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## Bioaccessibility of lead and arsenic in traditional Indian medicines

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### Abstract

Arsenic and lead have been found in a number of traditional Ayurvedic medicines, and the practice of *Rasa Shastra* (combining herbs with metals, minerals and gems), or plant ingredients that contain these elements, may be possible sources. To obtain an estimate of arsenic and lead solubility in the human gastrointestinal tract, bioaccessibility of the two elements was measured in 42 medicines, using a physiologically-based extraction test. The test consisted of a gastric phase at pH 1.8 containing organic acids, pepsin and salt, followed by an intestinal phase, at pH 7 and containing bile and pancreatin. Arsenic speciation was measured in a subset of samples that had sufficiently high arsenic concentrations for the X-ray absorption near edge structure analysis used. Bioaccessible lead was found in 76% of samples, with a large range of bioaccessibility results, but only 29% of samples had bioaccessible arsenic. Lead bioaccessibility was high (close to 100%) in a medicine (Mahayograj Guggulu) that had been compounded with *bhasmas* (calcined minerals), including *naga* (lead) *bhasma*. For the samples in which arsenic speciation was measured, bioaccessible arsenic was correlated with the sum of As(V)–O and As(III)–O and negatively correlated with As–S. These results suggest that the bioaccessible species in the samples had been oxidized from assumed As–S raw medicinal ingredients (realgar, As<sub>4</sub>S<sub>4</sub>, added to *naga* (lead) *bhasma* and As(III)–S species in plants). Consumption at recommended doses of all medicines with bioaccessible lead or arsenic would lead to the exceedance of at least one standard for acceptable daily intake of toxic elements.

### Keywords

Arsenic; Lead; Bioaccessibility; Ayurvedic; Speciation; Risk

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### Conflict of interest statement

The authors declare that there are no conflicts of interest.

## 1. Introduction

Ayurveda, Siddha, and Unani are fields of traditional medicine practiced in India and are considered a form of complementary and alternative medicine in western society. Treatments include herbal medicines, dietary interventions, and massage. A special subset of herbal medicines is *Rasa Shastra*, where metals, minerals or gems are intentionally added to herbs. Although important micronutrients such as iron and zinc are commonly used, elements widely considered toxic in the west, such as lead, mercury or arsenic, are also used. Detailed protocols for the manipulation of metals, minerals, and gems are described in ancient Indian medical texts in order for them to be converted into *bhasmas* (Shastri, 1979; Satpute, 2003). *Bhasmas* are purported to contain non-toxic forms of lead, mercury and arsenic, but to our knowledge these claims have not been rigorously tested.

In previous studies we have shown that approximately one-fifth of traditional Indian medicines from a variety of different sources contain detectable lead ( $\geq 5 \text{ mg.kg}^{-1}$ ), mercury ( $\geq 20 \text{ mg.kg}^{-1}$ ) and arsenic ( $\geq 20 \text{ mg.kg}^{-1}$ ). In most cases, the medicines that contained one or more of these elements exceeded one or more regulatory standards for their acceptable daily intake (Saper et al., 2004; Saper et al., 2008). In our previous work we identified that speciation of the elements, that is, the determination of their physicochemical form, in the medicines is a research area that has not yet been addressed. Non-toxic forms of lead may include species such as elemental lead, which is largely insoluble, whereas highly soluble species, such as lead acetate, are toxic (e.g.,  $\text{LD}_{50}$  is  $17 \text{ mg.kg}^{-1}$  in mice) (Park et al., 2001). Likewise, mineral forms of arsenic like realgar ( $\text{As}_4\text{S}_4$ ,  $\text{LD}_{50}$   $3.2 \text{ g.kg}^{-1}$  in mice) and orpiment ( $\text{As}_2\text{S}_3$ ,  $\text{LD}_{50}$  not available) show a much lower toxicity than the more soluble forms such as arsenic(III) oxide ( $\text{As}_2\text{O}_3$ ,  $\text{LD}_{50}$   $33\text{--}39 \text{ mg.kg}^{-1}$  in mice) (Liu et al., 2008) or sodium arsenate ( $\text{Na}_2\text{HAsO}_4$ ,  $\text{LD}_{50}$   $14\text{--}18 \text{ mg.kg}^{-1}$  in rats) (Franke and Moxon, 1936). Clearly, the solubility of the different arsenic and lead species is an important determinant of toxicity, since the fraction that becomes soluble upon ingestion, also known as the bioaccessible fraction, is that which can be absorbed into the body and potentially cause toxic effects.

The bioaccessible fraction can be measured using a laboratory-based extraction test that mimics the gastro-intestinal environment in such details as the chemicals and enzymes present, temperature and exposure time, and pH. Bioaccessibility testing can be used as a surrogate for animal experiments commonly used for bioavailability testing. Standardized versions of bioaccessibility tests are used frequently in the pharmaceutical field to measure, for example, dissolution of formulations (e.g., US Pharmacopeia) (Younis et al., 2009). To measure inorganic elements present as contaminants, a field of bioaccessibility research has emerged that focuses on soil samples, with frequent interest in arsenic and lead, e.g., (Ruby et al., 1993; Ruby et al., 1996; Schroder et al., 2004; Van de Wiele et al., 2007; Juhasz et al., 2009). Fewer studies are available for bioaccessibility of these elements in other media, e.g., (Laparra et al., 2004; Amiard et al., 2008), especially in natural or traditional medicines (Koch et al., 2007; Jayawardene et al., 2010). A method for lead bioaccessibility in soil has gained regulatory acceptance based on agreement with results from animal studies for a series of U.S. soils (U.S.EPA and Office of Solid Waste and Emergency Response, 2007). Other methods have also shown agreement for lead and arsenic with animal studies of soils from other areas (Ruby et al., 1993; Ruby et al., 1996; Schroder et al., 2004; Basta et al., 2007; Juhasz et al., 2009). In the present study, we used a method originally developed to model rabbit absorption of lead from soil, where this model was assumed to represent the gastro-intestinal conditions of human children (Ruby et al., 1993; Ruby et al., 1996). Our previous studies using this model yielded results in reasonable agreement with clinical measurements of arsenic bioavailability measured by urinalysis (adult human) following ingestion of an arsenic-containing traditional Chinese medicine (Koch et al., 2007). We also

found this method to be robust to changes in liquid to solid ratios for arsenic bioaccessibility measurements of soil and tailing materials (Meunier et al., 2010b), which is a desirable attribute for studies on new matrices in nonstandard liquid to solid ratios, as in the present study.

In the present study we aimed to refine the findings from previous studies reporting arsenic and lead concentrations that could pose risk following recommended ingestion regimes (Saper et al., 2008); in those studies only total concentrations were reported. Specifically, we focused on the following objectives: (1) determine the arsenic speciation in selected Indian traditional medicines, (2) measure the bioaccessibility of lead and arsenic in several medicines, and (3) recalculate the estimated daily intake of lead and arsenic from the medicines using the amount of bioaccessible metal rather than the total amount ingested.

