APPLICATION NOTE

LC-ICP-MS

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Speciation of Arsenic in Rice Flour Used as a Capsule Void Filler in Dietary Supplements

Introduction

The contamination of a variety of foods with arsenic has received significant attention recently. For food, regulatory limits have been introduced for rice which distinguish inorganic from organic arsenic forms in order to take into account their significant toxicity differences[1]. Rice flour, when used as a capsule filler material, may also constitute a major proportion of dietary supplements. Regulations that limit the amount of inorganic arsenic in dietary supplements are going to become effective under U.S. Pharmacopeia (USP) General Chapter <2232>. Inorganic arsenic in rice is a reasonably anticipated contaminant (RAC) and must be evaluated in rice powder or flour used as an excipient in dietary supplements to comply with the cGMPs for dietary supplements under 21 CFR Part 111.

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In this paper, we evaluate the speciation of arsenic (As) in several types of rice flour products marketed as excipients for the manufacture of dietary supplements. Any procedure used to assess the speciation of arsenic must be validated for suitability for the particular sample matrix investigated. We validate the methodology presented by analysis of a standard reference material (SRM) and using spike recoveries for the extracted excipient samples. In addition, we widened the range of tested materials to include samples which may not exclusively contain rice. This allows assessing method robustness for coping with sample matrices deviating from the norm. Furthermore, the efficiency of the extraction was assessed by comparison to total arsenic values obtained after sample digestion.

Experimental

Sample Preparation for LC-ICP-MS

Samples (1 g) were extracted in 50 mL Sarstedt tubes with 10 mL of 0.28 M HNO_3 (GFS Chemicals™, Veritas grade) on a PerkinElmer SPB 50-24 hotblock at 95 °C for 90 min[2]. Two distinguishing features of this extraction method are that it has been thoroughly validated for rice, and that it maintains species integrity for extracted species[3]. The cooled extracts were centrifuged at 6000 rpm for 5 min. A portion of the supernatant was taken up with a 20 mL NormJect all-plastic syringe and filtered through a 0.45 µm, 25 mm nylon syringe filter unit. The filtrate (3 - 7 mL) was collected in 15 mL Sarstedt tubes. The extracts were further diluted 10-fold (0.5 mL to 5 mL) with ultrapure water (ELGA PureLab 18.2 MΩ-cm).

Instrumental Conditions

The analysis was performed with a PerkinElmer Altus™ HPLC and NexION® 350D ICP-MS, controlled by Waters® Empower® 3 Software. The instrument conditions are given in Table 1.

Table 1. Instrument conditions.

LC	Altus HPLC
Mobile Phase	50 mM NH_4NO_3, 2 mM OSA, 2 mM malonic acid, pH 4.0, 1% MeOH
Column	Shiseido Capcell PAK, MG, C18, 4.6 x 250 mm, 5 µm
Column Temperature	50 °C
Vials	1.5 mL plastic
Injection Volume	10 µL
ICP-MS	**NexION 350D**
Nebulizer	Glass concentric
Spray Chamber	Glass cyclonic
RF Power	1600W
Plasma Gas	15 L/min
Auxiliary Gas	1.2 L/min
Isotope	^{75}As
Mode	Standard mode
Dwell Time	500 ms

The mobile phase was prepared using OSA, 1-octanesulfonic acid, sodium salt (98%, Sigma-Aldrich™) and malonic acid (99%, Acros Organics™). The desired NH_4NO_3 level was achieved using 3.125 mL/L nitric acid (Veritas grade, GFS Chemicals™) and addition of sufficient ammonium hydroxide (Optima grade, Fisher Scientific™) to achieve the final pH 4.0. Finally, methanol (Optima grade, Fisher Scientific™) was blended into the mobile phase.

