# A novel method to determine trimethylantimony concentrations in plant tissue.

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Environmental Context

Antimony (Sb) enters the soil mostly through mining and shooting activities and can thereafter be taken up by plants. In the soil, Sb may undergo several transformations, such as biomethylation, leading to the formation of trimethylantimony (TMSb). Here, we measured for the first time the uptake and translocation of TMSb in a plant using a new extraction and analysis method.

Abstract

Antimony (Sb) is a relevant pollutant that can be found in elevated concentrations in soils, near Sb mines and in shooting ranges. In soils, Sb occurs as trivalent Sb, Sb(III), pentavalent Sb, Sb(V) or trimethylantimony, TMSb ($(CH_3)_3SbO$) the latter being the result of microbial biomethylation. It is important to understand the transfer of Sb species from soil to plants to assess the role of Sb in the food chain. However, this research has historically been hampered by the lack of suitable extraction and analytical methods. In this study, we validated an efficient and reliable extraction technique using oxalic acid and ascorbic acid (72.6 ± 1.3 % of Sb extracted) as well as a HPLC-ICP-MS speciation analysis method to assess the uptake of TMSb in rye grass (*Lolium perenne* L.) a common pasture plant, in a hydroponics experiment. Our results show that TMSb and Sb(III) are not converted to other species

during extraction and that TMSb is taken up by rye grass roots and translocated to the shoots. Our study also points at specific methylation/demethylation mechanisms *in planta*. Moreover, an unknown Sb species was found in the shoots of TMSb-treated plants, highlighting the need for further research. These new extraction and speciation methods will enable researchers to study the soil-plant transfer of organo-Sb compounds in a reliable and consistent manner.

## Introduction

Antimony (Sb) has been much less studied than other relevant pollutants such as arsenic (As) or mercury (Hg) even though it is classified as a priority pollutant as well as a toxic pollutant by the US EPA[1]. Furthermore, Sb plant uptake and transport mechanisms are mostly unknown. Sb is the 9$^{th}$ most mined element with 78% of the world production in 2015 being extracted in China[2]. It is mostly used in plastics, as a fire retardant and in the production of ammunition. The latter is an important pathway of entry into shooting range soils, i.e. by weathering of the bullets[3] and can thus possibly pass into plants.

Recent studies show that Sb has a higher mobility than previously thought, especially in contaminated areas[4]. It is generally more mobile than other pollutants such as As and lead (Pb)[5,6], although this can change depending on the redox conditions[7]. Sb is however more available than As in mining soils[8] and can thus be taken up by microorganisms or plants more easily. Indeed, concentrations between 100 and 4000 mg.kg$^{-1}$ have been found in plants growing in mining areas[4]. Furthermore, shoots of clover (*Trifolium repens* L.), a common $N_2$-fixing pasture plant that can commonly grow on shooting ranges or near Sb-mining areas, was found to contain up to 770 mg.kg$^{-1}$ Sb when exposed to 200 µM Sb(V) in an hydroponic experiment[9]. However, it must be noted that this is possibly due to the lack of phytochelatins (PCs) in clover. Otherwise, PCs bind with metals and prevent them from being transported to the shoots or interacting with the plant metabolism. Recent work showed that in rice (*Oryza sativa* L.), that was grown in 1 mg.L$^{-1}$ of Sb(III) or Sb(V), most of the Sb remained in the roots due to the presence of the iron plaque, although a contribution of PCs to the immobilisation of Sb in the roots cannot be excluded[10-12]. The same authors reported that more Sb was found in all parts of the plant when Sb(III) was used and that Sb(V) was the dominant species in roots and shoots of rice. Recent work also showed that forage grasses such as rye grass (*Lolium perenne* L.) or velvet grass (*Holcus lanatus* L.) growing on shooting range soils take up more Sb when the soil is flooded[13]. This is of

importance since in Switzerland, shooting ranges with less than 1000 $mg.kg^{-1}$ Pb are commonly used as pastures[14].

Furthermore, in the environment, Sb can undergo biomethylation[15], a biological process by which inorganic Sb species are transformed to methylated Sb species. The most common methylated Sb species are mono-, di- and trimethylantimony (MMSb, DMSb and TMSb respectively). In the case of As and Hg, biomethylation plays an important role since the mobility and toxicity of the methylated species are very different from their inorganic counterpart[16,17]. Moreover, a recently published review on Sb in an agricultural context clearly shows that there is a lack of data with regard to Sb speciation and behaviour in soils and plants, especially for TMSb, and it further highlights the importance of studying methylated Sb to make better environmental risk assessments[18]. Unfortunately, MMSb and DMSb are not available commercially which makes their study very difficult by conventional methods. Nevertheless, some information can be found in the reviews of Bentley & Chasteen[19] and Filella[15]. For example, methylated species of Sb can be found in waters, soils, sediments and biota, just like methylated species of As. Furthermore, Filella states that Sb has been largely overlooked as an element of environmental concern and that the data on the physical and chemical properties of these organic compounds are fragmentary and old[15]. Nonetheless, recent studies were able to demonstrate that TMSb was more mobile than pentavalent methylated As species since it adsorbs two to three times less on soils, sediments or mine tailings[20]. In a Sb mining area in China, it was found that TMSb represented up to 80% of total Sb in some soils and that it was present mostly in the shoots of plants growing on this substrate[8]. TMSb was also found in water extracts of shoots of the As hyperaccumulator fern *Pteris vittata* L.[21]. Using more complex methods of analysis, DMSb could also be found in liverworts and mosses growing next to an abandoned Sb mine[22]. These previous studies illustrate the importance of TMSb in the environment and more specifically in plants. However, to our knowledge, no controlled studies looking at the uptake and translocation of TMSb in plants have ever been conducted and the limitations have always been the analytical tools available, especially the lack of appropriate extraction and speciation methods for methylated Sb compounds.

