**Determination of the total and bioaccessible contents of essential and potentially toxic elements in ayurvedic formulations purchased from different commercial channels**

# Determination of the total and bioaccessible contents of essential and potentially toxic elements in Ayurvedic formulations purchased from different commercial channels

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## Abstract

Ayurveda is a major traditional system of Indian medicine that is still being successfully used in many countries. It advocates the use of both herbal and metallic preparations, in which herbs are deliberately combined with metals, minerals and gems. This study is aimed to identify possible risks associated to the presence of toxic elements in ayurvedic formulations. We determined the concentrations of 25 elements (Al, As, Ag, Au, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, Pd, Pt, Sb, Si, Sn and Zn) in 17 ayurvedic products manufactured in India and sold in different distribution channels: Indian ayurvedic medical shops, an Italian pharmacy and on the Internet. After sample mineralization, concentrations were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). We calculated daily intake of each element and compared the obtained concentrations with the maximum tolerable intake levels or recommended nutrient amounts. Element content found in the products purchased on the Internet and in the Italian pharmacy was lower than the safety limits fixed by the international authorities. Five medicines purchased in India contained potentially dangerous amount of As, Cu, Hg, and Pb. Metal bioaccessibility was studied by extraction with solutions simulating gastric and intestinal fluids. The concentration of As potentially adsorbed during the digestion of two products was greater than the maximum admissible daily intake for this element. The obtained results were treated with chemometric techniques.

*Keywords:* Ayurvedic medicines; Potentially toxic elements; Metals; Bioaccessibility; Chemometrics

## 1. Introduction

There are many traditional systems of medicine in the world and each one is associated to a particular philosophy and culture. In particular, the ayurvedic system of medicine is the most widely practised in the Indian traditional medicine systems. In the last years, it has become increasingly popular in North America, Europe and Australia [1], so that ayurvedic formulations are available from local chemists, ethnic markets, practitioners, health food stores and Internet [1-7]. Ayurveda advocates both the use of herbal preparations (*kasthausadhi*), similar to other civilizations of the world, and metal-based preparations (*rasausadhi*), that are unique in Ayurveda and not present elsewhere [8]. In fact, *rasa shastra* is an ancient Indian practice in which herbs are deliberately combined with metals (e.g., mercury, lead, iron, zinc), minerals (e.g., mica) and gems (e.g., pearl) [9,10]. The metals and metalloids present in ayurvedic formulations are claimed to be detoxified by elaborate processing steps including many cycles of heating and subsequent cooling in herbal mixtures and animal products such as cow urine or butter (*ghee*) [11-14]. According to the tradition, these processes would eliminate the harmful effects of metals. Metallic herbal preparations offer advantages over plant-based drugs by virtue of their stability over a longer period, lower dosage, easy storability and sustained availability [8].

Furthermore, metals may be accidentally introduced into the herbal medicines: the distribution of inorganics in the end-products depends on a number of variables, such as climate, soil characteristics, conditions of transport, storage and preparation.

Generally, lead is widely distributed in spices and herbal plants; cadmium in foods is mostly derived from various sources of environmental contamination; high levels of arsenic can be due to use of fertilizers and pesticides [15-17]. Additional sources of heavy metal contamination can be rainfall, presence of vehicular traffic, use of oil or fossil fuels for heating. Pollution may also occur during half- and end-products manufacturing or during storage, by leaching of metals from containers [18].

Moreover, products sold as medications, whether “conventional” or not, should be licensed by the health authorities of each country in which they are marketed, and their quality and contents closely controlled [4]. Ayurvedic medicines are marketed as “dietary supplements”, which are regulated under a specific legislation for traditional herbal medicines in UE [19]. Traditional

formulations have some typical characteristics, in particular their long time of employment. For this reason the UE established an easy procedure for the license of these products, i.e. it is possible to register traditional formulations if they have been used for therapeutic purpose in the country of origin for at least thirty years, fifteen of which in UE countries. On September 2004 the Committee for Herbal Medicinal Products (HMPC) was established at the European Medicines Agency with the purpose of preparing a list of admitted formulations and plant-based products. However some of these products that do not satisfy the medicine requirements, can be marketed as "food" or as "food supplement" [20].

Also in USA, where ayurvedic formulations are regulated by the Dietary Supplement Health and Education Act (DSHEA), no proofs of safety or efficacy are required [9].

Over recent years, some metallic preparations used in the Indian system of medicine have been suspected to be harmful because some cases have been published detailing patients poisoned by heavy metals after the ingestion of these remedies [1-3,6-8,21-32]. Recently a study conducted on ayurvedic medicines by Harvard Medical School [14] also reported that some of these drugs had potentially harmful levels of lead, mercury and arsenic.

Hence it is very interesting to determine the element content in traditional ayurvedic medicines available on the market and consequently highlight the possible risks for the costumers.

Nonetheless, not all the amount of an element present in an ingested product is available for absorption by the organism; for this reason the knowledge of the total concentration is not sufficient to assess its potential harmful effects, but it is important to value the amount of each element potentially assimilated after ingestion. It is quite difficult to determine this parameter experimentally, since it would require expensive and ethically troublesome *in vivo* experiments, but it is possible make an *in vitro* estimation by measuring the bioaccessibility of metals and metalloids. Bioaccessibility has been defined as the fraction of a compound that is released from its matrix in the gastrointestinal tract, and thus becomes available for intestinal absorption [33]. Several *in vitro* approaches have been developed in attempts to mimic the effects of the human digestion process. They are commonly described in the scientific literature under the name of physiologically-based extraction test (PBET) or, more generally, simulated gastrointestinal extraction procedures [34]. These seek to mimic processes that occur in the different areas of the human digestive system and they can be applied to value the risk for human health associated to the fraction of different substances absorbed during the digestion of different matrices. For

example these types of study have been applied to study the bioaccessibility of lipophilic nutrients, nutrients, polyphenols and metals from foods and medicines and sediment [33,35-38].