Quantitation

Standards were prepared from 1000 ppm stock solutions for As3 (Spex Certiprep™) and As5 (PerkinElmer). DMA, dimethylarsinic acid (98%) was purchased from Sigma and MMA, monosodium methylarsonate (99%) was obtained from Chem Service. For the organoarsenicals, standards were based on total weight of the chemicals (excluding crystal water for MMA). For reporting purposes, organoarsenical results were converted to elemental As. Standards for LC-ICP-MS were prepared in mobile phase and, for comparison, additionally in the diluted extract matrix (0.028M HNO_3). Both matrices give the same arsenic response (Table 2). However, standards in 0.028M HNO_3 are not consistently stable. Species interconversion (As3 oxidation to As5) was observed in that matrix on repeated analysis of a check standard (1 ppb). Arsenic species proved to be stable in mobile phase (pH 4), consistent with previous observations[4]. The standards stability over time spanning longer than one day was not investigated in detail. We recommend, as a best practice, to prepare standards fresh each day for best accuracy, considering the low levels (ppt) involved.

Table 2. Comparison of 1 ppb standard response (ppb) in mobile phase vs. 0.028M HNO_3 – calibration was carried out with standards in mobile phase.

Sample	Injection	As5	MMA	As3	DMA
1 ppb 0.028M HNO_3	1	0.98	1.03	1.03	1.01
1 ppb 0.028M HNO_3	2	0.97	0.96	1.04	1.02
1 ppb Mobile Phase	1	1.02	0.98	1.05	1.02
1 ppb Mobile Phase	2	1.05	1.03	1.02	1.02

Samples

The tested excipient materials are identified in Table 3. Together with the SRM, NIST™ 1568b Rice Flour, their extracts were analyzed unspiked and spiked with mixed As standard.

Table 3. Sample identification of tested capsule filler materials.

ID#	Label	Description
FRL-2	Gluten-Free Mixed Flour	Gluten-free mix with rice flour representing complex solid blend[1]
FRL-3	Gluten-Free Mixed Flour	Gluten-free mix with rice flour representing complex solid blend
FRL-4	White Rice Flour	Basic white rice flour fill used in industry
FRL-5	Brown Rice Flour	Basic brown rice flour fill used in industry
FRL-8	White Rice Flour	Basic white rice flour fill used in industry
FRL-12	Rice Concentrate	"Rice extract" from China marketed as capsule filler (emerging material)

Total As After Microwave Digestion

For totals analysis, 0.25 g of sample material was digested with 10 mL HNO_3 using standard 75 mL vessels in the PerkinElmer Titan MPS™ Microwave Sample Preparation System, according to the temperature program given in Table 4.

Table 4. Microwave digestion program for total arsenic analysis.

Step	Temperature (°C)	Pressure Limit (bar)	Ramp (min)	Hold (min)	Power (W)
1	160	30	5	10	90
2	180	30	3	20	100
3	50	30	1	15	0

Digests were clear for all samples except FRL-12, which contained a substantial particulate residue. The digests were transferred to 50 mL Sartstedt tubes and filled to 50 mL with deionized water. Analysis was carried out after further 5-fold dilution with deionized water, addition of 2 ppb Ir as internal standard, and filtration through 0.45 µm syringe filters. Standards for totals analysis were prepared in 4% HNO_3 in the range 20 ppt - 1 ppb (six standards), and analysis was done in Reaction mode using O_2 as reaction gas (0.6 mL/min; RPq = 0.5).

Results and Discussion

The instrument was calibrated in the range 50 ppt - 5 ppb (corresponding to 5 - 500 ppb in the solid) to cover the expected range of arsenic concentrations for the unspiked and spiked samples (2 ppb spike level). The 5-point calibrations were linear with $r^2 > 0.999$ for all four species.

An example chromatogram for a mid-range standard prepared in mobile phase is shown in Figure 1, displaying adequate peak resolution within a short run time. Separation of the species is complete within 2.5 min. The benefits of a short run time include higher throughput and lower detection limits, the result of minimal dispersion experienced at short times (i.e. taller and narrower peaks).