Fortunately, new techniques helped to develop this specific research area. For example, extraction methods using citric acid (9.4-51.5% recovery) were found to be the best compared to water (3.4-45.2%) and methanol:water mixtures (3.5-26.1%), using either ultra-sonication or shaking[23,24]. However, citric acid extracted only 0.6 to 57.7 % of the total Sb in a range of plants growing near two Sb mines in two independent studies[4,23]. These results demonstrate that more effort must be undertaken to improve

recoveries of extracted Sb for speciation analysis. Furthermore, to our knowledge, there is no published Sb extraction method that could also extract TMSb in plants for which it was clearly demonstrated that there is no change in speciation between TMSb, Sb(III) and Sb(V) during extraction. With regards to speciation analysis, High Pressure Liquid Chromatography (HPLC) with a strong anion exchange column, such as the Hamilton PRP-X100, is usually preferred although Dionex AS4A, Phenomenex SAX-SB and the ICSep ION-120 have also been used in a few studies[24]. Generally, Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) is the detector of choice although HPLC can be easily coupled to an Atomic Fluorescence Spectrometer (AFS). In both cases, these couplings can be used in conjunction with hydride generation (HG) to improve sensitivity[24]. The successful separation by HPLC-ICP-MS of Sb(III), Sb(V) and TMSb has already been achieved using different mobile phases as eluents[21,25-27]. However, most studies used gradient elution which is not ideal, since gradients can modify HG efficiency as well as excitation in the AFS and plasma conditions, leading to sub-optimal quantification. Usually, isocratic elution is preferred, as it is the case for As analysis[28,29].

To the best of our knowledge, there are no published methods that combine a highly efficient Sb extraction method of plant tissues with a quantitative HPLC-ICP-MS analysis. Furthermore, no study has thoroughly investigated the issues that arise when TMSb is extracted from plants and when it is analysed by HPLC-ICP-MS. Therefore, in this study, we introduced a new method that allows for quantitative TMSb extraction in plants, in both shoots and roots and we also validated this method based on CRM extractions and Sb species spiking. This technique is based on a sediment extraction method using oxalic acid (OA) and ascorbic acid (AA)[30]. It is employed in conjunction with a recently developed isocratic HPLC-ICP-MS method developed by Ge and Wei where the mobile phase is ammonium tartrate (AT)[31]. This newly developed procedure was then used to quantify the amount of TMSb in shoots and roots of rye grass (*L. perenne*) exposed to solutions of 1 $mg.L^{-1}$ of Sb(III), Sb(V) and TMSb.

## Experimental

*Reagents.* Sb stock solutions were prepared from salts of potassium hexahydroxoantimonate (V) ($KSb(OH)_6$), potassium antimony (III) tartrate hydrate ($C_8H_4K_2O_{12}Sb_2 \cdot xH_2O$) and trimethylantimony (V) dibromide ($(CH_3)_3SbBr_2$) purchased from Sigma-Aldrich (Buchs, Switzerland). These compounds were used to validate the HPLC-ICP-MS method as well as the extraction recoveries. The Sb standard for total

analysis was purchased from Roth (1000 $mg.L^{-1}$, Karlsruhe, Germany). Extraction solutions were prepared with ascorbic acid (99.7%) and oxalic acid (99%) from Merck Millipore (pro analysi, Schaffhausen, Switzerland). HPLC mobile phases were prepared using ammonium tartrate ("AT", 99.5%, Fluka, Sigma Aldrich, Buchs, Switzerland), and methanol (99.9%, Merck Millipore, Schaffhausen, Switzerland). Hydrochloric acid (HCl, 35%, Supra Quality), and nitric acid ($HNO_3$, 69%, Supra Quality) used for digestions and dilution solutions were from Roth (Karlsruhe, Germany). Water from an ultrapure water system (MilliQ, Merck, Schaffhausen, Switzerland) was used throughout the study.

*Plant growth and Sb exposure.* The plant used for this experiment was rye grass (*L. perenne var. calibra*) obtained from Fenaco (Bern, Switzerland). The plants were grown in a growth chamber with a daily photo period of 16 h at 22 °C with a light intensity of 20000 Lux and a daily night period of 8h at 16°C. During the first 4 weeks, the plants were grown in a 1/5 strength Hoagland solution at pH 6 (Table S1). After this, 1 $mg.L^{-1}$ of either Sb(III) from $Sb_2O_3$, Sb(V) from $KSb(OH)_6$ and TMSb as $(CH_3)_3SbBr_2$ were added to the Hoagland solution for 8 days. The nutrient solutions were replaced every two days to prevent speciation change and potential changes were monitored using HPLC-ICP-MS. While the Sb(V) and TMSb treatment solution did not contain any other species (data not shown), the Sb(III) treatment solution was found to consistently contain 6.8 ± 1.9 % Sb(V) (n=11). Plants were grown in triplicate for each treatment. At harvest, plants were separated into shoot and roots, washed in ultrapure water and dried at 60 °C before grinding.