In this study we assessed the levels of Al, Ag, Au, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, Pd, Pt, Sb, Si, Sn and Zn in seventeen ayurvedic formulations purchased from different distribution channels: 7 from medical shops in India, 5 on the Internet, and 5 in an Italian pharmacy, to compare the safety of products from different sources.

We focused our attention on the potentially toxic elements such as As, Cd, Pb and noble metals to identify the traditional Indian ayurvedic medicines that were intentionally added with high concentrations of such elements and investigate the possible risks for health due to their consumption. We considered also macro- (e.g. Ca, Mg, Na, K, P) and micro- nutrients (e.g. Cr, Fe, Mn, Ni, Sn, Zn,…) to have a complete idea of the medicines inorganic composition.

In this way, we can verify if there are substantial differences between the samples commercially available in India, those available everywhere in the world from the Internet and those admitted by the European Legislation.

Concentrations were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) after sample mineralization in a microwave oven. This technique is largely used for the determination of metal content in ayurvedic medicines [4,7,13,32]. The results of the analysis are interpreted taking into account the meaning of the traditional names of the medicines. We treated the data with chemometric techniques to better understand similarities and differences among products and correlations among variables, i.e. the investigated elements, which might be more difficult to detect just from the dataset [39,40].

The estimated daily intake of each element was compared with reference values [41], considering the maximum tolerable intake levels or recommended nutrient amounts issued by national and international organizations.

To evaluate the bioaccessibility of the potentially toxic elements during digestion, we adopted the procedure suggested by the American Pharmacopeia, simulating the release of the elements in stomach and intestine [42]. To our knowledge, this is the first study in which this approach was adopted with ayurvedic medicines.

## 2. Materials and Methods

### *2.1. Samples and Sample Pretreatment*

The ayurvedic formulations analyzed were purchased from different commercial channels.

Products A, B, C, D, E, F and G were purchased in India: A, B, C, D, E and F from a medical shop in Chennay city (Tamil Nadu, South-East of India), while sample G in a village in Rajasthan region (North-West of India) by Italian tourists; samples H, I, J, K and L from the Internet website of an ayurvedic formulations producer; samples M, N, O, P, Q were bought in an Italian pharmacy in Torino (Piedmont region). The criterion of choice was the Indian origin of the raw materials used for the preparation of the medicines.

The names of the products, the ingredients declared on the label and the aspect of formulations are summarized in Table 1. The meaning of some Indian terms is explained in "Results and discussion" section.

The purchased samples appeared as powder (8 samples), tablet (8) or paste (1). The tablets were ground in a mortar in order to obtain powdered samples. The powders and the paste were analysed without any pretreatment.

### *2.2 Apparatus and reagents*

Sample dissolution was performed in polytetrafluoroethylene (PTFE) bombs, with a Milestone MLS-1200 Mega (Milestone, Sorisole, Italy) microwave laboratory unit.

The cations were determined with a Perkin Elmer Optima 7000 (Perkin Elmer, Norwalk, Connecticut, USA) inductively coupled plasma-optical emission spectrometer (ICP-OES).

Standard metal solutions were prepared from concentrated stock solutions (Merck Titrisol or Sigma Aldrich).

High purity water (HPW) produced with Millipore Milli-Q system was used throughout. The reagents adopted were of analytical grade.

The gastric juice was prepared dissolving 2.0 g of NaCl and 3.2 g of pepsin (3200-4500 UI/mg) in 7.0 mL of HCl. The solution was diluted to 1000 mL with HPW; the final pH is 1.2.

The intestinal medium was obtained dissolving 6.8 g of $KH_2PO_4$ in 250 mL of HPW; then 77.0 mL of 0.2 N NaOH and 10 g of pancreatin (protease 1.5 UI/mg; amylase 24.2 UI/mg and lipase 21.7 UI/mg) and 500 ml of HPW were added; finally pH was adjusted to 6.8 with the aid of 0.2 N NaOH or 0.2 N HCl and the solution was diluted to 1000 mL with HPW.

### *2.3 Procedures*

#### *2.2. 1. Dissolution and analysis for total element content*

Acid digestion in microwave oven was adopted to dissolve the samples. Aliquots of 200 mg were treated with 3 mL of 65 % $HNO_3$ and 3 mL of 30 % $H_2O_2$ in PTFE bombs. Six heating steps at a power of 250 (1 min), 0 (2 min), 200 (5 min), 350 (5 min), 550 (5 min), 250 W (5 min) respectively, followed by a ventilation step for 10 min, were applied. The resulting solutions were filtered on Whatman 5 filters and then diluted to 50 mL with HPW. The solutions were analysed by ICP-OES.

In order to check the accuracy of the experimental procedure, two quality control procedures were adopted. Firstly, synthetic solutions consisting of aliquots of sample blank added with known concentrations of all the considered analytes were prepared in the bombs of the digestion system and treated following the same procedure as for the samples. The final concentration of the analytes was 1 mg/L. Then, a Certified Reference Material (CRM), namely *Tomato Leaves* SRM 1573a, supplied by the National Institute of Standards and Technology (NIST), was analyzed to value the effect of a vegetal matrix on element determination. This CRM contains certified concentrations of Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Sb, Se and Zn

Initially, a semiquantitative analysis was conducted on the sample solutions to value the concentration levels of the above mentioned elements. Some elements, namely Ag, Au, Cd, Co and Sb, were found to be below the detection limits, so 20 analytes with concentration higher than the detection limit in most of the samples, or for a group of them, were subjected to quantitative analysis.

Taking into account the results of a preliminary semiquantitative analysis, the concentrations of all the analytes in the products from Internet and the pharmacy were determined without any dilution, while the quantification of As, Ca, Cu, Fe, Hg, K, P, Pb and Sb in some Indian medicine required a 100 (or 1000 in few cases) -fold dilution of the sample solutions. Aliquots of each sample solution were diluted with HPW to different dilution levels suitable for the determination of the different analytes. The calibrations were performed with standard solutions prepared in aliquots of sample blanks diluted in the same ratios as the sample solutions. Standard solutions were periodically analyzed and their signals were used to correct the sample signals for drift of instrumental sensitivity.