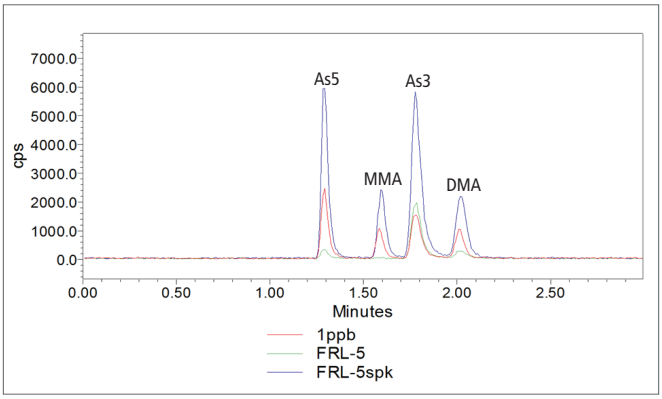

Figure 1. Separation of arsenic species achieved using ion interaction chromatography. Overlay of 1 ppb standard in mobile phase, and FRL-5 sample extract (brown rice flour) with and without 2 ppb spike.

Overlaid in Figure 1 are example chromatograms for one sample before and after spiking. Retention times for samples were identical to those observed for standards, and neither peak shape nor resolution was negatively affected by the sample/extraction matrix. Materials consisting not exclusively of rice flour (FRL-2, 3, and 12) behaved identically, demonstrating that the method is robust in terms of coping with some variation in the sample type.

To validate the methodology, the NIST™ 1568b reference material was analyzed. The NIST™ 1568b results show good agreement with certified values (Table 5). Inorganic As is recovered to 98%, proving reliability of the method for toxicity assessments.

Table 5. Standard reference material results in ppb (NIST 1568b) – analysis results are reported as elemental As and are corrected to dry weight (µg/kg).

Sample	As5	As3	Total As Inorganic	MMA	DMA	Sum of Species
NIST-E1	31.4	61.8	93.2	11.2	166	270
NIST-E2	32.9	55.1	87.9	10.6	161	260
Certified	-	-	92	11.6	180	285
Recovery (Avg)	-	-	98%	94%	91%	93%

In order to examine the time sensitivity of the analysis, diluted NIST™ extracts were reanalyzed after storage for two days at room temperature. Deviations are small and mostly random, averaging ± 1% change for most species (Table 6). For MMA, relative deviations were higher, which may be largely attributable to the low concentration of that species (compare Table 5), and absolute deviations are still small. Nevertheless, this could indicate degradation of DMA to MMA in very small amounts. Diluted NIST™ extracts, both unspiked and spiked with 1 ppb, were stored for seven days at room temperature and reanalyzed, resulting in spike recoveries of 98%, 103%, 97%, and 91% for As5, MMA, As3, and DMA, respectively (average of duplicates), demonstrating long-term species stability. The excipient materials were analyzed on the day of extract preparation and again on the following day with no major deviations observed; deviations averaged 6%, 2%, 0%, and 2% for As5, MMA, As3, and DMA, respectively, indicating that the diluted extracts of excipient samples are stable for one day.

Table 6. Aging of diluted NIST extracts for two days with changes reported as percentage relative to the initial value.

Sample	As5	MMA	As3	DMA	Sum of Species
NIST-E1 10x	-2%	7%	2%	-5%	-3%
NIST-E2 10x	0%	14%	-1%	4%	3%
Avg	-1%	11%	1%	-1%	0%

The speciation results for samples are reported in Figure 2. Results for individual species range from 2 - 287 ppb with sharp differences in the distribution patterns observed. The dominant individual species could either be organic (FRL-3 and FRL-8) or inorganic (remaining FRL samples). Within the group of inorganic species, As3 was mostly dominating over As5, with one notable exception (FRL-12). This observation demonstrates that it is valuable to collect information on the individual inorganic species. Whilst a summary of inorganic concentration is mostly required for toxicity assessment, information on the nature of the sample would be lost without the ability to distinguish between As3 and As5. In this case, the more detailed information provided by the distinction between As3 and As5 enabled singling out FRL-12 as an unusual sample in line with its identification as 'concentrate' rather than flour (Table 3). As for the organic species, DMA was the dominant form in all cases, with MMA being mostly absent.

