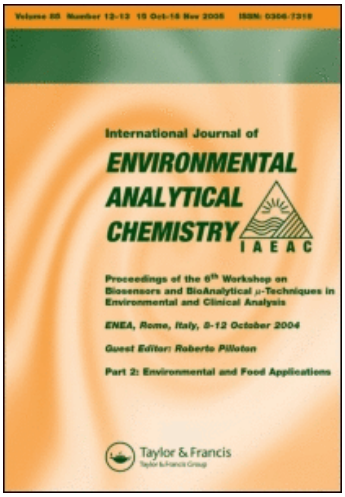

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Tack, Filip M. G. , Bogart, Nicolas , Verloo, Marc G. , Hendrickx, Frederik , Maelfait, Jean-Pierre and Mertens, Johan(2000) 'Determination of Cd, Cu, Pb and Zn in Woodlouse (*Oniscus Asellus*)', International Journal of Environmental Analytical Chemistry, 78: 2, 149 — 158
To link to this Article: DOI: 10.1080/03067310008044407
URL: http://dx.doi.org/10.1080/03067310008044407

Intern. J. Environ. Anal. Chem., Vol. 78(2), pp. 149-158
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DETERMINATION OF Cd, Cu, Pb AND Zn IN WOODLOUSE (*ONISCUS ASELLUS*)

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(Received 16 June 1999; In final form 15 May 2000)

The aim of this study was to assess the performance of selected destruction methods for the determination of heavy metals (Cd, Cu, Pb and Zn) in woodlouse (*Oniscus asellus*). A vigorous total analysis involving microwave destruction with HF, HCl and HNO_3 (method 1) was used as a reference method. Consistently low values for the dry ashing method may indicate incomplete dissolution of the elements and/or losses through volatilisation. Method 3 (concentrated HNO_3) that frequently is used in literature, produced erroneous values for Cd, Cu and Pb. Results were consistent with the microwave digestion, provided H_2O_2 was used during digestion (method 4, HNO_3/H_2O_2). Method 5 ($HNO_3/HClO_4$, one destruction step) yielded low recoveries when only one destruction step was applied. Applying two destruction steps (method 6) resulted in values consistent with the microwave method, but was at the expense of reproducibility and rendered the method more lengthy and laborious. Because of the very good performance combined with speed and simplicity, destruction with HNO_3/H_2O_2 (method 4) emerged as the most convenient method.

Keywords: Heavy metals; woodlouse; invertebrates; analytical method

INTRODUCTION

Some arthropods are known to be very active heavy metal accumulators [1–3]. They may therefore play an important role in the transfer of these elements from soil or sediment substrates into the food chain. Also of interest is that they could be useful indicators for metal pollution [4]. For these reasons, there is currently a great interest for the analytical determination of heavy metals in arthropods.

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Matrix destruction for the determination of heavy metals in arthropods is usually accomplished using wet destruction methods. In related literature, details of the procedures followed for analysis are rarely given or referred to. Most procedures adopted involve a wet digestion using nitric acid, alone [5,6], or in combination with perchloric acid [7,8] or hydrogen peroxide[9]. It appears to be no papers published where the performance of analytical methods applied for heavy metal analysis of arthropods is compared. The aim of this study was to assess the performance of ashing and wet destruction methods for the determination of heavy metals (Cd, Cu, Pb and Zn) in woodlouse (*Oniscus asellus*).

EXPERIMENTAL

About 200 woodlice (*Oniscus asellus*) were collected in September 1998 by hand picking in the field. The sampled area was located in a forest of poplar trees in Bourgoyen-Ossemeersen, near Ghent, Belgium. Animals were collected in test tubes and frozen upon arrival in the lab [10]. Animals were then dried at 70°C for 48 hrs. The biologic material was thoroughly crushed using a mortar and a pestle, and subsequently mixed by coning and quartering to assure good homogeneity.