## 2. Materials and methods

### 2.1. Traditional medicine samples

Forty-two traditional Indian medicine samples were studied. Of these, 40 were selected from a larger set of 193 products randomly purchased in 2005 via the Internet for one of our previous studies, and results were reported for 35 of them (Saper et al., 2008). These 40 were chosen because they contained detectable arsenic ( $>10 \text{ mg. kg}^{-1}$ ) and/or lead ( $>5 \text{ mg. kg}^{-1}$ ) by X-ray fluorescence (XRF) analysis. Two additional products containing lead and arsenic purchased via the Internet that were not included in the previous study were also analyzed. The samples used in the study are listed in Table 1, where information is also given about their status as *Rasa Shastra* medicines. *Rasa Shastra* medicines are elaborately prepared compounds combining herbs with metals, minerals, and gems. An Indian-trained Ayurvedic physician classified medicines as *Rasa Shastra* (Saper et al., 2008) if they contain metals, minerals, or gems traditionally used according to two classic *Rasa Shastra* texts (Shastri, 1979; Satpute, 2003). Details of the medicines' contents, according to information on their labels, are given in the supporting information.

### 2.2. Total arsenic and lead

All samples were analyzed by X-ray fluorescence (XRF) analysis and several of the results (35 of 42) were reported previously in Saper et al. (2008). For XRF results reported here for the first time, methods and quality control procedures, as well as quality control results, are described in detail in Saper et al. (2008). Samples that had arsenic and lead concentrations below the XRF limits of detection ( $10 \text{ mg. kg}^{-1}$  for arsenic and  $5 \text{ mg. kg}^{-1}$  for lead) were reanalyzed by the methods described in the following paragraphs.

Samples 6 and 7 were powdered samples and did not require further sample preparation. The rest of the samples were first prepared by grinding/homogenizing pills using mortar and pestle, since an early trial run revealed that large differences in bioaccessible arsenic and lead content were apparent between individual pills and capsules. Since the pills and capsules were compounded from ingredients that had been previously ground, and were dissolved/suspended in the extraction solution by the end of the gastric phase, this additional homogenization step was not considered to substantially reduce the particle size or surface area of the samples. A minimum of three pills were used, and sufficient pills were used to obtain the mass required to complete the extraction and digestion analyses.

Total arsenic and lead contents in the ground pills and powders were obtained by digesting a 0.50 g portion of each in pyrex boiling tubes with 9 mL *aqua regia* (concentrated hydrochloric and nitric acids in a ratio of 3:1), followed by inductively coupled plasma-mass spectrometry (ICP-MS) analysis in collision cell mode (to reduce interferences from

chloride) for all samples except 11. The m/z monitored were 75 (As), 206, 206 and 208 (all Pb and results were averaged); more instrumental details can be found in Smith et al. (2008).

Sample 11 was also subjected to the *aqua regia* digestion method because the lead concentration was very high (in weight percent levels) and exceeded the arsenic concentrations by more than two orders of magnitude. In this case the XRF values for arsenic were not accurate because of interference of lead on the arsenic analysis (United States Environmental Protection Agency (USEPA), 2007; Stark et al., 2008). The *aqua regia* digestion method was modified to ensure maximum digestion since the concentrations were very high; *aqua regia* to sample ratios greater than 250 were used (see supporting information for quality assurance/quality control details), and the digests were analyzed by ICP-optical emission spectrometry (OES) using a Perkin Elmer Optima 5300 V instrument, outfitted with a Perkin Elmer GemCone nebulizer and Perkin Elmer Baffled Cyclonic Spray Chamber. The emission lines monitored were as follows (where more than one emission line was monitored, the results were averaged): 188.979, 193.696, 197.197 (As); 220.353 (Pb) nm.

Quality control tests were carried out to accompany total arsenic and lead determinations. Specifically, a procedural blank, a standard reference material, and a duplicate sample were analyzed along with every batch of 10–12 samples. MESS-3 (NRC Canada), a sediment matrix, was used as the standard reference material to monitor dissolution of mineral components of the samples, which were thought to be the most challenging to dissolve, and also because this allowed us to ensure comparability to an accredited laboratory (who uses the same standard reference material). During the analysis of digested samples by ICP-MS, a five point calibration curve was used, and run before and after each batch of analysis and every 36 samples if the number of samples in a batch exceeded that number. Calibration check standards, prepared from a source separate from the calibration standard, were analyzed every 12 samples and the results were accepted only when the results were within 20% of the known value. Internal standards Sc, Y, Rh, In, Tb, Ho, Bi were included and interpolation (provided by the software) was used to correct for plasma drift and matrix effects. Internal standard elements appearing in a specific sample matrix were deleted from the calculation for the specific sample. For ICP-OES analysis, Y was used as an internal standard. The digested samples were diluted 10–10,000 times to ensure that the acid matrix was the same as the matrix of the calibration standards and concentrations were within the calibration curve.

### 2.3. Speciation analysis of arsenic

X-ray absorption near edge structure (XANES) spectra of the arsenic K-edge (11,868 eV) were recorded in fluorescence mode at the bending magnet beamline of the Pacific Northwest Consortium X-ray Sciences Division (PNC/XSD) facility, sector 20, Advanced Photon Source (APS) in Argonne, Illinois; and an arsenate–glycerol standard was analyzed at the hard X-ray microanalysis beamline, at the Canadian Light Source (CLS; Saskatoon, SK, Canada). Si(111) double-crystal monochromators used at both beamlines were calibrated using the first inflection point of the gold L<sub>III</sub> absorption edge (11919.7 eV) and a reference gold foil was measured simultaneously with samples. At the CLS, the monochromator was detuned to 50% of the maximum intensity for harmonic rejection. The arsenate–glycerol standard was maintained at 50 K using an Oxford liquid helium cryostat (CF 1208). Incident and transmitted intensities were measured using straight ion chamber detectors filled with 20% Ar and 80% He. As K<sub>α</sub> data were collected in fluorescence mode using a 32-element solid state Ge detector (Canberra, 32 element Ultra LEGe Array). The configuration of APS is described elsewhere (Smith et al., 2005).

All samples were ground in a ball mill and mounted as a thin film on Kapton™ tape. Four layers of sample were affixed to a sample holder. Analyses were performed at room temperature. Three or six scans were collected and averaged for each sample.

XANES spectra were fit within  $-20$  to  $+30$  eV from  $E_0$  using ATHENA software (Ravel and Newville, 2005). Data were compared to linear combinations of solid As(III) ( $As_2O_3$ , Fluka reagent grade), solid As(V) ( $KH_2AsO_4$ , Fluka reagent grade), a synthesized arsenate–glycerol standard, and a synthesized amorphous orpiment standard. As(III) and As(V) samples were prepared and measured in the same way as the samples; the orpiment standard was measured as product on filter paper encased in Kapton™ tape and the arsenate–glycerol standard was measured as a liquid absorbed in a Kimwipe™. All fits were constrained to sum to 100% of measured arsenic. Error is reported both as the reduced  $\chi^2$  and as three times the estimated standard deviation of the fit.