The limits of detection (LoD) and quantification (LoQ) were estimated as three and ten times the standard deviation of the blank respectively.

### *2.3.2. Study of bioaccessibility*

The efficacy of the extraction of gastric and intestinal media after the same contact time with the sample was valued: 200 mg of sample were put in contact with 25 mL of each extractant for 2 h maintaining the solutions at the temperature of 37 °C and shaking periodically to mimic peristaltic motions of stomach and intestine.

In a separate experiment, each solution was left in contact for the time suggested by the Pharmacopeia [40]: 2h and 6h for gastric and intestinal media respectively. After extraction the suspensions were centrifuged at 4000 rpm for 10 minutes. The solutions were filtered on Whatman 5 filters.

All experiments were performed in triplicate.

### *2.3.3 Chemometric data processing*

A chemometric analysis of the experimental results was performed by Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) and Factorial Discriminant Analysis (FDA) with the aid of XLSTAT 4.4 software package, used as a Microsoft Excel plug-in. Unscrambler X 10:2 software package was used for data preprocessing and for some tests by Discriminant Analysis.

The values below detection limit were substituted with a random number between zero and that limit. The data were preprocessed by column standardization, i.e. by mean-centering (for each variable) and dividing by the corresponding standard deviation.

PCA is the multivariate technique most extensively used for processing the results of single and sequential extractions. This happens because it is relatively easy to apply, using the commercial software packages, the interpretation of the data is relatively simple and provide useful information.

PCA is an unsupervised pattern recognition technique, i.e. a technique for classifying objects into classes that are not established a priori. It is based on variable reduction through the calculation of the so-called principal components (also named “factors” or “latent variables”), which are linear combinations of the original variables. The first two PCs were considered in PCA. The interpretation of the loading plots was aided by the examination of the Pearson's correlation matrix (not reported).

The aim of cluster analysis is grouping samples or variables; the most frequently used form of cluster analysis is HCA. It is an unsupervised pattern recognition technique, in which the clusters are formed during the calculation and their number is not decided a priori. The groups are

obtained stepwise with an agglomerative (starting from single objects and joining them in successively larger groups) or divisive (starting from one cluster comprising all objects and dividing it into successively smaller and more homogeneous clusters) approach. The clusters are formed during the calculation and their number is not decided a priori. HCA was carried out using the Euclidean distance and Ward's agglomeration method; the results were reported in a dendrogram.

Linear discriminant analysis (LDA) is a supervised pattern recognition technique, in which objects are grouped in classes established by the researcher a priori. It is based on the calculation of new variables, called discriminant functions, obtained from linear combinations of the original variables. Such linear combinations are derived so as to best indicate the differences between the classes in contrast with the variance within the classes [39,40].

## *3.* Results and discussion

### *3.1. Analytical approach*

To verify the accuracy of both digestion and analysis procedures, the concentrations of the considered analytes in synthetic solutions a were preliminarily determined and the concentrations found were compared with the expected ones: the recoveries obtained were in the range 93 (for P) ÷ 101 (for Cu) % for all the considered elements, as reported in Table 2; this table also collects the LoD and LoQ values. Some of considered analytes, namely Ba, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, and Zn were also determined in CRM NIST 1573a: the recoveries range between 85 and 113% for all considered analytes (Table 2).

Those results can be considered satisfactory in view of the concentrations involved, so ayurvedic drugs were analysed following the same procedure. Unfortunately, the commercially available plant based-CRMs contain some elements, such as As, Hg, Pd and Pt in concentration lower that the ICP-OES detection limits or are not certified for other elements, as Pd, Pt and Sn. For these reasons we periodically checked the goodness of our procedures also with other CRMs (e.g. BCR 279, BCR 176) and with commercially available Multi-Element Standards for ICP. The recovery obtained are generally on the order of ± 15 % for all elements in a range of concentration of 1-100 mg/L. The uncertainty of the experimental results, evaluated from the standard deviation estimates, ranges between 0.5 (Na) and 6.0 (Cu)%.

### *3.2 Total element content*

Total element contents in the different batches of samples (purchased in India, on the Internet and in an Italian pharmacy) will be discussed separately and then compared (paragraph 3.1.4).
The concentrations of Ag, Au, Cd, Sb are lower than the LoQ in all investigated samples.

### *3.2.1 Samples from India*

Medicines purchased in India were bought in a traditional Indian medical shop in Chennay city (samples A, B, C, D, E and F) and in a village in Rajastan region (G).
These products belong to the group of ayurvedic drugs called *rasausadhi*: they are traditional multi-ingredients formulations containing herbs and minerals.
Part of the name of *rasausadhi* ayurvedic medicines is traditionally referred to the type or to the aspect of the preparation: for example "bhasma" means that the formulations contain incinerated metals or minerals; "sinduram" that the drugs are prepared by sublimation; "parpam" that they are powders, "chenduram" that they are nano-sized particles powders.
Product G contains "Guggulu" that is "*commiphora mukul*", one of the most largely used plants in Indian medicine, generally present in the formulations in combination with several other herbs to enhance its effects. There are different preparations of "guggulu-based medicines", namely mineral-herbal and herbal only. In sample G the term "yograj" means "formulation of the king" denoting that metals have been added during the preparations to increase the effect of the medicine, as indicated by the superlative "maha".
The obtained results are reported in Table 3.
We can see a great elemental composition variability among the formulations that reflects the high variability of the ingredients adopted. Some elements that are present in trace levels in some drugs show very high concentration in others.
Aluminum, Ca, Fe, Hg, K, Mg, Na, Pb, Si and Zn are the most abundant elements in the group of ayurvedic formulations purchased in India: they are present at concentrations greater than 100 mg/kg in most of these products. Al, Ca, Fe, K, Mg, Na, Si, and Zn are nutrients for humans and/or for plants and can derive from natural sources: for instance, the natural herbals composition is conditioned by characteristics of the soil and by the water with which the herbs were in contact during their growth. Different considerations have to be made for Hg and Pb that stand out among the elements with highest concentration. Five samples contain very high level of mercury (from 100 to 50,000 mg/kg). Four drugs contain Pb in concentrations greater than 100 mg, and in one sample the concentration reaches $1.3 \times 10^5$ mg/kg.