Each time before a sample was taken, the sample bottle was thoroughly shaken. To mimic the situation where individual animals are analysed, aliquots between 20 – 30 mg were weighed to the nearest 0.01 mg on a Mettler Toledo AT 21 Comparator (Nänicon, Switzerland) analytical balance. This weight is within the range for a typical dried woodlouse.

Destructions were replicated eight times except for methods 3 and 5. When it was observed that these methods were inappropriate (see *Results and Discussion*), no further replicates were undertaken. Four of the replicates were re-analysed at different times in order to discriminate between variability associated with the destruction procedure and that associated with the analytical determination.

Microwave destruction (method 1)

Following is a US-EPA method for the determination of total metal contents in environmental matrices [11]. Samples were weighted into 45 ml teflon destruction bombs. Three ml 65% HNO_3 and 1 ml 37% HCl were added. The recipients were placed during 15 minutes in an ultrasonic bath to evacuate nitrous vapours. Closed bombs were then heated in a microwave, subsequently at 250 Watt during 5 minutes, at 400 Watt during 5 minutes and at 600 Watt during 4 minutes. Recipients were then cooled and the destruate was filtered over a 0.45 µm mem-

brane filter in a 50 ml volumetric flask. The membrane filter with the solid residue was transferred again into the destruction vessel and 1 ml 48% HF, 1 ml 37% HCl and 1 ml 65% HNO_3 were added. This mixture was subjected to the same heating program and cooled. The solution was combined with the filtrate and diluted to 50 ml.

Dry ashing (method 2)

This method is adopted from a procedure commonly used for plant analysis [12,13]. Samples were weighed into porcelain crucibles. The crucibles had been washed in a laboratory dishwasher and were subsequently soaked in 5% ultrapure 65% HNO_3 and rinsed with deionised water. The samples were pre-ashed in a muffle furnace at a temperature of 250°C and subsequently ashed during 3 hours at 450°C. The crucible was transferred to a hot plate, 5 ml of 6 mol/L HNO_3 was added and the mixture was evaporated to a low volume. The residual was dissolved in 5 ml of 3 mol/L HNO_3, filtered (S&S, blue ribbon) and diluted to 50 ml.

Wet destruction with HNO_3 or HNO_3/H_2O_2 (methods 3 and 4)

A method described by Marinussen and Van Der Zee (1997)[9] was used (method 4). Samples were weighed into 100 ml pyrex beakers and treated with 5 ml ultra-pure 65% HNO_3. The beaker was covered with a watch-glass and the suspension was heated up to 130°C for 1 h. A total amount of 4 ml 20% H_2O_2 was added in aliquots of 0.5 ml. After cooling, the solution was quantitatively transferred to a 50-ml volumetric flask and diluted to the mark. For the destruction involving only HNO_3, the same procedure was followed without addition of H_2O_2 (method 3).

Wet destruction with $HNO_3/HClO_4$ (methods 5 and 6)

This method is adapted from that of van Straalen and van Wensem[7]. Digestion was performed in watch-glass-covered 100 ml pyrex beakers by means of 5 ml 7:1 mixture of ultra-pure 65% nitric acid and 70% perchloric acid. The suspension was heated at 85°C and then at 160°C, each during 45 minutes. The watch-glass was removed and the mixture was evaporated at 170–180°C until no more white fumes evolved. The method indicates that this destruction procedure should be repeated on the residue when it is observed that destruction is not complete, i.e., when the residue is not white. If destruction is evaluated to be com-

plete, the residue is dissolved in 0.1 mol/L HNO_3, heated during 30 minutes and diluted to 50 ml. Our samples were subjected to either one destruction (method 5) or two destructions (method 6).

Chemical analysis

Flame atomic absorption (SpectrAA-10, Varian, Palo-Alto, CA) equipped with deuterium background correction was used for the determination of Zn in the extracts. Cd, Cu and Pb were analysed by graphite furnace atomic absorption (SpectrAA-100, Varian, Palo-Alto, CA), equipped with Zeeman background correction.