The amorphous orpiment standard was synthesized using the procedure described in detail elsewhere (Rochette et al., 2000). A 1.0 M acetate buffer solution, pH adjusted to 4.0, was deoxygenated by bubbling nitrogen gas through the solution for 10 min. Stock solutions of 0.133 M sodium sulfide and 0.0133 M arsenate were prepared. Sodium sulfide and arsenate were added to the acetate buffer solution in an amber jar at an As:S ratio of 1:2, and were shaken at 150 rpm for 72 h. Subsequently, the solution was filtered through a Whatman glass microfibre filter (45  $\mu$ m) to collect the precipitated product.

A arsenate–glycerol standard solution was prepared by adding 0.7 mL of 1000  $mg.L^{-1}$  arsenate aqueous solution ( $KAsH_2PO_4$ , > 98%, Fluka) to a large excess (8 mL) of glycerol ( $\geq 99\%$ , Sigma), acidified by concentrated HCl (37%, OmniTrace, EMD), and adding 1.0 M NaOH ( $\geq 95\%$  EMD) dropwise until pH 6 was reached, measured using pH paper.

#### 2.4. Bioaccessibility of arsenic and lead

The bioaccessibility method was designed for a human receptor and consisted of a two phase (stomach and intestine) physiologically based extraction test (PBET). Two 0.1–0.2 g portions per sample were weighed into 50 mL plastic centrifuge tubes and 10–40 mL of simulated gastric solution, prewarmed to 37 °C, was added to each sample. The gastric solution was based on the physiologically based extraction test (PBET) first developed by Ruby et al. (1993), modified using variables proposed by Rodriguez et al. (1999) (pH and salt). It consisted of 1.25  $g.L^{-1}$  pepsin (P7000 from porcine stomach mucosa, Sigma-Aldrich), 0.5  $g.L^{-1}$  sodium citrate (Caledon), 0.5  $g.L^{-1}$  malic acid (Sigma-Aldrich), 1  $mL.L^{-1}$  glacial acetic acid (Fisher), 0.15 M NaCl (puriss p.a. Fluka), adjusted to pH  $1.8 \pm 0.05$  with HCl (Fisher). Exact masses and volumes for each sample are summarized in Table 2 and are based on a worst case scenario of ingesting the maximum recommended dose per day all at once in a fasted human stomach that was assumed to have a liquid volume of 417 mL over 5 h (2000 mL per day, adjusted for 5 h of extraction per 24 h). This scenario represents a higher liquid-to-solid ratio than would be calculated based on volumes obtained from either fasted stomach volumes (around 25 mL) (Scarr et al., 1989; Kong and Singh, 2008) or from a gastric basal secretion rate of 60 mL per h (300 mL over 5 h) (Deshpande et al., 1996; Kong and Singh, 2008), and is therefore more conservative (Richardson et al., 2006). Assuming fasted conditions with respect to pH (Kong and Singh, 2008) in the stomach compartment is also the most conservative assumption, and was used even when drug information recommended administration with meals (i.e., samples 12 and 19; sample 9 is with milk). Gastric extraction proceeded for 1 h, with a pH check and adjustment if necessary at 0.5 h, at 37 °C at 275 rpm on a temperature-controlled flatbed rotation incubator (New Brunswick Scientific Innova 4230). After 1 h, the gastric phase solutions were removed and centrifuged at 2970 g for 15 min, and the supernatant was filtered (0.45  $\mu$ m, PVDF membrane, Millipore). To simulate intestinal conditions the remaining samples

that had been subjected to the stomach extraction described above were neutralized to pH  $7\pm 0.2$  with a saturated solution of  $\text{Na}_2\text{CO}_3$  (Fluka), and  $1.875 \text{ g.L}^{-1}$  bile extract (B8631 porcine, Sigma) and  $0.5 \text{ g.L}^{-1}$  pancreatin (P1500 porcine, Sigma) were added. Intestinal extraction conditions were applied for 4 h at  $37^\circ\text{C}$  with 275 rpm shaking, and samples were centrifuged and filtered in the same way as the gastric samples.

Each extract was kept refrigerated for a maximum of 1 week before analysis. Samples were first diluted with 2% v/v nitric acid ( $\text{HNO}_3$ , Fisher) solution by 10–10,000 $\times$  as needed and analyzed for total arsenic and lead concentrations by ICP-MS (Thermo Electron Corporation X-SeriesII) as described elsewhere (Smith et al., 2008), except that collision cell mode was used to reduce interference from chloride in the sample. Bioaccessibility results are expressed as a concentration, which is the quantity of a substance ( $\text{mg.kg}^{-1}$  dry weight) extracted during a bioaccessibility test, or as a percentage, which is the bioaccessible concentration divided by the total concentration in a given sample and multiplying by 100.

Quality control tests were carried out to accompany bioaccessibility tests. Specifically, a procedural blank, a standard reference material, and a duplicate sample were analyzed along with every batch of 9 samples. NIST 2710, a soil sample, was used to ensure quality control of the bioaccessibility tests because our laboratory has established control limits for this standard based on over 2 years of testing (mean $\pm 3$  times the standard deviation). Instrumental quality control (ICP-MS and ICP-OES) was as described in Section 2.2.

### 3. Results

All results of quality control tests were within control limits, meaning they were acceptable according to established criteria, and quality control results are described in detail in the supporting information or, for XRF results, in Saper et al. (2008).

#### 3.1. Total arsenic and lead concentrations

Total arsenic and lead concentrations are included in Table 1 and all samples had detectable values of lead and/or arsenic; previously published XRF values were used where available and are included for clarity in the present study. Samples that were reanalyzed using *aqua regia* digestion with ICP-MS analysis mostly had arsenic concentrations below the XRF detection limit of  $10 \text{ mg.kg}^{-1}$  and in a few samples lead values less than the XRF detection limit of  $5 \text{ mg.kg}^{-1}$  were obtained. As mentioned previously, sample 11 was reanalyzed (ICP-OES was used) because the XRF analysis was inaccurate for the high concentrations of lead and arsenic present. In these cases, and for sample 11, the results obtained by using the *aqua regia*-ICP method are used for further calculations in this study; otherwise XRF values were used.