Arsenic concentrations are lower than the LoQ in two products, at mg/kg level in other two, while reach g/kg amounts in the other ones.

Copper shows a high concentration (1.9 and $2.5x10^2$ mg/kg) in two samples and a very high content ($4.5x10^5$ mg/kg) in another sample.

Also Pd and Sn, generally absent or present in low concentrations respectively, are contained with high concentration in one sample.

These results reflect the fact that metals and metalloids are intentionally added to some ayurvedic formulations to improve the therapeutic effect of the medicine, according to tradition.

In some cases we obtained very high standard deviations: this is caused by the heterogeneity of the materials present in each sample, even if the drugs were grounded in very fine powder.

For *rasausadhi* medicines, the high content of some elements, in particular of potentially toxic ones, could have been predicted from the names of the formulations:

Sample A shows high Ca content due to the presence of a shell ("suththi seitha kadal sangu") and CaO ("kilinchal chunnambu") as ingredients; this formulation also presents high concentration of Na (probably derived from the NaCl deposited on the shell) and Zn.

Sample B ("ekaguna sindura" or "rasa sindura") contains lower amounts of elements in comparison with the others, with the only exceptions of Hg and Pd. "Rasa" means Hg, showing that it was intentionally added.

Samples C and F, both named "thalaka or talaka" that means orpiment ($As_2S_3$), present high concentrations of As. With the exception of As content, these two products have different composition. In particular sample C shows a higher amount of Hg.

Sample D, called "thambira parpam" (from "tambra"= Cu) contains a high concentration of Cu. This drug is also characterized by high concentrations of Fe ($3.0x10^4$ mg/kg) and Pd ($1.2x10^2$ mg/kg), which is present at levels lower than the LoQ in all the other drugs. The high content of Pd cannot be connected to the drug name, but the addition of noble metals it is a typical practice of "rasa sastra".

Sample E is named "vel vanga parpam": "vanga" means Sn, whose content in this formulation is 25.5 mg/kg; anyway other samples (D and G) contain higher concentrations of this element.

Sample G contains a lot of herbs, as listed in Table 1. However, this medicine contains also Sn ("vanga bhasma"), Ag ("raupya bhasma"), Pb ("naga bhasma"), Fe ("loha bhasma"), mica ("abhrak bhasma"), rust ("mandura bhasma") HgS ("rasa sindur") and

auxiliary exipients ("shesh"). This composition is well represented by the results obtained, in particular sample G contains the maximum amount of Pb ($1.3x10^5$mg/kg) and a very high concentration of Hg ($4.8x10^3$ mg/kg). The high concentration of As is not related to the drug ingredients. The sample also contains a high level of K, probably deriving from the herbs adopted.

### *3.2.2 Samples from the Internet*

The samples purchased on the Internet belong to the ayurvedic formulations called *kasthausadhi*, that is they only contain herbs. They are declared as "metal free" by the producer on the web site and the ingredients are declared to have Indian origin. All the medicines are multi-herbs products, with the only exception of sample K, that contains only *Aloe vera* (for this medicine the composition is not reported on the label but only on the Internet site).

Also for these samples part of the name defines the aspect of the preparations according to the Indian tradition: the words "vati", "podi" and prash" mean "tablets", "powder" and "paste" respectively. But the characteristic of the *kasthausadhi* formulations is that they are named after their benefits or action towards the body parts or disease and not after their ingredients. For example "vaira" (=diamond) means that the drug enhances sturdiness; "shanti" (=peaceful) that it induces relax; "niragada" is a Sanskrit name and we were not able to translate it; "kumari" (=young lady) indicates that the medicine is supposed to help to remain young.

The elements showing the highest content in the medicines purchased on the Internet are Al, Ca, Fe, K, Mg, Na; P and Si.

The presence of Si and Mg are declared on the label for products H, I and J and derives from the excipients contained in them, namely silica and magnesium stereate; the high concentrations of P and K derive from the plants present as major constituents in these formulations.
Arsenic, Cr, Hg and Pb are always present at concentrations lower than 5 mg/kg.
The amounts of Pt and Pd are not reported because they are always below the LoQs.

### *3.2.3 Samples from Italian pharmacy*

The drugs purchased from a pharmacy in Torino belong to the *kasthausadhi* group, with the exception of sample P in which also minerals are present. Also in this case, we selected commercial products containing ingredients coming from India. The total concentrations are reported in Table 5.

All the selected products are well known traditional ayurvedic formulations.
In sample M "nidra" means meditation and "shanti" peaceful; in sample O "rakta" reinforces "shanti". Both these products are indicated to reduce blood pressure.
Unlike sample G, purchased in India (paragraph 1.1.1), sample N, "kaishor guggulu", is a type of guggul preparation that belongs the *kasthausadhi* family since it is only herbal-composed. It is traditionally used to treat a great variety of problems (from weak digestion to cancer).
As to sample P, "chandraprabha" means moonlight, to define its effect on the health problems influenced by moon cycles.
In sample Q, "praval moti" means "refresh": it is a strong mineral salts supplement used to balance depletion of bodily fluids.
The medicines purchased in Torino present great amounts of Ca, K, Mg, Na, Fe, Si and P. As observed above, the high concentrations of K and P come from the plants adopted for these preparations. The high concentration of Ca observed in sample Q derives from the presence of calcined coral and pearl in the product.
The concentrations of As, Hg, Pd and Zn were lower than the instrumental LoQ in all samples with the only exception of sample M, in which As was detected at very low level. In all the products Cu, Cr, Ni and Pb show concentrations lower than 5 mg/kg.
The amounts of Hg, Pd, Pt and Zn are not reported because they are always below the LoQs.