Standard reference materials for quality control of heavy metal analysis in arthropods are not currently available. Quality control was performed by analysing a reference plant material (ryegrass, CRM 281)[14]. These data allow for quality control (Table I), but do not allow to draw conclusions with respect to the analysis of metals in arthropods using these methods. Not only is the matrix different, but metal levels are a factor 10 to 100 lower than in woodlouse.

TABLE I Duplicate analyses in mg/kg dry wt. of rye grass reference material (CRM 281[14]) using destruction methods 2 (dry ashing), 4 (HNO_3/H_2O_2) and 5 ($HNO_3/HClO_4$, 1 destruction). Uncertainty intervals of certified values are 95% confidence intervals of the grand mean

	Method 2	*Method 4*	*Method 5*	*Certified*
Cd	0.134/0.135	0.110/0.120	0.062/0.075	0.12 ± 0.11
Cu	8.77/8.79	9.29/8.99	6.22/6.22	9.65 ± 0.38
Pb	2.41/2.18	2.38/2.92	1.57/1.42	2.38 ± 0.11
Zn	29.6/28.8	29.0/28.3	31.9/30.0	31.5 ± 1.4

Using method 5, it was easily observed that one destruction step was insufficient to achieve complete destruction. As a result, recoveries for Cd, Cu and Pb were incomplete, while Zn was quantitatively recovered (Table I). The two other methods, including dry ashing and destruction with HNO_3/H_2O_2, yielded good recoveries for Cd, Pb and Zn, although the latter elements tended to show a negative bias. Cu recovery was good for method 4 involving destruction with HNO_3/H_2O_2, but slightly low for the ashing method.

Statistical analysis

The significance of differences between results of the microwave destruction and other methods was evaluated using the t-test for equality of means[15]. Equality

of variances between two groups was assumed unless the Levene's test for equality of variances was significant at the 5% level.

To discriminate between variability due to the destruction and to the analytical measurement, analysis of variance with a nested design was performed[15]. Only methods that were considered appropriate (methods 1, 2, 4 and 6) were included. The factor in the nested design were the different methods (4 levels) and replicate destructions (four levels). Residual variance is associated with variability in the analytical determination of metals in the extracts (2 replicates). This procedure allowed to discriminate between variabilities associated with the use of different destruction methods, with the destruction procedure and heterogeneity of the sample, and with the analytical determination.

RESULTS AND DISCUSSION

Metal contents obtained using different destruction procedures were compared with the microwave destruction method (method 1) (Table II). This is a vigorous destruction method proposed by US-EPA for the determination of total metal contents in a variety of matrices[11]. As it involves the use of hydrofluoric acid, it is capable of dissolving silicates as well[11,16]. It can therefore be considered as a reference to check recoveries of other methods. In the following discussion, recoveries are defined with respect to the microwave destruction method.

Differences with results from the microwave destruction methods were in many cases significant (Table II). Overall, methods 4 (HNO_3/H_2O_2) and 6 ($HNO_3/HClO_4$, two steps) compared best to the microwave destruction method. The other methods usually yielded significantly lower values. Occasionally, values were systematically higher than the values obtained using microwave destruction.

Dry ashing methods still are frequently applied for trace element analysis in biological matrices because of convenience. Although dry ashing generally takes a long time, it is attractive because operator involvement is low, large sample sizes can be employed, and, unless reagent addition is necessary, contamination due to reagents is low[17]. Many sample can readily be ashed together in appropriate muffle furnaces with little labour[18].

With the exception of Zn, recoveries with respect to the microwave destruction method were consistently too low. Low recoveries can be due to incomplete dissolution of the element from the ash. Besides, and in particular for Cd, volatilisation of the element may occur during dry ashing and it is usually not possible to apply higher ashing temperatures than 450°C to avoid excessive losses of Cd[12].