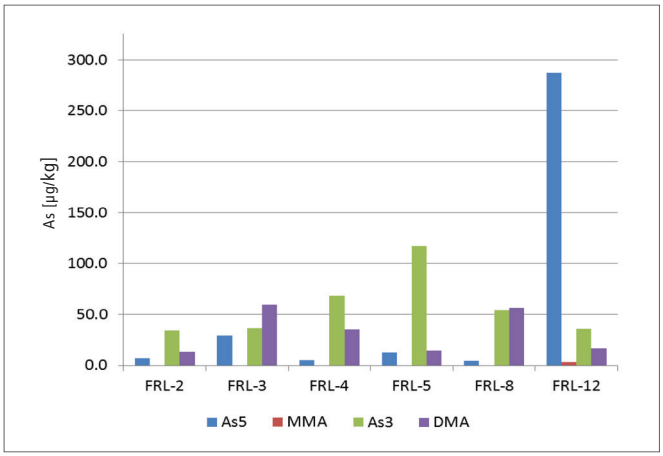

Figure 2. As species distribution for excipient samples showing sharp differences in the distribution of individual species.

Inorganic arsenic dominated As speciation for most samples (Figure 3). For two samples (FRL-3 and FRL-8), only half of the arsenic was in inorganic form. Those samples benefit most from toxicity assessment via speciation, as opposed to assessment by total arsenic content. Furthermore, speciation analysis clearly identifies FRL-12 as being of concern with its high arsenic level being mostly attributable to inorganic forms.

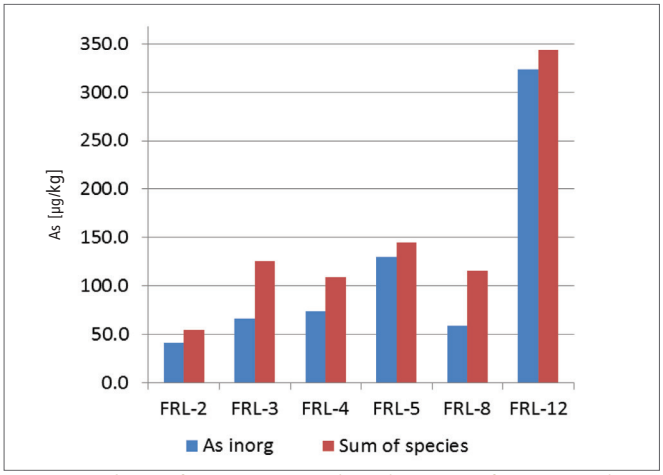

Figure 3. Contribution of inorganic arsenic to the total As content of excipient samples.

To further validate the accuracy of the speciation analysis, a spike recovery experiment was carried out. Spike recoveries on diluted extracts averaged 99 - 107% for individual species, confirming that species were recovered quantitatively and demonstrating that species were stable and did not interconvert (Table 7).

Table 7. Spike recoveries obtained on diluted extracts of samples and NIST™ reference material (extracted in duplicate) spiked with 2 ppb.

Sample	As5	MMA	As3	DMA
FRL-2	100%	112%	101%	102%
FRL-3	96%	107%	98%	98%
FRL-4	102%	113%	99%	104%
FRL-5	101%	109%	99%	106%
FRL-8	99%	107%	105%	102%
FRL-12	99%	103%	101%	102%
NIST-E1	97%	103%	99%	101%
NIST-E2	94%	104%	97%	99%
Avg	99%	107%	100%	102%

The effectiveness of the extraction of arsenic species was assessed by measuring total arsenic by ICP-MS after microwave digestion of the samples. Analysis of the NIST™ SRM 1568b digest showed close agreement with the certified value (101% recovery), confirming the accuracy of the analysis. Arsenic totals obtained after digestion are compared with the sum of As species obtained by LC-ICP-MS in Figure 4. The close agreement between the two methods confirms the quantitative extraction of arsenic species.