#### 3.2. Speciation analysis

Arsenic species were determined by X-ray absorption spectroscopy (XAS) only in samples with arsenic contents greater than  $10 \text{ mg.kg}^{-1}$ ; the list of samples and results are summarized in Fig. 1 and Table 3, which also include the labeled ingredients of the samples (details for all samples can be found in the supporting information). The fluorescence signal from these traditional medicines was low, resulting in some fits that gave  $\chi^2$  values higher than 0.003 (<0.003 is considered to be a good fit). However, the purpose of the XANES analysis for this paper was to investigate the oxidation state of arsenic within the pills with respect to the bioaccessibility results, rather than to unequivocally identify the specific arsenic compounds present. The speciation of arsenic in most samples is a mixture of arsenic-sulfur species (amorphous orpiment  $\text{As}_2\text{S}_3$  was selected as the likeliest match and is from herein referred to as As-S), arsenic trioxide ( $\text{As}_2\text{O}_3$ , referred to as As(III)-O) and an

arsenate ( $\text{As}^{\text{V}}$ ) species (i.e.,  $\text{As}(\text{V})\text{-O}$ ). In five samples (samples 5, 11, 24, 30, and 33), an arsenic species appeared that was shifted 1.0 eV higher than the arsenate compound used for fitting ( $\text{KH}_2\text{AsO}_4$ ) and this species matched  $\text{As}(\text{V})$ -glycerol.

### 3.3. Bioaccessibility

The bioaccessibility results (Table 4) are included for both the gastric (G) phase and the gastric followed by intestinal (G+I) phase for arsenic, since the added extraction time and higher pH during the intestinal phase has been observed to increase arsenic bioaccessibility for certain kinds of samples (Meunier et al., 2010a; Meunier et al., 2010b). For lead, because the gastric phase results were always higher, only these results are included, which is consistent with standard risk assessment practice. Only detectable values are reported in Table 4. Excluded samples (those for which bioaccessible concentrations of both lead and arsenic were lower than the detection limit of  $1 \text{ mg}\cdot\text{kg}^{-1}$ ) are samples 6, 7, 17, 18, 20, 21, 23, 28, and 29.

The bioaccessibility results for both arsenic and lead were comparable to each other in terms of range and means, but almost all samples (32 out of 42, or 76%) had bioaccessible concentrations of lead, whereas the majority of samples were too low in arsenic to measure bioaccessibility (12 out of 42 samples, or 29% had detectable bioaccessible arsenic in the gastric phase and 9 out of 42, or 21% in the gastric+intestinal phase).

### 3.4. Comparison to acceptable daily intakes (ADIs)

The daily amounts of lead and arsenic that would be ingested if medicines were taken according to manufacturers' recommendations, based only on the amount that is bioaccessible (Fig. 2a and b), were calculated. The risk assessment approach used in the present study, that is, comparing estimated daily intakes with acceptable daily intakes (rather than the more conventional practice of calculating hazard quotients), was the same as that used in Saper et al. (2008) to facilitate comparisons with the results in that work. Standards for acceptable daily intakes were similar to those used in Saper et al. (2008), except that comparisons to Food and Agricultural Organization/World Health Organization Joint Expert committee on Food Additive (FAO/WHO JECFA) provisional tolerable daily intakes were not included, since these values for lead and arsenic have recently been withdrawn by FAO/WHO JECFA (Joint FAO/WHO Expert Committee on Food Additives, 2010a; Joint FAO/WHO Expert Committee on Food Additives, 2010b). Comparisons to Canadian standards (toxicological reference values, or TRVs) were added (Health Canada Contaminated Sites Division Safe Environments Programme, 2009).

The daily amount of lead in all of the medicines that contained bioaccessible lead exceeded at least one regulatory standard (California Proposition 65), but the daily amounts of only two medicines (sample 40 Ayu-Nephro-Tone and 11 Mahayograj Guggulu) exceeded the Health Canada TRV. However, the California Proposition 65 daily intake standard ( $0.5 \mu\text{g}\cdot\text{d}^{-1}$ ) is exceeded even at the detection limit of the bioaccessibility test, and thus lower detection limits are needed to assess the safety of the medicines that did not have detectable bioaccessible lead (samples 6, 7, 17, 18, 20, 21, 23, 28, 29), when this regulatory standard is considered in combination with the dosing regimes.

The arsenic daily amounts exceeded the lowest arsenic regulatory standard, American National Standard Institute/National Sanitation Foundation International Dietary Supplement Standard 173 (ANSI 173) for three medicines (samples 11 Mahayograj Guggulu, 30 Mahasudarshan, and 38 Ayu-Hemoridi-Tone) of the 13 that had bioaccessible arsenic. The maximum intakes of arsenic for a 70 kg adult ingesting Mahayograj Guggulu

(sample 11) and Mahasudarshan (sample 30) exceeded the United States Environmental Protection Agency reference dose (USEPA RfD).

#### 4. Discussion

In a previous study several Ayurvedic medicines with elevated arsenic and lead exceeded intake guidelines if ingested as recommended (Saper et al., 2008), but nothing was known about their speciation and associated solubility. Thus the present study addressed these issues in samples that were specifically selected to represent the products in the previous study with the highest concentrations of arsenic and lead, in addition to several others that had detectable amounts of these elements, including some that have not been studied previously. The process used to produce several of the products, known as *Rasa Shastra*, combines herbs with metals, minerals and gems, and is believed to produce medicines that are safe and therapeutic (Shastri, 1979; Satpute, 2003). Additionally, the processing of the minerals (*shodhana* or 'purification') is claimed to eliminate their harmful effects, rendering them non-toxic (Kumar et al., 2006). In the current study, sample 11 (Mahayograj Guggulu) contains a high concentration of lead, around 5% by weight ( $50,000 \text{ mg.kg}^{-1}$ ), which was likely added as *naga bhasma* (lead ash) (Raza, 1975). *Bhasmas* are calcined or ashed minerals or gems; in chemical terms, the *bhasma* process is mainly oxidative roasting. The lead species in the sample could not be detected by X-ray diffraction analysis, likely because only amorphous forms were present, but the lead form was nearly 100% bioaccessible in the final product. This suggests that the *bhasma* process may actually increase the bioaccessibility of the lead starting material, most likely elemental lead, which is insoluble in water. Of note, Mahayograj Guggulu is the traditional Indian medicine most frequently associated with reported lead poisoning cases (Ernst, 2002; Centers for Disease Control and Prevention, 2004; Saper et al., 2008).

In the present study we report a reasonably high concentration of arsenic (around  $90 \text{ mg.kg}^{-1}$ ) in Mahayograj Guggulu (sample 11). This arsenic was likely introduced as part of a *bhasma*, since arsenic minerals like orpiment and realgar are often incorporated into the *bhasma* process (Thatte et al., 1993). These minerals may also be contaminants of other metal starting materials. The arsenic speciation in this sample was primarily As(V)-O along with a solid As(III)-O compound and an As-S compound (likely remaining from added arsenic-sulfide mineral such as orpiment or realgar) (Table 3). The high proportion of oxidized (and likely soluble) species As(III)-O and As(V)-O (89%) resulting from a likely starting material of insoluble orpiment or realgar gives additional evidence that the *bhasma* process changed the arsenic from a less soluble to a more soluble form. The speciation in starting materials and the end product *naga bhasma* should be studied directly to further examine this point.