### *3.2.4 Comparison among the different groups of medicines*

Figure 1 shows the percentages of samples, divided for each group, containing element concentrations higher than 100 mg/kg.
Aluminum, Ca, Fe, K, Mg, Na, P and Si are generally the most abundant elements in all the medicines, showing no difference between *rasausadhi* and *kasthausadhi* drugs. The concentration level of Mn is randomly higher than 100 mg/kg in the three groups of drugs. These elements derive from the vegetal components of the formulations or from the excipients.

The presence of As, Cu, Hg, Pb, Pd, Sn and Zn at high concentration levels is a characteristic of the *rasaushadi* preparations.

The obtained results show great differences between the *kasthausadhi* and *rasausadhi* drugs: in the latter, intentional addition of As, Hg, Pb and noble metals is still adopted, according to the tradition. This causes the presence of high concentrations of potentially toxic elements, as demonstrated by our measurements.

We have to underline the important difference between the two "guggulu preparations", G ("maha yograj guggulu") and N ("kaishor guggulu"). Sample G, purchased in a village in India, contains very high amounts of As, Hg and Pb, that are absent in sample N purchased in the Italian pharmacy. To avoid health risks, the customers have to be aware of the drug composition that they are assuming, since a medicine named after a herbal component can belong to both *katsausadhi* and *rahsausadhi*. For instance a tourist from a Western country, who usually consumes a product in his nation of origin, during a trip to India, might buy a product with a similar name, but a different composition, also taking into account that labels may be written in foreign languages, with Sanskrit fonts. At the same time, practitioners should have an extensive knowledge of ayurvedic medicine tradition and of the feature of each formulation, even in the presence of similar names, and indicate the right dosage to avoid health risks.

### *3.2.5 Chemometric data processing*

PCA and HCA are multivariate techniques that take into account simultaneously the behaviour of more variables (in our work the variables are the element concentrations).

Processing the data on total concentrations of all considered analytes with these techniques, as we can see (Figures 2.1 and 3.1) the samples are grouped all together with the exception of formulations D and G, the first characterized by high concentrations of Cu, Fe and Pd, and the second (the only formulation purchased in an Indian village) containing high levels of Pb, As and K. The three groups of medicines (i.e. from India, Italy and Internet) are not separated, probably because of the high variability of the compositions of the formulations, even among products belonging to the same group, and the presence of a vegetal matrix that characterizes all the considered medicines. In order to better investigate the effect of the presence of the herbal matrix we treated separately the data on total concentrations of i) the elements that have very high values only in the samples purchased in India (As, Cu, Hg, Pb, Pd, Sn and Zn) and of ii) the most abundant elements (Al, Ca, Fe, K, Mg, Na, P and Si) (see paragraph 3.1.4). We expected that only

in the first case the group of *rasaushadi* medicines would be well separated from the other samples, but in both cases we observed a differentiation between the *rasausadhi* and *kasthausadhi* drugs, with the exception of sample B. In particular, Figure 2.2, reporting PCA for case i), shows that all the drugs are grouped together with the exception of nearly all samples purchased in India, i.e. A, C, D, E, F and G; instead the score of sample B (characterized by lower amounts of considered analytes with the exception of Hg) and the medicines purchased in Internet and in Italy are overlapped. An interesting result comes from Figure 2.3, showing PCA for case ii): the scores of all the formulations are generally more separated, but also in this case it is possible to distinguish the scores of the samples A, C, D, E, F, G which are in the upper area of the plot, while the others are in the lower region.

HCA confirmed these trend: considering all the analytes together, all the samples are mixed, while considering cases i) and ii) separately, in Figure 3.2 and 3.3 we observe a more clear separation between *rasausadhi* and *kasthausadhi* products. We can underline that in the case i) all the *rasausadhi* formulations are grouped with the exception of sample E (instead of the product B).

As to the loadings, the following positive correlations can be observed: Fe and Mn, Zn, Pd and Cu; Pb and Sn. Moreover, two negative correlations are present: Ca with K, P and Na; Si with Mg. On the other hand, As and Hg have no significant correlations with other elements. The observed correlations probably arise from the chemical properties of the elements as well as from their sources.

We also treated the data with Factorial Discriminant Analysis (FDA).

Unfortunately, Unscrambler X 10:2 software package did not allow us to apply DA to our data, because of the limited number of samples in each group. On the other hand, XLStat 4.4 performed the calculations even in these conditions, and the results are reported hereafter: anyway, owing to the different outputs of the two software packages, such results should be considered as approximate. Figure 4 shows that the three groups of ayurvedic products are well separated, in particular we can observe a clear separation between *rasausadhi* and *kasthausadhi* groups.

*3.3. Daily intake and reference values*

One of the major concerns for *kashausadhi* drugs, is the nature of the source materials.

Many papers report the determination of As and heavy metal concentrations in different types of plants including vegetables, cereals and fruits etc., but less work has been reported on medicinal

plants [43-47]. Herbal drugs can contain potentially toxic elements deriving from the environment, in particular from polluted water, soil and atmospheric particular matter. For instance, some areas of India are severely affected by As pollution, since soils and natural waters contain high level of this element causing a serious contamination of the cultivars. The safety and quality of herbal drugs depend on various factors, and contamination by metals and pesticides during any stage of production can occurred. So, it is important to verify the concentrations of potentially toxic elements both in raw materials and in the end-products, which are used more widely in Western countries as compared to crude drugs.