*Digestion method.* Samples were digested using a closed-vessel microwave-assisted extraction technique to prevent losses through volatilization of Sb-Cl compounds[32]. The instrument used was an Ethos contFLOW 1600 (Milestone, Shelton, USA). Briefly, 200 mg of oven-dried and ground plant samples were weighed and transferred in Teflon pressure vessels. Then, 7.5 mL of $HNO_3$ and 2.5 mL of HCl were added. The microwave program for the digestion is described in Table S2. The vessels were cooled before being opened (30 min inside the microwave oven and 30 min under a fume cupboard). The digests were then filtered (Grade 589/2, Whatman) and diluted 5-fold with ultrapure water. The resulting solution was stored at 4°C until analysis. The Certified Reference Material (CRM) used for quality control was citrus leaves (China National Analysis Center for Iron and Steel, NCS ZC73018 (GSB-11)) that contained 200 ± 60 $\mu g.kg^{-1}$ Sb. For each digestion batch including 10 samples, a CRM and a blank were also digested and treated in the same manner as the plant tissue samples. The limit of detection for the whole digestion method (n=15 blanks, average + 3 x standard deviation) is 17.7 $\mu g.kg^{-1}$.

147 *Extraction method.* The plant samples were extracted using a newly developed method inspired
148 from Potin-Gautier *et al.* in 2005[30]. First, 100 mg of dried and ground plant material were weighed and
149 transferred to a borosilicate glass vial (20 mL Wheaton liquid scintillation vials, Sigma Aldrich, Buchs,
150 Switzerland). Then, 10 mL of a solution of oxalic acid and ascorbic acid (OA/AA, 200mM and 100mM
151 respectively) was added to the powder and the resulting mixture was shaken vigorously for 1 minute.
152 The vials were placed in an ultrasonic bath for 30 min. After the ultrasonic bath, the extraction solution
153 was separated from the plant material using a centrifuge at 3500 rotations per minute (relative
154 centrifugal force: 6000) for 5 min and then filtered at 0.45 µm. Finally, the supernatant was diluted with
155 a solution of 150 mM AT and stored in the fridge at 4°C to prevent Sb(III) oxidation[33].

156 *Total analysis.* The total Sb concentrations in samples was measured with an ICP-MS (7700x ICP-MS,
157 Agilent Technologies, Santa Clara, USA). The settings for total Sb analysis by ICP-MS are presented in
158 Table S3. The internal standard (10 $\mu g.L^{-1}$ Indium, m/z 115) was injected online through a T-piece. The
159 measured mass for Sb was m/z 121. The instrumental LOD for Sb is 0.003 $\mu g.L^{-1}$, however Sb is very
160 persistent in the injection line (auto-sampler, nebulizer and spray chamber) during total analysis by ICP-
161 MS. Therefore, a strong cleaning procedure was adopted to rinse the injection line between each
162 sample using a solution of 5% HCl and 5% $HNO_3$ for 40 seconds, followed by two solutions of 1% $HNO_3$
163 for 30 seconds each. Although the carry-over was not fully eliminated, this procedure allowed us to
164 lower it to < 0.2 $\mu g.L^{-1}$ after a 100 $\mu g.L^{-1}$ solution.

165 *Speciation analysis.* Concentrations of Sb species were measured by coupling an HPLC (1200 Series,
166 Agilent Technologies, Santa Clara, USA) to the ICP-MS. The settings for HPLC-ICP-MS analysis are
167 presented in Table S3. The same internal standard was injected online through a T-piece as for the total
168 Sb analysis. The mobile phase used for separating Sb(III), Sb(V) and TMSb on the HPLC column (Hamilton
169 PRP-X100 10µm, 4.6 x 150mm (PEEK), Reno, USA) was 150 mM of AT at pH 5 with 4% methanol. It was
170 run isocratically at a flow of 1 $mL.min^{-1}$ and the column compartment temperature was set at 20°C. This
171 method was initially developed by Ge and Wei[31]. Due to the high salt content of the mobile phase, a
172 10/32" micro-splitter (IDEX H&S, Middleboro, USA) was used to remove 50% of the HPLC flow. This
173 smaller flow was further diluted online with 1% $HNO_3$ solution through a T-piece prior to mixing with the
174 internal standard solution.

175

176

## Results and discussion

### *Speciation analysis.*

Firstly, the method developed by Ge and Wei[31] was tested with our HPLC-ICP-MS system. However, due to the large quantity of salts being deposited in the torch and on the sampling and skimmer Ni cones, leading to a complete clogging of the torch after only 3 hours analysis, it had to be modified as described in the Experimental section. The use of a micro-splitter to divert 50% of the post-column HPLC flow to the waste produced a remaining flow of 0.5mL.min$^{-1}$ and resulted in a chromatogram with low sensitivity and high noise since the flow was too low for our nebulizer. Therefore, a T-piece was used to mix a 1% $HNO_3$ solution with the HPLC flow, using a peristaltic pump at 0.3 RPS. The resulting chromatogram (Fig. 1) showed less noise while the addition of 1% $HNO_3$ helped greatly to improve the sensitivity with a LOD for Sb(V) of 0.1 µg.L$^{-1}$. The method could thus be used for 12 hours non-stop without causing any blockage in the torch or in the orifices of the cones. However, 50% of the Sb is lost in this process and a different mobile phase, with less salt content, might possibly allow for an even better limit of detection. We nevertheless chose this method because of its high sensitivity. Furthermore, we preferred an isocratic elution which allows for quantification of all the Sb species using a standard consisting of a single Sb species for calibration. Because of the specific effects of different eluents on the plasma conditions, gradient elution would require several calibrations in different matrices complicating the analysis considerably.