TABLE II Grand means (Avg in mg/kg dw.), standard deviations (Std in mg/kg dw.) and coefficients of variation (CV%) for the determination of Cd, Cu, Pb and Zn using different destruction procedures (method 1: Microwave destruction with HNO_3/HCl/HF; 2: dry ashing; 3: HNO_3; 4: HNO_3/H_2O_2; 5: $HNO_3/HClO_4$, 1 destruction; 6: $HNO_3/HClO_4$, 2 destructions)

Method		*1*	*2*	*3*	*4*	*5*	*6*
Cd	Avg	19.0	15.0	16.1	17.9	21.9	17.4
	Std	1.6	2.3	1.0	1.7	1.3	3.2
	CV%	8.2%	15.1%	6.3%	9.6%	5.8%	18.4%
			***	***	–	**	–
Cu	Avg	155	140	167	149	98	152
	Std	8	5	10	8	6	17
	CV%	5.3%	3.9%	5.8%	5.2%	6.6%	11.0%
			***	**	–	***	–
Pb	Avg	22.2	18.6	14.7	22.7	13.7	21.4
	Std	2.1	3.2	7.7	2.9	3.4	2.7
	CV%	9.5%	17.4%	52.7%	12 8%	24.5%	12.6%
			**	*	–	***	–
Zn	Avg	294	284	329	273	289	282
	Std	13	15	31	18	3	17
	CV%	4.4%	5.3%	9.3%	6.4%	1.1%	6.0%
			–	*	**	–	–

The significance of the difference with the microwave destruction method (method 1) is designated with ns, not significant, *: significant at the 5% level of confidence, **significant at the 1% level, *** significant at the 0.1% level (t-test for equality of means)

The method involving HNO_3 was performed without (method 3) and with addition of H_2O_2 (method 4). When no H_2O_2 was used, recovery of Pb amounted to only 66% of the amount recovered using microwave digestion. In contrast, values obtained for Cu and Zn were too high, approx. 110% of the microwave destruction method. The brown colour of the solutions suggested that destruction of organic matter was incomplete when no H_2O_2 was used. This may result in incomplete dissolution of elements such as Pb. High values may be related to interference during analytical determination. These observations are very important because many authors used HNO_3 destruction as a relatively fast and convenient method to determine metals in biological matrices. Our results suggest that this method is inappropriate for the determination of Cd, Cu, Pb and Zn, at

least in arthropods and likely other biota. The simple modification of adding H_2O_2 during digestion was effective and yielded good results for all elements except Zn, which was 7% lower than the result of the microwave destruction method.

The method involving destruction with $HNO_3/HClO_4$was performed applying a single destruction (method 5) and applying two consecutive destructions (method 6). The method prescribes that a subsequent destruction of the residue of a destruction is to be carried out when it is judged by visual inspection that destruction is incomplete[7]. This should be observed by the colour of the residue not being white. In our case, the colour of the residue remaining after one destruction was a very pale yellow, which was easily mistaken as indicating complete destruction. From the results, it became clear that destruction in fact had not been complete. Values for Cu and Pb were much lower than for the other methods and Pb exhibited a much higher variability than that observed with the other methods (Table II). Analyses were then repeated applying two consecutive destructions (method 6) and this approach yielded values consistent with the other methods.

This experience illustrates the danger of relying an analytical procedure on a visual criterion. It would be advisable to adapt this method to prescribe two consecutive destructions in all cases. With this modification, the method performed equally as the microwave destruction method, except for the higher variability between replicates. Another drawback is that the need for two consecutive destructions renders the method even more lengthy and labour intensive.

Optimum reproducibility was in the range of 5 – 8%. For the weaker performing methods, reproducibility was around 15% and for Pb using methods 3 and 5, reproducibility was very low, at 50 and 25%, respectively. These variation coefficients involve variability associated with both the destruction and the analytical determination.

Low recoveries of Pb in methods 3 and 5 were accompanied by a high variability between the individual measurements, probably mostly related to incomplete destruction of the organic matrix. Reproducibility may also be adversely affected for these methods that involve numerous manipulations. When comparing method 5 with method 6, it is observed that the extra destruction step involved in method 6 increased the variation coefficient from around 6% to more than 11% for Cu and Cd, and from 1% to 6% for Zn.