The daily intake for each considered element upon consumption of ayurvedic medicines was calculated taking into account the posology suggested on the package leaflet, when present, or indications from literature. As expected from the high variability of the ingredients present in these medicines, the intake is different for each sample. These amounts were compared with the daily limits suggested by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for a standard human of 60 kg calculated from *Provisional Maximum Tolerable Daily Intake (*PMTDI, mg/bw/d), *Provisional Tolerable Weekly Intake (*PTWI, mg/ bw/w) and *Provisional Tolerable Monthly Intake (*PMTI mg/bw/m) values, with bw = body weight [41].

Table 6 reports the range of estimated daily intake upon consumption of ayurvedic medicines of the three considered groups (mg/d, min–max) and the corresponding admissible value for each element (referred to an individual of 60 kg). The values for the single medicines are reported in the Supplemental data (Appendix A).

All the elements determined in the formulations sold from the Internet and the Italian pharmacy presented concentrations lower than the safety limits for all considered elements. These results reflect the good quality of the Indian plants adopted for the preparation of these formulations and the lack of contaminations during the manufacturing steps.

Among the medicines purchased in India, five samples presented concentration levels exceeding the maximum daily intake for the following elements: Hg (B: Ekaguna Sindura, 4,94-9,88 mg/ d), Hg and As (C: Thalaka Chenduram 19.4 mg/ d for Hg and $2.4 \times 10^{-2}$ mg/ d for As), Cu (E: Vel Vanga Parpam, 22.9-45.8 mg/ d), As (F: Talaka Bhasma 45.2-84.3 mg/ d) and Pb (G: Maha Yograj Guggulu 135 mg/ d) (data reported in supplementary material).

*3.4 Study of bioaccessibility*

Bioaccessibility studies are carried out in order to estimate the portion of an element, present in ingested material (food, medicine,…), which is available for uptake by the organism [47].

Since some investigated products revealed a possible toxic level of As, Cu, Hg and Pb, it is important to know to what extent such elements can be assimilated, since the total concentrations might lead to an overestimation of the real risk for health. For this reason we applied extraction procedures simulating the effect of gastric and intestinal juices to samples C, D and F. Unfortunately, it was not possible to make these experiments with samples B and G since the available amounts of these products were not sufficient for the test.

The element release in the mouth was not considered, because in literature it is demonstrated that saliva has only a negligible effect on the level of mobilization of metal contaminants, since the pH of saliva is close to neutral [33].

We adopted the procedure suggested by the American Pharmacopeia; like most of the studies on bioaccessibility; this procedure considers the addition of enzymes to the extractant solutions to mimic the natural fluids [34]. For the sake of completeness, we determined the release of all the investigated elements, with the exception of K, Na and P, which were present in the adopted reagents.

Initially, we assessed the capacity of extraction of the two considered media (gastric and intestinal) using the same contact time (2h). Then, we left each sample in contact with the solutions for the time indicated in the methods, 2 and 6 h for gastric and intestinal media respectively. No differences were observed in the percentages of extraction obtained at different times for intestinal juice, demonstrating that after 2h the extraction in the intestinal solution can be considered complete.

Table 7 reports the results obtained treating samples C, D and F with the two considered media. In the table we also show the daily intake calculated from the total amount of each element released during a hypothetic digestion and the maximum posology for each sample.

Generally, the release of most of the analytes in the gastric medium was lower than that in the intestinal one. The acid pH of the stomach juice permits a greater dissolution of the hydroxides, oxides, other salts (such as sulfides) and organic species (complexes between elements and the organic components present in the plants) of the elements contained in the medicines. In particular, Al, Cr, Cu, Fe, Mn, Ni, Pb, Si and Zn present higher percentages of extraction in the gastric juice than in the intestinal one for all the considered samples, with the only exception of

Cu and Pb in sample C that show similar release in both media. Ca and Mg are quantitatively extracted into intestinal media from samples C and F.

The leachability of As is similar in both media; Hg is always present in solution in concentration lower than the instrumental LoQ.

We observed a great variability in the extracted percentages of the same analyte from the different samples: the release of an element depends on the form in which it is present in the considered material, which determines how strongly it is bound to inorganic or organic components contained in the matrix. The great differences of elements behavior in the samples reflects the great assortment of adopted ingredients in the ayurvedic formulations.

For example, As is present in samples C and F as orpiment (thalaka): this mineral is insoluble in water, in acid and alkaline media and in fact a low release of As is observed from these drugs. On the contrary As is quantitatively extracted from sample D in both media, suggesting that it is not strongly bound to the medicine matrix. Its total concentration in this sample is low and we were not able to identify a possible source. These considerations lead us to suppose that a pollution of the drug took place, presumably during the growing, irrigation and/or manufacturing steps.

From the comparison between the maximum daily intake and reference admissible values, we can observe possible risks associated to the consumption of samples C and F, even if the percentages of release of As are low. The concentration of As in the considered “talaka formulations” is so high that even a very low release is sufficient to exceed JECFA limits; the maximum acceptable percentage of release would be 0.67 % for sample C and 0.31 % for sample F.

The amount of Cu released from sample D (0.51 mg/d) is much lower than the maximum admissible value (4.3 mg/d), so no risks seem to be associated to the consumption of this drug.

As expected, the total amounts potentially released during digestion for all the other analytes were lower than daily limits.

## 4. Conclusions

The great variability in inorganic compositions of the investigated ayurvedic medicines derives from the great assortment of the ingredients adopted.

In particular, the high content of As and metals as Cu, Hg and Pb in the drugs purchased in India is mainly caused by the intentional addition of these elements during the medicine preparation following the *rasa shastra* tradition.