### *CRM extractions.*

Citrus leaves containing Sb (200 ± 60 µg.kg$^{-1}$, mean ± SD) were used as a CRM to test the microwave digestion procedure as well as the extractive power of oxalic acid in ascorbic acid (OA/AA, 200 mM and 100 mM respectively). Fig. 2 shows that our closed vessel digestion technique, using ultrapure $HNO_3$ and HCl as acids, was adequate to digest plant samples for total Sb analysis since the recovery was 99.4 ± 1.8 % (n=3). The same CRM was used for testing the extraction procedure for Sb species. The extract was analyzed by ICP-MS to measure the total Sb concentration extracted and by HPLC-ICP-MS to measure the column recovery. The extraction method yielded a recovery of 72.6 ± 1.3 % Sb (n=3) when measured directly with ICP-MS while when analyzed for speciation it yielded a similar recovery of 71.9 ± 7.2 % Sb (n=3, Fig. 2). These results demonstrate that the extraction is efficient, since a high value of more than 70% of the Sb present in the CRM was extracted. Although only one plant was tested, the results are consistently higher than previously published recoveries of 0.6 to 57.7 % of the total Sb concentrations

207 in a range of plants from two distinct Sb mining areas using citric acid extraction[4,23]. Furthermore, the
208 Sb species present in the extract were not lost on the HPLC column since there was no difference of the
209 recoveries between the HPLC method in which the three analyzed Sb species were separately quantified
210 and summed up and the total concentration measurement of Sb with ICP-MS. However, these results
211 alone are not enough to assess species interconversion during extraction, dilution and storage since the
212 CRM is only certified for total Sb and not for Sb species.

213 Due to a lack of CRM with certified concentrations of Sb species, blank extraction solutions, prior to
214 the extraction procedure, and extracted solutions of the CRM were spiked with 50 $\mu g.L^{-1}$ of each species
215 of interest in triplicate. This technique allowed us to assess if the extraction itself was causing a
216 conversion of Sb species or if extracted plant compounds could be responsible for speciation changes.
217 ICP-MS results show that all of the Sb spiked to the extracting solution was recovered (Fig 3a). The
218 recoveries for total Sb yielded 100.2 ± 1.1%, 103.2 ± 1.1% and 97.7 ± 1.1% (n=3) for extracting solutions
219 spiked with Sb(III), TMSb and Sb(V) respectively. Moreover, the recoveries for the Sb speciation by HPLC-
220 ICP-MS analysis (sum of species) of the same spiked extracts were 99.1 ± 1.3 %, 97.4 ± 1.7 % and 98.8 ±
221 2.5 % (n=3) for solution spiked with Sb(III), TMSb and Sb(V) respectively (Fig. 3a). This confirms that all
222 the extracted species were successfully separated with our HPLC method and that none was lost during
223 the separation or the transfer to the ICP-MS. Furthermore, the speciation analysis by HPLC-ICP-MS
224 analysis revealed that no change in speciation occurred during the extraction and analysis for the
225 samples spiked with Sb(III) and TMSb since all the chromatograms only showed one peak for the spiked
226 compounds (100%). However, our results also clearly show that Sb(V) was consistently converted to
227 Sb(III) during extraction since only 31.6 ± 0.5 % (n=3) of the spiked Sb(V) remained in the solution after
228 extraction (Fig 3a). This example illustrates how difficult it is to preserve inorganic Sb species during an
229 extraction. To our knowledge, no studies showing preservation of inorganic Sb species during extraction
230 of plants have been previously published.

231 Our HPLC-ICP-MS results of spiked CRM extracts again confirmed that no species conversion
232 occurred for Sb(III)- and TMSb-spiked CRM extracts (Fig. 3b). Indeed, only a single peak corresponding to
233 the spiked compounds was observed in the chromatograms (100%). However, it was also found that
234 only 27.6 ± 3.1% (n=3) of the spiked Sb(V) remained in the solution, all the rest was converted to Sb(III).
235 In both cases (with extractant only and with plant CRM extracts), Sb(V) was reduced to Sb(III) to almost
236 the same extent. This is due to the reducing nature of the extracting solution: oxalic acid and ascorbic
237 acid. However, this phenomenon did not occur when 20 or 600 $\mu g.L^{-1}$ of Sb(V)were spiked to a CRM of a

marine sediment[30]. Finally, we could also show that the plant extract had a small positive impact on spiked TMSb since the recovery (sum of species / spiked amount) was 119.2 ± 9.6% (n=3) while it was perfectly acceptable for Sb(III) and Sb(V) with 102.1 ± 14.5% and 99.4 ± 5.6 % respectively (n=3). It is very difficult to ascertain exactly why. Indeed polyatomic interference with m/z 121 is only possible with $^{105}Pd^{16}O^{+}$ and it is unlikely that a Pd compound would co-elute with TMSb. Another possibility could be that carbon-rich compounds co-elute with TMSb and increase the torch temperature and thus the sensitivity[34]. However, this would be visible on the internal standard chromatogram, too.