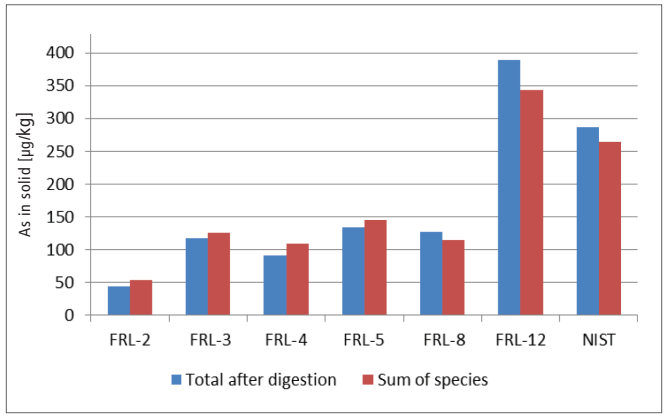

Figure 4. Comparison of sum of species obtained by LC-ICP-MS with totals obtained after microwave digestion.

Replicate performance was assessed on two extracted samples analyzed in duplicate. The results show good agreement between the replicates (Table 8).

Table 8. Duplicate analysis shows adequate replicate performance. Values are reported as elemental As (µg/kg).

Sample	As5	MMA	As3	DMA	Inorganic As	Sum of Species	% Inorganic As
FRL-8 a	3.7	< 2.0	56.8	59.3	60.5	120	51
FRL-8 b	4.5	< 2.0	54.1	56.6	58.6	115	115
FRL-12 a	284	2.0	34.0	13.6	318	334	95
FRL-12 b	287	3.4	36.0	16.6	323	343	94

Conclusion

This study examined the performance of the PerkinElmer Altus HPLC coupled to the NexION 350D ICP-MS for the analysis of arsenic species in rice flours used as filler material in dietary supplement capsules. The method employs an acidic extraction with subsequent analysis by ion interaction chromatography. Good agreement of the obtained speciation data with totals measured after microwave digestion confirms that the species are extracted quantitatively. The toxicologically important sum of both inorganic arsenic species was in close agreement with the certified value, as were the other data obtained for the reference material (sum of species, totals is digests). Those data together with spike-recovery experiments confirm that quantitation was accurate in the matrices examined, and that the species are stable once extracted. Distinction between the inorganic arsenic forms proved useful for sample characterization based on differences in the species distribution, highlighting the usefulness in employing a procedure which maintains the integrity of the extracted inorganic species. Samples were stable at least for one day after extraction. The sample/extract matrix did not impact the chromatography as evidenced by unaffected retention times and good peak shape throughout. Performance was equally good for samples off the norm, demonstrating method robustness. In addition to those benefits, the analysis by ion interaction chromatography yields desirable low detection limits and fast analysis times.

References

1. *Commission Regulation (EU) 2015/1006 Amending Regulation (EC) No 1881/2006 as regards Maximum Levels of Inorganic Arsenic in Foodstuffs.*
2. Huang, J. H.; Ilgen, G.; Fecher, P. Quantitative Chemical Extraction for Arsenic Speciation in Rice Grains. *J. Anal. At. Spectrom.* 2010, 25, 800–802.
3. Huang, J. H.; Fecher, P.; Ilgen, G.; Hu, K. N.; Yang, J. Speciation of Arsenite and Arsenate in Rice Grain - Verification of Nitric Acid Based Extraction Method and Mass Sample Survey. *Food Chem.* 2012, 130 (2), 453–459.
4. Ernstberger, H.; Neubauer, K. *Accurate and Rapid Determination of Arsenic Speciation in Apple Juice;* PerkinElmer Application Note; 2015.

Consumables Used

Component	Part Number
0.45 µm Nylon Syringe Filter Units	02542905
Column: C18, 4.6 x 250 mm, 5 µm	N8145326
Autosampler Vials, clear, 1.5 mL (package of 100, with caps)	N9301736
PEEK Tubing, 0.007" ID x 1/16" OD (5 feet)	N9302678
PEEK Finger Tight Fittings	09920513
PEEK Solvent Filter, 10 µm	N8122249
PEEK In-line Filter, 10 µm	N8122250
Nebulizer Connector for HPLC	WE024372
Connector for Peristaltic Pump Tubing to PEEK Tubing	N8122258
Finger Tight Connector for 1/16" OD PEEK Tubing	09920513
50 mL Sarstedt Tubes	N8145124
15 mL Sarstedt Tubes	N0777471

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