To be marketed in Western countries, ayurvedic medicines have to be declared “metal free”. For this reason it is possible to purchase only *kasthausadhi* products. Drugs sold in pharmacies assure more safety for the customers in comparison to other market channels. On-line shopping might present more risks associated to the difficulty to control the quality of the available products. For this reason we checked and compared metal content in formulations purchased on the Internet and in pharmacies: no remarkable features were observed for the products bought in our study from these two commercial channels. The inorganic contents of the medicines purchased online were lower than the maximum admissible level so there are no risks from the point view of the presence of metals, in the investigated products. It must be pointed out that we did not analyze the organic components of the medicines, so we cannot make any statement on the overall quality of the formulations.

It is interesting to note that the names of ayurvedic formulations are often indicative of their main component, or of the procedure used for their preparation. On the other hand, two drugs with a similar name may have a different content of potentially toxic elements.

The chemometric data processing showed that the medicines purchased in India were differentiated from the other products, owing to the higher concentrations of potentially toxic elements.

Bioaccessibility studies showed that the amount of metals actually released during the ingestion is lower than the total amount, but in some cases is still higher than the maximum admissible level.

Due to the great compositional differences observed among the ayurvedic formulations, our study underlines the importance: i) to know very well the traditions according to which the formulation are prepared; ii) to understand and to respect the dosage of assumption, in particular avoiding the unlabeled products, and translating the leaflet if written in foreign languages; iii) to consume medicines only under medical counsel and iv) to purchase them from controlled commercial channels.

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**References**

[1] P.I. Dargan, I.B. Gawarammana, J.R.H. Archer, I.M. House, D.M. Wood, Heavy metal poisoning from Ayurvedic traditional medicines: an emerging problem?, Int. J. Environ Health, 2 (2008) 463-474.

[2] S.N. Kales, C.A. Christophi, R.B. Saper, Hematopoietic toxicity from lead-containing Ayurvedic medications, Med Sci Monit, 13 (2007) 295-298.

[3] M.J. Martena, J.C.A. Van Der Wielen, I.M.C.M. Rietjens, W.N.M. Klerx, H. N. De Groot, E. J.M. Konings, Monitoring of mercury, arsenic, and lead in traditional Asian herbal preparations on the Dutch market and estimation of associated risks, Food Addit.Contam. 27 (2010) 190–205.

[4] D.M. Wood, S. Athwal, A. Panahloo, The advantages and disadvantages of a 'herbal' medicine in a patient with diabetes mellitus: a case report, Diabetic Medicine, 21 (2004) 625-627.

[5] S. Costantini, G. Mazzanti, F. Menniti-Ippolito, Medicine tradizionali ayurvedica e cinese: qualità e sicurezza di alcune preparazioni, Rapporti ISTISAN 04/33, ISSN 1123-3117, Istituto superiore di sanità.

[6] J. Liu, Y. Lu, Q. Wu, R.A. Goyer, M.P. Waalkes, Mineral Arsenicals in Traditional Medicines:Orpiment, Realgar, and Arsenolite, JPET 326 (2008) 363–368.

[7] R.P. Choudhury, A. Kumar, A.V.R. Reddy, A.N. Garg, Thermal neutron activation analysis of essential and trace elements and organic constituents in Trikatu: An Ayurvedic formulation, J. Radioanal. Nucl. Chem. 274 (2007) 411–419.

[8] A. Kumar, A.G.C. Nair, A.V.R. Reddy, A.N. Garg, Availability of essential elements in bhasmas: Analysis of Ayurvedic metallic preparations by INAA, J. Radioanal. Nucl. Chem., 270 (2006) 173–180.

[9] R.B. Saper, R.S. Phillips, A. Sehgal, N. Khouri, R.B. Davis, J. Paquin, V. Thuppil, S.N. Kales, Lead, Mercury, and Arsenic in US- and Indian-Manufactured Ayurvedic Medicines Sold via the Internet, JAMA. 300 (2008) 915-923.

[10] B. Kumar, M. Vijayakumar, R. Govindarajan, P. Pushpangadan, Ethnopharmacological approaches to wound healing—Exploring medicinal plants of India, J. of Ethnopharmacol. 114 (2007) 103–113.

[11] U.M. Thatte, N.N. Rege, S.D. Phatak, S.A. Dahanukar, The flip side of Ayurveda, J. of Postgraduate Medicine, 39 (2003) 179–182.

[12] A. Ravinarayan, K.P. Skandhan, Lead preparation in Ayurvedic medicines, Postgrad Med J. 71 (1995) 251.

[13] Ministry of Health and Family Welfare 2000, 2003.
[14] R.B. Saper, S.N. Kales, J.Paquin, M.J. Burns, D.M. Eisenberg, R.B. Davis, R.S. Phillips, Heavy Metal Content of Ayurvedic Herbal Medicine Products, JAMA, 292 (2004) 2868-2873.
[15] U. Divrikli, S. Saracoglu, M. Soylak, L. Elci, Determination of trace heavy metal contents of green vegetable samples from Kayseri-Turkey by flame atomic absorption spectrometry, Fresen Environ Bull 12 (2003) 1123-1125.
[16] Adriano DC (2001) Trace elements in the terrestrial environments: Biogeochemistry. Bioavailability and risk of metals, Springer- Verlag, New York.
[17] Sierra-Alvarez R, Yenal U, Field JA, Kopplin M, Gandolfi AJ, Garbarino JR (2006) Anaerobic biotransformation of organoarsenical pesticides monomethylarsonic acid and dimethylarsinic acid. J Agri Food Chem 54 (2006) 3959–3966.
[18] Alabdula´aly AI, Mujahid AK, Heavy metals in cooler waters in Riyadh, Saudi Arabia. Environ Monit Assess 157 (2009) 23–28.
[19] Legislative Decree 2004/24/EC.
[20] Legislative Decree 2002/46/EC; EC guideline N. 1924/2006.
[21] S. Khandpur, A.K. Malhotra, V. Bhatia, S. Gupta, V.K. Sharma, R. Mishra, N.K. Arora, Chronic arsenic toxicity from Ayurvedic medicines, Int. J. Dermatol. 47 (2008) 618–621.
[22] E. Lynch, R. Braithwaite, A review of the clinical and toxicological aspects of 'traditional' (herbal) medicines adulterated with heavy metals, Expert Opin. Drug Saf. 4 (2005) 769-778.
[23] L. Borgese, A. Zacco, E. Bontempi, M. Pellegatta, L. Vigna, L. Patrini, L. Riboldi, F.M. Rubino, L.E. Depero, Use of total reflection X-ray fluorescence (TXRF) for the evaluation of heavy
metal poisoning due to the improper use of a traditional ayurvedic drug, J Pharmaceut Biomed 52 (2010) 787–790.
[24] R. Garnier, J. Poupon, Intoxication par le plomb resultant de l'utilisation d'un remède traditionnel indien, Press Med. 35 (2006) 1177-1180.
[25] E. Ernst, Heavy metals in traditional Indian remedies, Eur. J. Clin. Pharmacol.57 (2002) 891–896.
[26] L.G. Miller, Herbal medicinals: selected clinical considerations focusing on known or potential drug-herb interactions, Arch. Intern. Med. 158 (1998) 2200–2211.
[27] Ernst, E.b, Toxic heavy metals and undeclared drugs in Asian herbal medicines, Trends Pharmacol. Sci. 23 (2002) 136–139.