*Growth experiment*

Overall, our method was satisfactory for the extraction and measurement of TMSb in plant samples, so we continued with a TMSb ryegrass uptake experiment. The extraction recovery (sum of species / total digestion) was not the same for the rye grass roots and shoots. The shoot recovery was similar to the recovery for the CRM (63.1 ± 12.7 %, n=8), which is also shoot material while the root recovery was much lower (37.6 ± 5.5 %, n=7). Each treatment led to very distinct results in terms of concentration and speciation for shoots and roots and this is reflected by the representative chromatograms shown in Figure 4 while the percentage of species in shoots and roots as well as concentration are shown in Figure 5.

First of all, we found that most of the Sb in the plants was present in the roots (Fig. 5a). This has already been reported in previous studies on other plants and seems to be a common feature of non-hyperaccumulator plants. Ren *et al.*[10] reported that the highest amount of Sb, found in exposed rice, was in the roots. Furthermore, our results show that Sb(III)-treated plants took up 100 times more Sb in the roots than Sb(V)- or TMSb-treated plants as shown in Figure 5a and Table 1 (1029 ± 191.7 $mg.kg^{-1}$ for Sb(III) vs. 9.9 ± 1.5 $mg.kg^{-1}$ for Sb(V) and 9.0 ± 2.3 $mg.kg^{-1}$ for TMSb). Several studies found that Sb(III)-exposed rice took up more Sb than Sb(V)-exposed rice[10,11,35]. The most likely explanation for the higher uptake of Sb(III) than of Sb(V) is that Sb(III) enters microorganisms and plants through aquaglyceroporins due to its similarity with glycerol[36,37], while for Sb(V) and TMSb up to now no transporters are known[38]. If the aquaglyceroporin-facilitated Sb(III) uptake was more efficient than that of Sb(V) and TMSb, it could explain the difference in uptake with Sb(V) and TMSb. Of interest is the similarly low Sb concentration in shoots of Sb(V)-, Sb(III)- and TMSb-treated plants of 1.1 ± 0.7, 1.0 ± 0.4 and 0.8 ± 0.2 $mg.kg^{-1}$, respectively (Tab. 1), while in white clover, another common pasture plant 770 $mg.kg^{-1}$ Sb was found in shoots of plants treated with Sb(V)[9]. We interpret this difference as an indication of the presence of

PCs, which immobilized the Sb in the vacuole of rye grass roots while the white clover does not produce PCs allowing for a more pronounced transfer of Sb from root to shoot.

In roots and shoots of the Sb(III)-treated plants, Sb (III) contributed 45.0 ± 4.2 and 25.8 ± 32.9 % to the respective total Sb concentrations in plant tissue (Fig. 5b&c, Tab. 1). The rest of the Sb mostly consisted of Sb(V), except in the roots where a contribution of 0.4 ± 0.1 % of TMSb (n=3) to the total Sb was measured. These results are in line with previous studies showing that rice plants exposed to Sb(III) contained mostly Sb(V)[10]. Unfortunately, although not detected by our analysis, the presence of a small amount of TMSb in the nutrient solution could not be completely excluded; therefore we cannot be sure that TMSb was formed in the roots. Moreover, we showed that Sb(III) was consistently stable during the extraction, while Sb(V) was reduced to Sb(III) (Fig. 3). Therefore, Sb(V) was really present in the roots and shoots of the exposed plants and was not an artifact due to the extraction procedure. However, its presence could be attributable to the oxidation of Sb(III) to Sb(V) during the processing of the plant prior to the extraction. Furthermore, since the nutrient solution contained a small amount of Sb(V) (6.8 ± 1.9 % of the total Sb in solution, n = 11) it cannot be excluded that some of it entered the plant, although it is unlikely that all the plant Sb(V) results from the nutrient solution, since the percentage of Sb(V) present in the plants is much higher than in the solution.

In the Sb(V) treatment, only 59.8 ± 2.1 % (n=3) of the Sb in the roots was found to be Sb(V) while the rest was Sb(III) as shown in Figure 5c. It is not possible in this case to assess whether Sb(III) was indeed present in the plant or simply a result of the extraction procedure (due to the conversion of Sb(V) to Sb(III) mentioned previously) although the nutrient solution contained exclusively Sb(V). Interestingly, no Sb(III) could be found in the shoots (Fig. 5b). The latter result suggested that rye grass shoot extracts stabilized the Sb(V) and prevented its reduction by the extraction solution. Furthermore, and most importantly, TMSb was found in small quantities in the shoots of the Sb(V) treated plants but not in the roots, suggesting *in planta* methylation of Sb by rye grass or a fast transport of TMSb from roots to shoots, where TMSb accumulates to a detectable concentration. This interesting result is novel and does not parallel recent studies on plant methylation of As. Indeed, it was found that plants do not have the capacity to methylate As directly but rather take up methylated As from the soil by roots and transport it to the shoots[39].