Differences in total arsenic and lead, as well as percent bioaccessibility of these two elements, were noted between results in the present study and those reported recently for a sample of Mahayograj Guggulu (total lead and lead bioaccessibility were lower in the previous study, and arsenic was not detected) (Jayawardene et al., 2010). These differences probably reflect the heterogeneity of this sample from commercial sources, and the same heterogeneity probably exists for traditionally prepared preparations. Therefore, whether our findings with commercially prepared Mahayograj Guggulu are generalizable to such preparations requires further research.

Arsenic speciation was also determined in several other samples, chosen because their arsenic content is around or above  $10 \text{ mg.kg}^{-1}$  (Table 3). Most of these samples, including Yog-Raj (Yograj) Guggulu (samples 5, 36), Trifala Guggulu (sample 24), Mahasudarshan (sample 30), and Sugar Fight (sample 34) have not been classified as *Rasa Shastra*



medicines, which means that they have no mineral ingredients. Consequently, the likeliest arsenic source is plant ingredients. The three other samples analyzed for arsenic speciation, Ayu-Hemoridi-Tone (sample 38), Shilajeet (sample 10) and Shilajit (sample 33), have been classified as *Rasa Shastra* medicines, indicating that they contain metals, minerals, or gems, although they are also derived from plant material (Shilajeet(jit) is made of “mineral pitch”, the dried exudate of the plant *Asphaltum punjabinum* from rock).

Plants have been observed to contain elevated arsenic concentrations when harvested from areas that have naturally or anthropogenically elevated soil arsenic concentrations, e.g., (Koch et al., 2000) and some plants used in Ayurvedic medicines have been shown to contain arsenic (Singh and Garg, 1997). Roots especially are susceptible to contamination, e.g., (Aldrich et al., 2007), and if these were included in the compounding of the medicines, they might have contributed to the arsenic content. The speciation of the arsenic in the samples indicates that an As–S compound is found in all samples except for Ayu-Hemoridi-Tone (sample 38) but the quality of the spectra was not sufficiently high to identify the As–S compound with certainty (Fig. 1, Table 3 Red  $\chi^2$  values). As(III)–S compounds are commonly found in fresh and live plant samples using mass spectrometric (Bluemlein et al., 2009) and XAS methods (Pickering et al., 2000; Smith et al., 2008), although these compounds appear to be relatively unstable to processing steps like harvesting, drying and aging in air (Smith et al., 2008; Bluemlein et al., 2009). In some plants, however, As–S compounds have been observed to survive air-drying and grinding steps (Mir et al., 2007). Similar mild preparation techniques were likely used for all the medicines (e.g., cleaning, drying and grinding plants, aqueous extractions at 60–70 °C of guggulu), as described for Yogaraga Guggulu (Simha et al., 2008). In the absence of the knowledge of any mineral forms of arsenic added to the products that have not been classified as *Rasa Shastra* medicines, we propose that it is possible that the As–S species in these samples are derived from plants. This may be a possibility for Shilajeet (jit) (samples 10, 33) as well, but the possibility of the As–S compound as a mineral form, scraped from rocks that may have contained arsenic minerals, cannot be discounted.

In five samples, Yog-Raj Guggulu (sample 5), Mahayograj Guggulu (sample 11), Trifala Guggulu (sample 24), Mahasudarshan (sample 30), and Shilajit (sample 33), the As<sup>V</sup> compound present had a white line position that was 1.0 eV higher than solid KH<sub>2</sub>AsO<sub>4</sub> (Fig. 1). Arsenic white lines that are approximately 1.0 eV higher than arsenate have been seen previously in dried plant and insect residues (Mir et al., 2007; Andrahennadi et al., 2009) and the compound with this energy has been identified as an octahedrally coordinated As(V)–glycerol compound (Andrahennadi et al., 2009). When the As(V)–glycerol was included in the fittings, the higher energy compounds in the present study were tentatively identified as the glycerol compound. The presence of this compound may be attributable to the dried plant residues in the medicines.

When the bioaccessibility results are compared with the speciation results, it appears that percent bioaccessibility increases with the proportion of pentavalent arsenic (Fig. 3) (n=9, r=0.82, p<0.05). The correlation is even better when As(III)–O is summed with As(V)–O (n=9, r=0.93, p<0.05). At the same time, a negative correlation is observed between the bioaccessibility and the proportion of the As–S species suggesting that these may be the arsenic species that are not bioaccessible (Fig. 3) (n=9, r=–0.93, p<0.05). Thus the appearance of oxidized As(III)–O and As(V)–O in these medicines, either introduced as an ingredient or resulting from processing, may make the arsenic more available for uptake into the human body, where toxic effects can be exerted. Since it is difficult with XANES to distinguish As(V)–O as a soluble species, such as the potassium arsenate (KH<sub>2</sub>AsO<sub>4</sub>) used as a standard in the present study, from insoluble species such as scorodite (FeAsO<sub>4</sub> · 2H<sub>2</sub>O), we cannot state with certainty that the As(V)–O species found in these samples are

indeed the forms dissolved in the bioaccessibility test; further research involving XANES testing of residues from the bioaccessibility test would be required. No organoarsenic species were detected in any samples, although they may be present at very low concentrations below the technique's detection limit.

The implications of using the studied medicines were determined by comparing estimated daily intakes with acceptable daily intakes. Of the fourteen medicines included in the present study that previously exceeded the ANSI standard for lead in Saper et al. (2008), only five exceed this standard in Fig. 2a. Likewise, the three medicines that exceeded arsenic standards in Saper et al. (2008) no longer exceed any standards in the present work. However, one sample (sample 38, Ayu-Hemoridi-Tone) exceeds the ANSI arsenic standard in Fig. 2b, but was not included in the Saper et al. (2008) evaluation because its arsenic concentration was not detectable by the XRF method used. Incorporating bioaccessibility into the intake calculations therefore resulted in a decrease in the number of samples exceeding standards, compared with results using total concentrations.

## 5. Conclusions

The speciation for arsenic in a medicine that likely had a *bhasma* added to it indicates changes to more soluble forms, likely attributable to *bhasma*-type preparation procedures, and the high bioaccessibility of lead in the same material supports this theory. Arsenic appearing as As(V)-O and As(III)-O appears to be associated with bioaccessible arsenic and the remaining arsenic is as less-bioaccessible As-S compounds. These speciation trends are specific to the samples analyzed in the present study, and their interpretation in the context of other samples must be undertaken carefully. Nevertheless, the results suggest that increases in bioaccessibility, and likely toxicity (lead and arsenic are likely inorganic species in the bioaccessibility extracts), are associated with the processing steps used to compound these medicines (i.e., oxidation of arsenic minerals and elemental lead in *naga bhasma*, and oxidation of As-S in plants, (Smith et al., 2008)).