[28] P.A. De Smet, Health risks of herbal remedies: an update, Clin. Pharmacol. Ther. 76 (2004) 1–17.

[29] G.J. Garvey, G. Hahn, R.V. Lee, R.D. Harbison, Heavy metal hazards of Asian traditional remedies, Int. J. Environ. Health Res. 11 (2001) 63–71.

[30] S. Srivastava, V. Rai, M. Srivastava, A.K. S. Rawat, S. Metherotra, Estimation of heavy metals in different Berberis species and its market samples, Environ. Monit. Assess. 116 (2006): 315–320.

[31] P. Mahawatte, K.R. Dissanayaka, R. Hewamanna, Elemental concentrations of some Ayurvedic drugs using energy dispersive XRF, J. Radioanal. Nucl. Chem., 270 (2006) 657–660.

[32] A. Giacomino, O. Abollino, M. Malandrino, M. Karthik, V. Murugesan, Determination and assessment of the contents of essential and potentially toxic elements in Ayurvedic medicine formulations by inductively coupled plasma-optical emission spectrometry, Microchem J 99 (2011) 2–6.

[33] M. Intawongse, J.R. Dean, In-vitro testing for assessing oral bioaccessibility of trace metals in soil and food samples, TrAC, 25 (2006) 876-886.

[34] A.G. Oomen, A. Hack, M. Minekus, E. Zeijdner, C.Cornelis, G. Schoeters, W. Verstraete, T. Van de Wiele, J. Wragg,C.J.M. Rompelberg, A.J.A.M. Sips, J.H. Van Wijnen, Comparison of Five In Vitro Digestion Models To Study the Bioaccessibility of Soil Contaminants, Env. Sci. Tec. 36 (2002) 3326-3334.

[35] J.P. Day, G. Rago , K.F. Domke, K.P. Velikov, M. Bonn, Label-free imaging of lipophilic bioactive molecules during lipid digestion by multiplex coherent anti-Stokes Raman scattering microspectroscopy, J Am Chem Soc. 132 (2010) 8433-8439.

[36] V. Fogliano, M.L. Corollaro, P. Vitaglione, A. Napolitano, R. Ferracane, F. Travaglia, M. Arlorio, A. Costabile, A. Klinder, G. Gibbson, Polyphenols from food In vitro bioaccessibility and gut biotransformation of polyphenols present in the water-insoluble cocoa fraction, Molecular Nutrition & Food Research. 55 (2011) 44-55.

[37] D. H. Yang; Y. Joo, Determination of cadmium bioaccessibility in herbal medicines and safety assessment by in vitro dissolution and ICP-AES, Microchim. Acta 167 (2009) 117-122.

[38] A. Bignasca, C. Ianni, E. Magi, P.Rivaro, Using proteolytic enzymes to assess metal bioaccessibility in marine sediments, Talanta 86 (2011) 305–315.

[39] O. Abollino, M. Malandrino, A. Giacomino, E. Mentasti, The role of chemometrics in single and sequential extraction assays: A review. Part I. Extraction procedures, uni- and bivariate

techniques and multivariate variable reduction techniques for pattern recognition. Anal Chim Acta 688 (2011) 104–121.

[40] A. Giacomino, O. Abollino, M. Malandrino, E. Mentasti, The role of chemometrics in single and sequential extraction assays: A Review. Part II. Cluster analysis, multiple linear regression, mixture resolution, experimental design and other techniques, Anal Chim Acta 688 (2011) 122–139.

[41] JECFA, database: http://apps.who.int/food-additives-contaminants-jecfa-database/search.aspx

[42] American Pharmacopoeia, 24th editions

[43] R. Chizzola, C. Franz, Metallic trace elements in medicinal and aromatic plants from Austria, J Appl Bot-Angew Bot 70 (1996) 52-56.

[44] V. Rai, P. Kakkr, S. Khatoon, A.K.S. Rawat, S Mehrotra Heavy metal accumulation in some herbal drugs, Pharmaceut Biol 39 (2001) 384-387.

[45] V. Rai, P. Kakkar, C. Misra, S.K. Ojha, N. Srivastava, Metals and Organochlorine Pesticide Residues in Some Herbal Ayurvedic Formulations, Bull Environ Contam Toxicol 79 (2007) 269–272.

[46] V. Ray, P.Kakkar, J. Singh, C. Misra, S. Kumar, S. Mehrotra, Toxic metals and organochlorine pesticides residue in single herbal drugs used in important ayurvedic formulation - 'Dashmoola', Environ. Monit. Asses. 143 (2008) 273-277.

[47] P. Artursson, K. Palm,K. Luthman, Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv. Drug Del. Rev. 46 (2001) 27–43.