Remarkably, the roots of the plants exposed to 1 $mg.L^{-1}$ of TMSb contained a TMSb contribution of 75.8 ± 20.4 % of the extracted Sb while the rest was exclusively Sb(V) (Fig. 5c). Since no Sb(V) or Sb(III) could be found in the nutrient solutions, this suggests that demethylation occurs in the roots or at their

interface with the nutrient solution since we also showed that no conversion of TMSb occurred during the extraction procedure (Fig. 3). This new result shows that plants could be equipped with specific mechanisms to demethylate TMSb. Furthermore, our extraction did not lead to the reduction of Sb(V) to Sb(III) as shown in the extract spiking experiment (Fig. 3). In the shoots, the results were consistent between plants and a similar contribution of TMSb (76.4 ± 0.1 %, n=3) to the amount of extracted Sb was found while Sb(V) represented only 15.4 ± 0.5 %, n=3 (Fig. 5b). An unknown species was also found in the shoots and made up 8.2 ± 0.5 % (n=3, 0.05 ± 0.005 $mg.kg^{-1}$) of the extracted Sb. This unknown peak could be DMSb or MMSb or maybe an organo-Sb compound formed from TMSb. It is unlikely that this compound is formed from Sb(V) since no such compound was found in Sb(V) or Sb(III) treated plants. It is unfortunately impossible to identify the compound since MMSb and DMSb standards are not commercially available. Ideally, a molecular mass spectrometer (e.g. electrospray ionization MS: ESI-MS) could be used to identify the structure of such compounds. However, the mobile phase used in this study is not compatible with ESI-MS and the concentration in the extract would be too low to achieve a successful identification.

*Conclusions*

An efficient extraction method coupled with a quantitative HPLC-ICP-MS method used in our study showed that TMSb could be taken up and transported to the shoots of rye grass as well as possibly being formed in the plant. Although previous studies showed the presence of TMSb in plants using other extractions methods[8,23,24,31], this is the first study to provide a sufficient level of quality control to ensure that TMSb is not formed during the extraction or analysis of these samples. Also, it is the first time that TMSb uptake and transfer is demonstrated under controlled conditions. Unfortunately, the method does not allow to completely discriminate between Sb(III) and Sb(V) because of the artificial reduction of Sb(V) to Sb(III) during extraction. However, to our knowledge, there is up to now no published plant extraction method that avoids the inter-conversion of Sb species. Nonetheless, we are convinced that our proposed method will be useful in studies of the fate of TMSb in the soil-plant system which need to be conducted to allow for a safe use of Sb-contaminated sites such as mining areas and shooting ranges.

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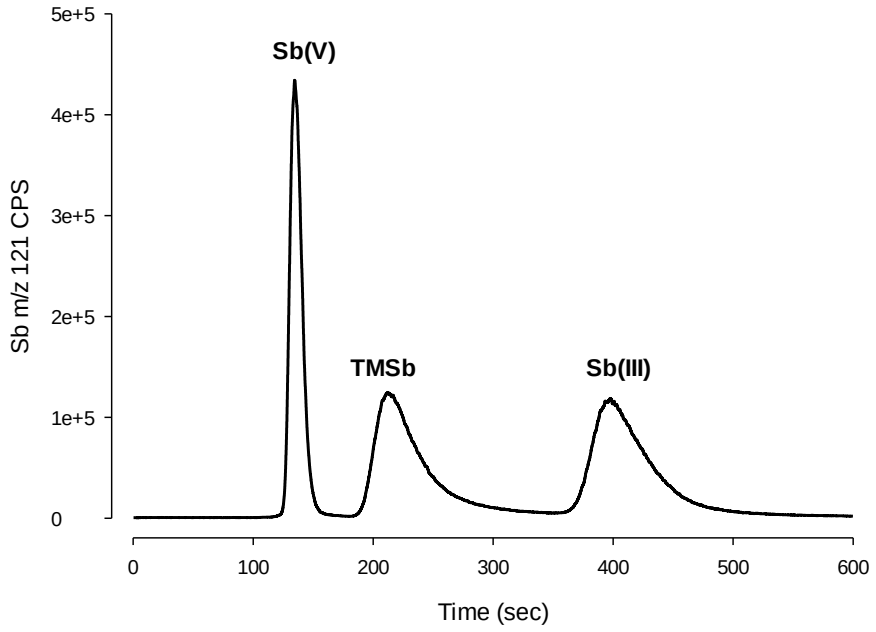
472 Figures:




473

474 Figure 1: HPLC-ICP-MS chromatogram of a mixture of three Sb species in deionized water (200 µg.$L^{-1}$).
475 The analysis was performed with isocratic elution using 200 mM ammonium tartrate buffer at pH 5 with
476 4% methanol (1 mL.$min^{-1}$).

