eryngii	117 ± 9	118 ± 3	235 ± 4	322 ± 84
	Legista nuda	290 ± 21	<LOD	290 ± 21	292 ± 68
Cultivated from Spain	*Pleurotas eryngii*	264 ± 29	246 ± 36	510 ± 5	610 ± 70
	Agaricus bisporus	81 ± 14	59 ± 23	140 ± 6	130 ± 7

Goessler, & Irgolic, 1997; Slejkovec et al., 1998; Slekovec, Goessler, & Irgolic, 1999; Smitht et al., 2007; Soeroes et al., 2005) (see Fig. 1) it can be seen that samples considered in this study contain in all the cases very low concentrations of As (III) and As (V) as compared with other mushroom types.

3.4. Total arsenic in mushroom samples

Results obtained for total As determination in the mushroom samples considered in this study are shown in Table 7. These results are in good agreement with the sum of values found by HG-AFS for As (III) and As (V), thus indicating that there is no presence of organic species except for *L. giganteus*, for which the main part of As was not recovered by the developed procedure, thus indicating that organic As and not As (III) nor As (V) is the main species present in this sample.

The certified reference material (NIST-SRM 1573a) was also analysed by ICP–OES in order to assess the accuracy of the procedure proposed for the determination of total arsenic in mushroom samples. Total arsenic obtained in reference material tomato leaves was 109 ± 12 ng g^{-1}, being the recovery of As obtained by the ICP–OES method 97% of the average certified value.

4. Conclusion

The developed procedure offers a fast easy and low cost alternative to the available methods for As speciation in mushrooms. It is true that methods based on the use of atomic absorption or mass spectrometry detection after a chromatographic separation are able to see directly both, inorganic and organic species, but concerning the screening of food samples in order to verify their availability for human consumption, a simple determination of toxic As (III) and As (V) seems enough for application laboratories.

On the other hand, concerning the mean analytical features of the method it must be taken into account that the proposed methodology is more sensitive than previous ones (Koch et al., 1999) and it has been fully validated. Additionally the extraction step involves only 10 min and provides quantitative recoveries.

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