Daily intakes calculated with bioaccessible arsenic and lead concentrations are lower than previously estimated (Saper et al., 2008) but many are still above acceptable levels. Given our findings for Mahayograj Guggulu's high level of bioaccessible lead and arsenic and case reports suggesting toxicity, its ongoing use should be carefully scrutinized. Mercury is an element of concern in the medicines that was not addressed in the present research, but future work is planned to measure the bioaccessibility of this element. Information about the elemental content should ideally be provided with medicines so practitioners and consumers can make informed decisions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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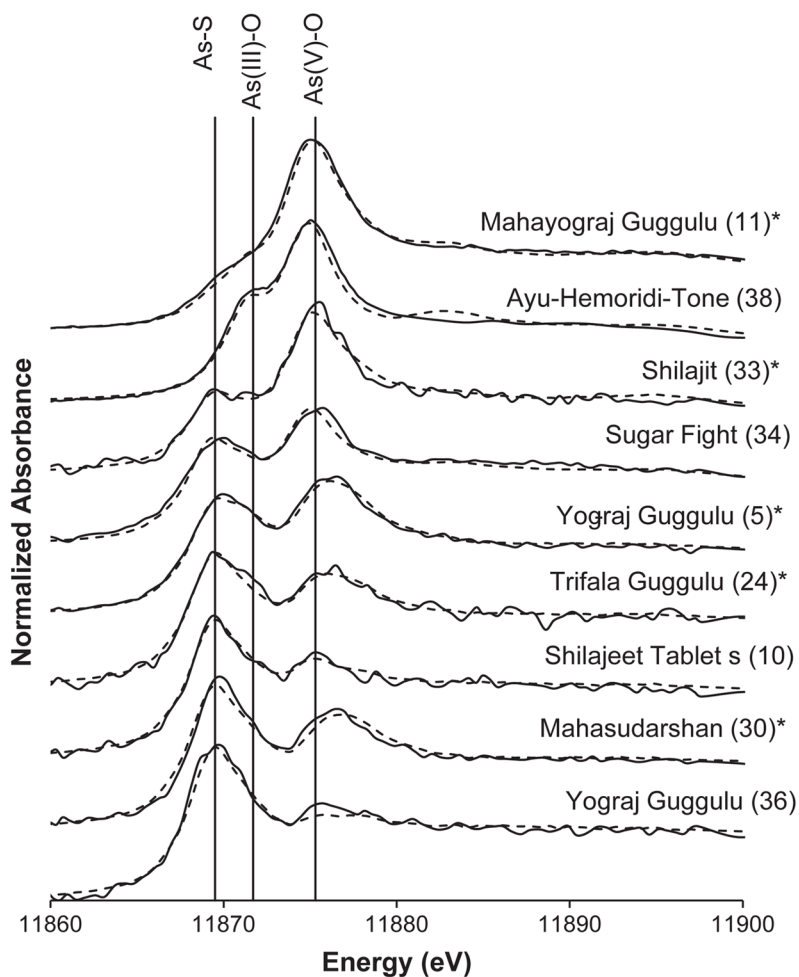
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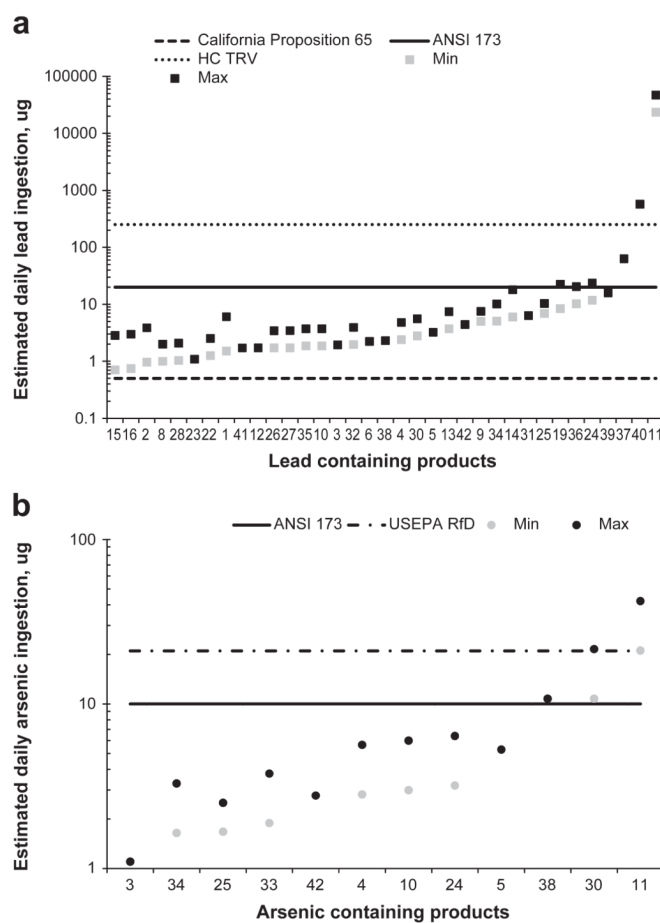
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## Appendix A. Supplementary data

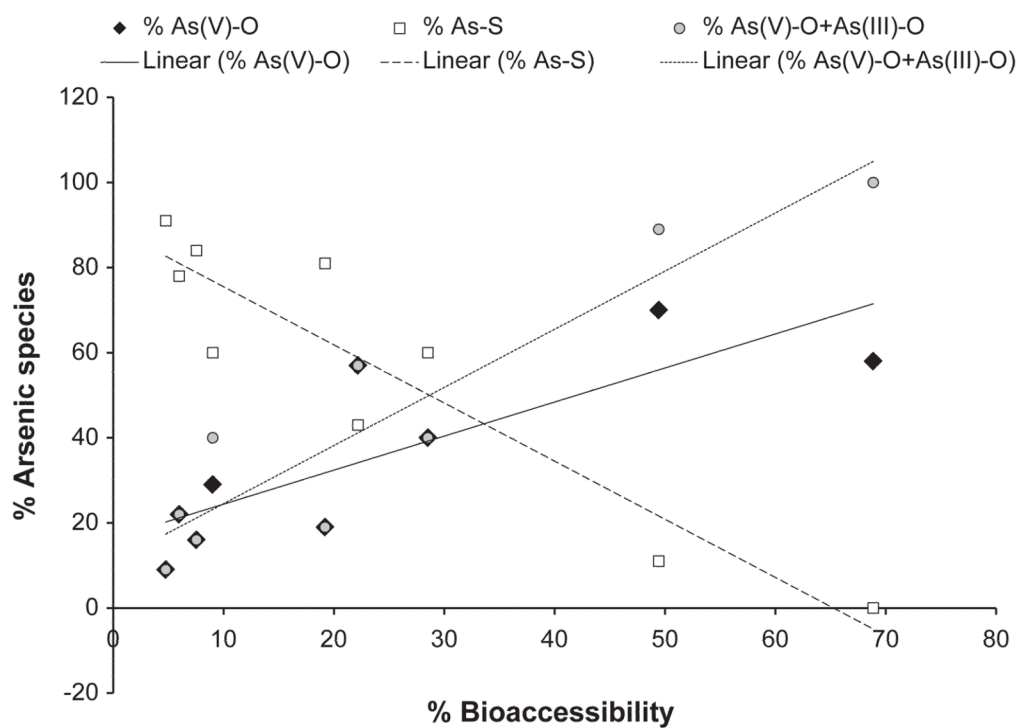
Supplementary data to this article can be found online at doi:10.1016/j.scitotenv.2011.07.059.