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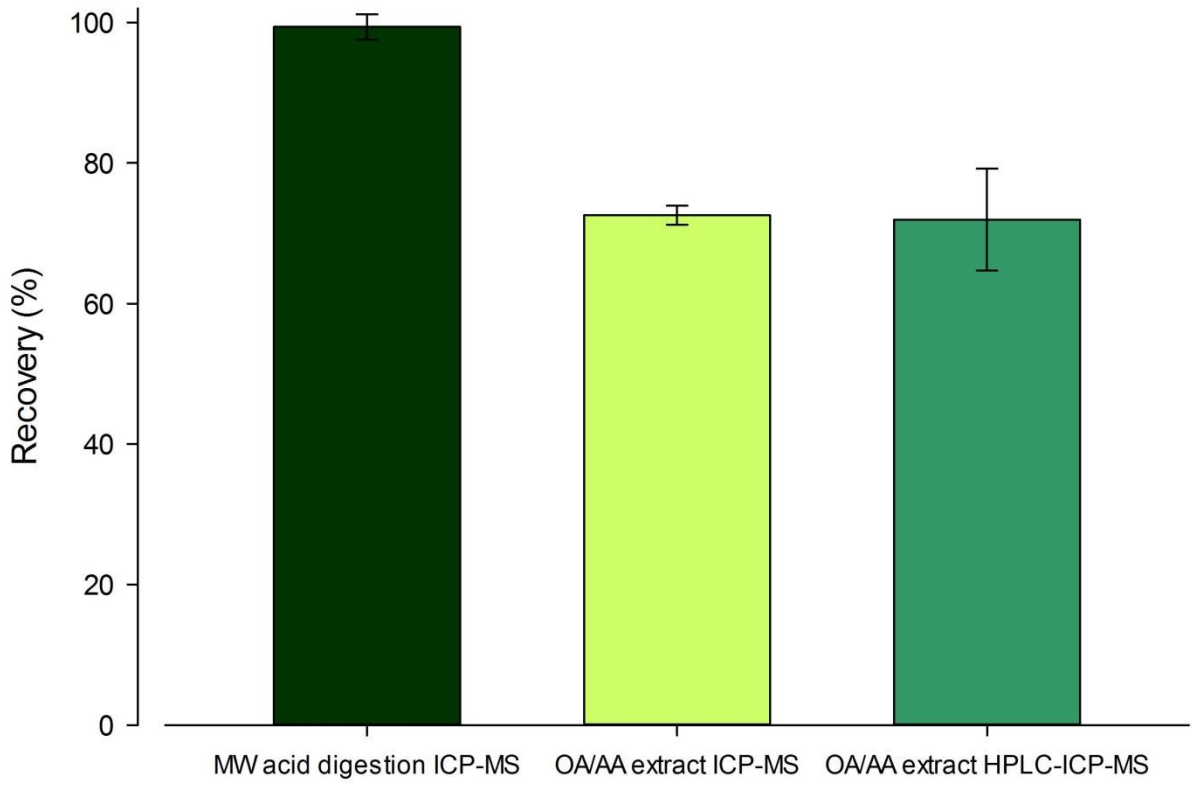


479

480 Figure 2: Recovery of Sb from the Certified Reference Material (CRM) “Citrus Leaves” when digested by
481 microwave, extracted with oxalic acid and ascorbic acid (OA/AA) and analyzed by ICP-MS and by HPLC-
482 ICP-MS (sum of species). The recovery is calculated from the CRM value of 200 ± 60 µg.kg$^{-1}$ (mean ± SD,
483 n=3).