Analysis of variance with nested design was used to partition the sum of squares into different sources of variability (Table III). Sources of variability were the use of different destruction methods, the destruction procedure and the analytical determination. Sample heterogeneity also may constitute a significant

source of variability, because small sample sizes between 20–30 mg were used. Methods 3 and 5 were not further considered.

TABLE III Analysis of variance with nested design (DF: degrees of freedom; SS: Sum of Squares; MS: mean Square, p: probability of *F* statistic; s^2: estimated variance associated with factor; %CV: estimated variation coefficient associated with factor)

Source of variation (factor)	*DF*	*SS*	*MS*	*p*	s^2	*%CV*
Cadmium						
Method	3	104.5	34.9	0.000	4.19	11.7%
Destruction/sample	12	42.6	3.5	0.007	1.31	6.6%
Analytical detn.	16	14.8	0.9		0.92	5.5%
Copper						
Method	3	1031	343.5	0.000	41.0	4.3%
Destruction/sample	12	758	63.2	0.100	15.7	0.0%
Analytical detn.	16	509	31.8		31.8	3.8%
Lead						
Method	3	140.8	52.3	0.000	6.26	12.6%
Destruction/sample	12	98.5	7.6	0.054	2.20	7.5%
Analytical detn.	16	48.5	3.2		3.20	9.0%
Zinc						
Method	3	1645	548.3	0.000	46.7	2.4%
Destruction/sample	12	4704	392.0	0.000	174.6	4.6%
Analytical detn.	16	683	42.7		42.7	2.3%

Mean squares were used to estimate standard deviations associated with the different factors that determine variability[15]. For example, the mean square associated with the outer level factor *Method* is an estimate of $s^2 + ns_b^2 + bns_a^2$. Here, s^2 represents residual variance, s_b^2 is the variance associated with the nested factor (replicate destruction in our case) and s_a^2 is the variance associated with the outer level factor. The parameters *n* and *b* represent the number of replicate determinations and the number of levels of the nested factors. The standard deviation is given by the square root of the variance and was used to calculate a coefficient of variation (CV%), which would give the reproducibility of the analytical results if there were no other sources of variability (Table III).

For all elements except Zn, the most important contribution to the overall variability of the analytical results was caused by differences between destruction

methods. This is mostly due to the differences between the ashing procedure and the other procedures. For Zn, replicate destructions/sample heterogeneity was the most important source of variability.

The variation coefficients associated with analytical determination were at or below 5% for Cd, Cu and Zn (Table III). This is acceptable for analytical determinations in biological matrices. It reflects that metal concentrations in the extracts were relatively high and well within the reach of flame atomic absorption for Zn (0.10–0.13 mg/l) and graphite furnace atomic absorption for Cd (6–9 μg/l), Cu (50–70 μg/l). For Pb, that variation coefficient was larger, at 9%. This is in agreement with concentrations of Pb in the extracts, 6–9 μg/l, being already in a lower range for determination with graphite furnace atomic absorption. Relatively low variation coefficients for the factor destruction/sample heterogeneity suggest that sample heterogeneity was not limiting, despite the low sample size. A thorough homogenisation of the sample was a prerequisite for the purpose of method testing. In practical field work, whole animals are usually analysed separately, an approach that avoids the problem of proper sample homogenisation.

In conclusion, three of the tested methods were appropriate for the determination of Cd, Cu, Pb and Zn in woodlouse. Of these, the method involving HNO_3/H_2O_2 is by far the easiest and fastest to carry out in the laboratory. At the same time, it allows to obtain recoveries and reproducibilities comparable with the two other more laborious approaches.

Acknowledgements

This work was supported by the Research Fund of Ghent University as part of the research programme "Transfer van zware metalen in terrestrische ecosystemen: speciatie, bioaccumulatie en effecten op populatieniveau" (BOF 01110498).

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