**Fig. 1.** Arsenic K-edge XAS spectra of traditional medicines containing arsenic around  $10 \text{ mg} \cdot \text{kg}^{-1}$  or higher (product name is followed by ID in parentheses). Medicines are ordered from top to bottom from most % As(V)-O to least, and dotted lines indicate best fit. Solid vertical lines indicate locations of As-S, As(III)-O, and the usual position of As(V)-O white line. Samples marked with an \* contained As(V)-O partially as As(V)-glycerol.



**Fig. 2.** Estimated daily ingestion amounts of (a) lead and (b) arsenic for Ayurvedic medicines containing bioaccessible lead and arsenic. Min = minimum amount based on minimum dose; max = maximum amount based on maximum dose. The California Proposition 65 maximum allowable dose level for lead is  $0.5 \mu\text{g}\cdot\text{d}^{-1}$ ; the American National Standard Institute/ National Sanitation Foundation International Dietary Supplement Standard 173 (ANSI 173) states that dietary supplements should not contain undeclared metals that would cause intakes greater than  $20 \mu\text{g}\cdot\text{d}^{-1}$  for lead and  $10 \mu\text{g}\cdot\text{d}^{-1}$  for arsenic; the Health Canada Toxicological Reference Value (HC-TRV) for lead is  $0.0036 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  corresponding to  $252 \mu\text{g}\cdot\text{d}^{-1}$  for a 70 kg person; the United States Environmental Protection Agency reference dose (USEPA RfD) for chronic oral intake of arsenic is  $0.3 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ , corresponding to  $21 \mu\text{g}\cdot\text{d}^{-1}$  for a 70 kg adult.



**Fig. 3.** Percent arsenic species vs. percent bioaccessibility for ◆ As(V)-O species (sum of species matching  $\text{KH}_2\text{AsO}_4 + \text{As(V)-glycerol}$ ), ● As(V)-O+As(III)-O, and ◻ As-S, based on samples (n=9) analyzed by XANES. The correlations of the data are significant ( $p < 0.05$ , see text for  $r$  values), showing that the As(V)-O and As(III)-O species are likely the bioaccessible ones, whereas the As-S species are not.



**Table 1**Products tested and arsenic and lead contents (mg.kg<sup>-1</sup>).

ID	Product name	Rasa Shastra <sup>a</sup>	Total As <sup>b</sup> (mg.kg <sup>-1</sup> )	Total Pb <sup>b</sup> (mg.kg <sup>-1</sup> )
1	Mahasudarshan specialty tablets		1.4	8.5 <sup>c</sup>
2	Shilajit specialty tablets	Yes	3.2	10 <sup>d</sup>
3	Vital Lady	Yes	2.1	5.5 <sup>c</sup>
4	Worry Free	Yes	<1.0	7.0 <sup>c</sup>
5	Yog-Raj Guggulu		31 <sup>d</sup>	6 <sup>d</sup>
6	Commiphora mukul		<1.0	2.3
7	Bacopa monniera		<1.0	6 <sup>c</sup>
8	Yogaraj Guggulu		2.2	2.0
9	Ezi Slim		1.4	3.0 <sup>c</sup>
10	Shilajeet Tablets	Yes	21 <sup>d</sup>	10 <sup>d</sup>
11	Mahayograj Guggulu	Yes	89	52,000
12	Brahmi Capsules		<1.0	6.5 <sup>c</sup>
13	Amoebica		3.8	11.0 <sup>c</sup>
14	Arogyavardhini Bati	Yes	4.6	125 <sup>c</sup>
15	Kanchanar Guggulu		<1.0	7.5 <sup>c</sup>
16	Shilajit	Yes	1.6	10.5 <sup>c</sup>
17	Lean Plus		<1.0	6.0 <sup>c</sup>
18	Neem Plus		<1.0	10.5 <sup>c</sup>
19	Prana: Breath of Life		2.1	9.0 <sup>c</sup>
20	Bakuchi		<1.0	3.0 <sup>c</sup>
21	Brahmi		1.1	6.5 <sup>c</sup>
22	Chairata		<1.0	6 <sup>c</sup>
23	Cold Aid		<1.0	5.5 <sup>c</sup>
24	Trifala Guggulu		27 <sup>c</sup>	20.5 <sup>c</sup>
25	Heart Plus		2.0	7.5 <sup>c</sup>
26	Jatamansi		3.6	12.0 <sup>c</sup>
27	Kanta Kari		<1.0	20.5 <sup>c</sup>
28	Lavan Bhaskar		3.4	<1.0
29	Licorice — Glycyrrhiza Glabra		1.0	5.5 <sup>c</sup>
30	Mahasudarshan		140 <sup>d</sup>	15 <sup>d</sup>
31	Praval Pisti	Yes	27.5 <sup>c</sup>	7.5 <sup>c</sup>
32	Prostate Rejuv	Yes	1.2	11.5 <sup>c</sup>
33	Shilajit	Yes	12 <sup>d</sup>	1.6
34	Sugar Fight		6.4	7.5 <sup>c</sup>

ID	Product name	Rasa Shastra <sup>a</sup>	Total As <sup>b</sup> (mg.kg <sup>-1</sup> )	Total Pb <sup>b</sup> (mg.kg <sup>-1</sup> )
35	Tagar		2.3	12.0 <sup>c</sup>
36	Yograj Guggulu		10.5 <sup>c</sup>	17.5 <sup>c</sup>
37	Ayu-Arthri-Tone	Yes	<1.0	63 <sup>c</sup>
38	Ayu-Hemoridi-Tone	Yes	7.5	3.9
39	Ayu-Leuko-Tone	Yes	2.6	33 <sup>c</sup>
40	Ayu-Nephro-Tone	Yes	<1.0	340 <sup>c</sup>
41	AyurRelief™		<1.0	5.5 <sup>c</sup>
42	Glucorite™		1.8	6.0 <sup>c</sup>

<sup>a</sup> Products were classified as *Rasa Shastra* medicines (elaborately prepared compounds combining herbs with metals, minerals, and gems) by an Indian-trained Ayurvedic physician.

<sup>b</sup> Unless otherwise noted, results were determined by *aqua regia*-ICP methods.

<sup>c</sup> XRF results, previously published with significant figures as reported in Saper et al. (2008).

<sup>d</sup> XRF results, reported for the first time in the present study.

**Table 2**

Liquid to solid ratios used in bioaccessibility extractions and recommended dosing information.

Samples (IDs)	Ratio (mL:g) <sup>a</sup>	Minimum daily dose <sup>b</sup> (g)	Maximum daily dose <sup>b</sup> (g)
4, 24, 26, 28, 35, 36	100:1	2.6	4.1
1, 2, 5, 8, 10, 14, 15, 16, 20, 21, 22, 25, 27, 29,30, 31, 32, 33, 34, 37, 38, 39, 40, 42	200:1	1.5	2.8
3, 11, 12, 13, 17, 18, 23, 41	400:1	1.0	1.4
Powders 6, 7	100:1	Standard ratio <sup>c</sup>	
9	200:1	Maximum dose of 6.1 g per day in 2000 mL <sup>d</sup>	
19	400:1	Maximum dose of 11 g per day in 2000 mL <sup>d</sup>	

<sup>a</sup> Ratios are based on the daily gastric juice volume (2000 mL) corrected to the total extraction time (×5 h per 24 h=417 mL) to the maximum dose (g) of each medicine, except where noted.

<sup>b</sup> Minimum and maximum doses are within the group of samples. All doses for individual samples were calculated as maximum, based on label information.

<sup>c</sup> Dose could not be estimated so standard ratio was used.

<sup>d</sup> Large daily masses resulted in ratios that were impractical for bioaccessibility extraction; 2000 mL used instead of 417 mL.

Table 3

Arsenic concentration ( $\text{mg}\cdot\text{kg}^{-1}$ ) and speciation (proportion of arsenic species, %, by X-ray absorption near-edge structure spectra) in a subset of samples; only the samples that contained arsenic at approximately  $10 \text{ mg}\cdot\text{kg}^{-1}$  or greater could be analyzed.

ID	As ( $\text{mg}\cdot\text{kg}^{-1}$ )	Product name	Ingredients	Amorphous orpiment%			Red $\chi^2$ <sup>a</sup>
				As(V)-O %	As(V)-glycerol %	As(V)-O %	
5	31	Yog-Raj Guggulu	Resin and plants	60(5)	11(4)	8(3)	21(2) 0.001
10	26	Shilajeet Tablets	Bitumous substance collected from rocks	81(3)		19(3)	0.003
11	89	Mahayograj Guggulu	Resin, plants, seven <i>bhasmas</i> <sup>b</sup>	11(6)	19(4)	59(4)	11(2) 0.002
24	27	Trifala Guggulu	Resin and plants	78(3)		11(5)	11(6) 0.004
30	140	Mahasudarshan	Plants, including roots	84(3)			16(3) 0.004
33	12	Shilajit	Bitumous substance collected from rocks	43(4)		48(5)	8(6) 0.005
34	6.4	Sugar Fight	Plants, including roots	60(2)		40(2)	0.002
36	11	Yograj Guggulu	Resin and plants	91(3)		9(3)	0.004
38	7.5	Ayu-Hemoridi-Tone	Plants, sodium carbonate		42(2)	58(2)	0.003

Numbers in parentheses indicate three times the standard deviation for the fit achieved. Arsenic species are named as those to which data were fitted.

<sup>a</sup>Red  $\chi^2$  indicates the goodness of the fitting to obtain the proportions of arsenic species (smaller values are better;  $<0.003$  indicates a good fit).

<sup>b</sup>The seven *bhasmas* used in Mahayograj Guggulu preparation are naga (lead), yanga (tin), rajata (silver), loha (iron), abhraka (mica), mandura (oxidized iron), and rasa sindura (red sulfide of mercury). *Bhasma* preparation often uses arsenic containing compounds. For example, in the classical preparation of *naga* (lead) *bhasma*, realgar ( $\text{As}_4\text{S}_4$ ) is used. arsenate compound used for fitting ( $\text{KH}_2\text{AsO}_4$ ) and this species matched As(V)-glycerol.

Table 4

Bioaccessible (BA) arsenic (As) and lead (Pb) in concentration units ( $\text{mg}\cdot\text{kg}^{-1}$ ) and percent of total (%).

ID	BA As ( $\text{mg}\cdot\text{kg}^{-1}$ )		% BA As (%)		BA Pb ( $\text{mg}\cdot\text{kg}^{-1}$ )		% BA Pb (%)	
	G	G+I	G	G+I	G	G	G	G
1	<1.0	<1.0	<71	<71	3.2	3.2	37	
2	<1.0	<1.0	<31	<31	2.2	2.2	22	
3	1.1	<1.0	52	<48	1.9	1.9	35	
4	1.4	1.1	100	100	1.2	1.2	17	
5	1.6	2.8	5.1	9.0	1.7	1.7	28	
8	<1.0	<1.0	<45	<45	1.0	1.0	52	
9	<1.0	<1.0	<71	<71	1.2	1.2	41	
10	3.8	3.9	19	19	2.5	2.5	25	
11	40	44	45	49	49,000		94	
12	<1.0	<1.0	N/a	N/a	1.4	1.4	21	
13	<1.0	<1.0	<26	<26	6.0	6.0	55	
14	<1.0	<1.0	<22	<22	10	10	8	
15	<1.0	<1.0	N/a	N/a	1.5	1.5	21	
16	<1.0	<1.0	<63	<63	1.6	1.6	15	
19	<1.0	<1.0	<48	<48	2.0	2.0	23	
22	<1.0	<1.0	N/a	N/a	1.3	1.3	22	
24	1.4	1.6	5.3	6.0	6.0	6.0	29	
25	1.3	<1.0	64	<50	5.4	5.4	72	
26	<1.0	<1.0	<28	<28	1.2	1.2	10	
27	<1.0	<1.0	N/a	N/a	1.4	1.4	6.7	
30	10	11	7.1	7.5	2.7	2.7	18	
31	<1.0	<1.0	<3.6	<3.6	2.5	2.5	33	
32	<1.0	<1.0	<83	<83	2.0	2.0	17	
33	2.4	2.6	20	21	<1.0	<1.0	<63	
34	1.8	1.4	29	21	5.6	5.6	75	
35	<1.0	<1.0	<43	<43	1.3	1.3	11	
36	<1.0	<1.0	<9.5	<9.5	5.2	5.2	30	

ID	BA As ( $\text{mg}\cdot\text{kg}^{-1}$ )		% BA As (%)		BA Pb ( $\text{mg}\cdot\text{kg}^{-1}$ )		% BA Pb (%)	
	G	G+I	G	G+I	G	G	G	G
37	<1.0	<1.0	N/a	N/a	25		40	
38	5.2	5.1	69	68	1.1		29	
39	<1.0	<1.0	<38	<38	7.1		21	
40	<1.0	<1.0	N/a	N/a	270		79	
41	<1.0	<1.0	N/a	N/a	1.3		23	
42	1.5	<1.0	86	<56	2.5		41	

G = gastric phase and G+I = gastric followed by intestinal phase; both are shown for arsenic since both phases are relevant, but only the gastric phase results are shown for lead since these are the highest results. N/a = not applicable, because bioaccessible concentration detection limit is greater than total concentration, both bioaccessible concentration and total concentration are below detection, or total concentration is below detection.