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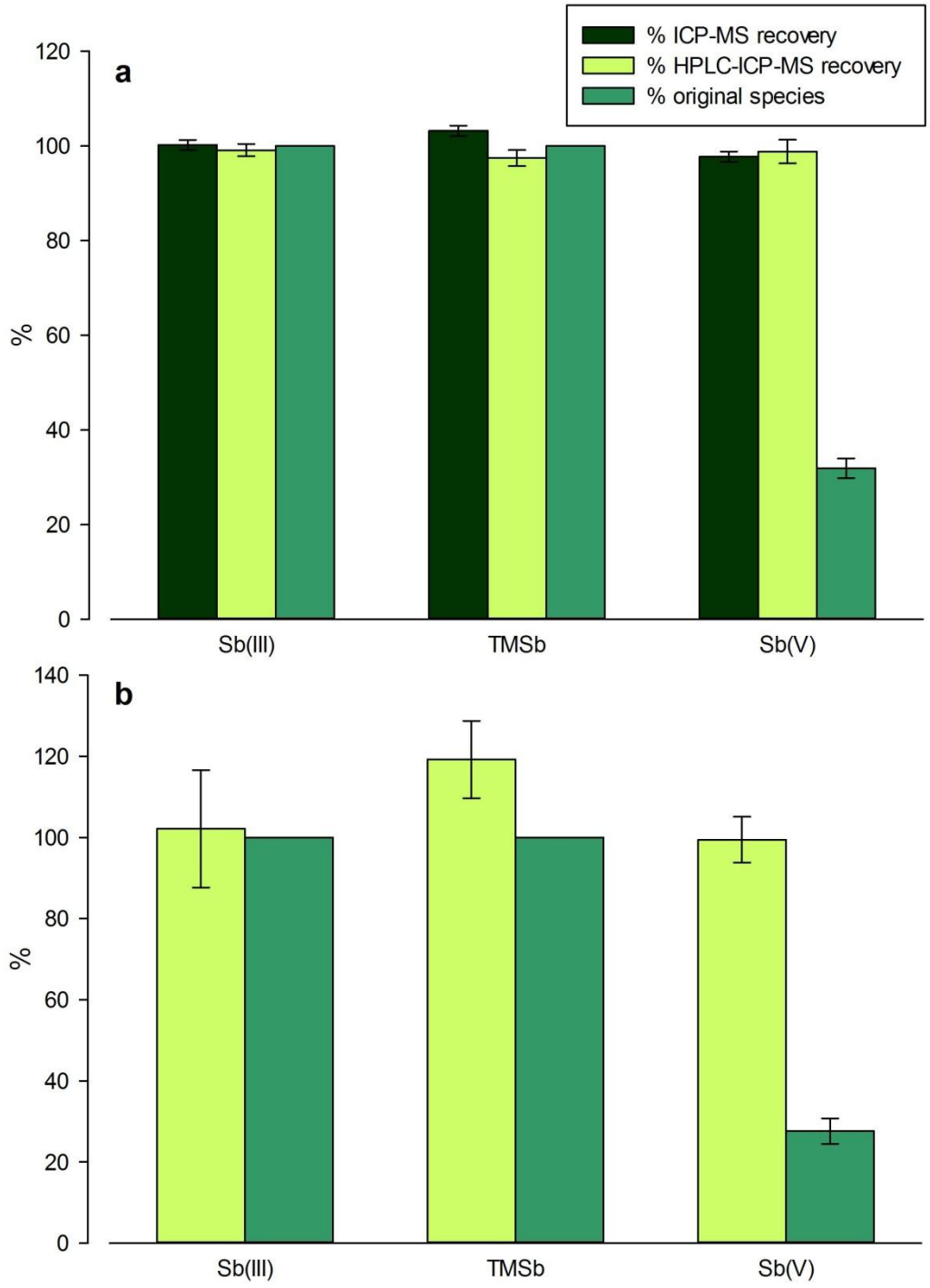
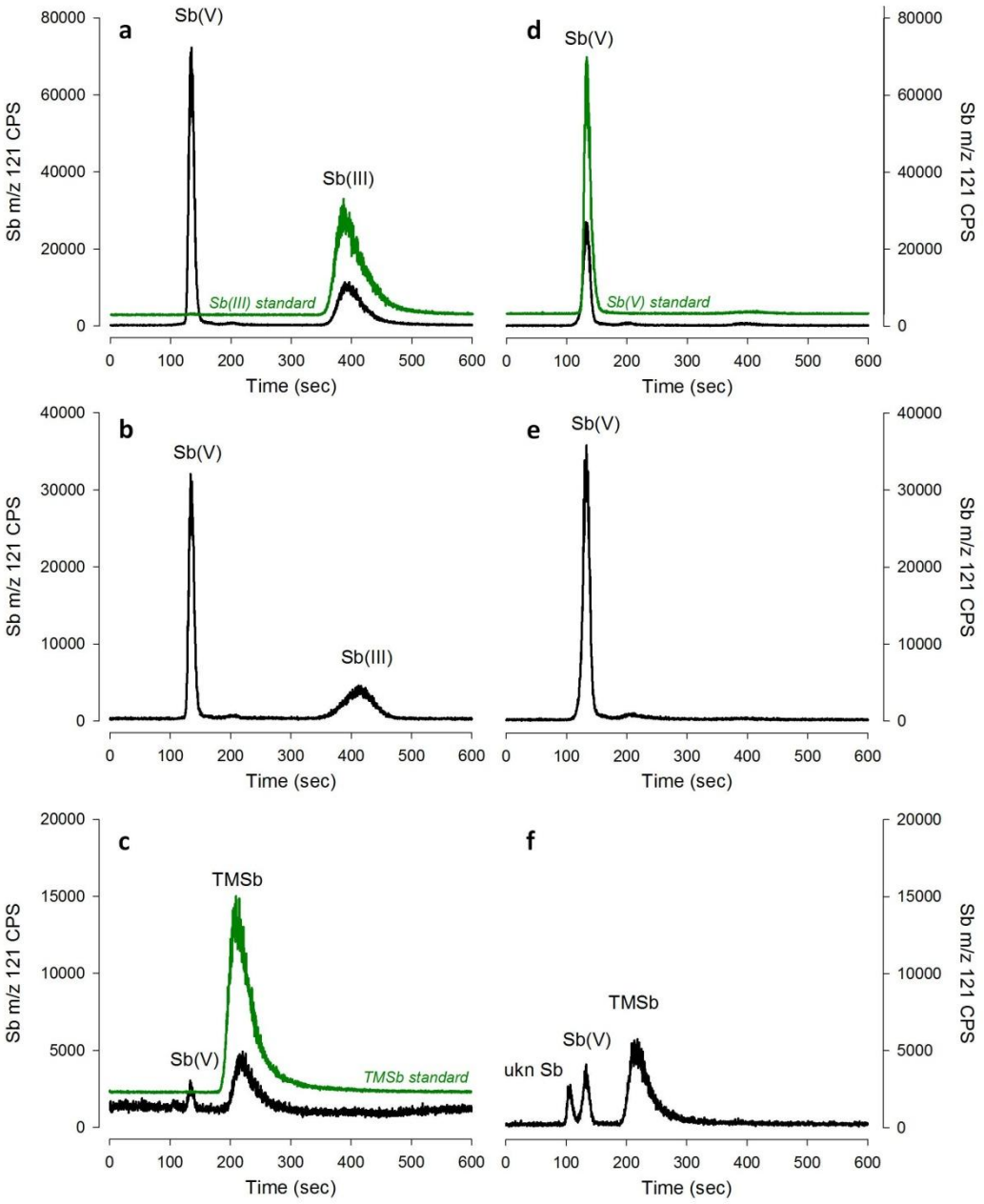
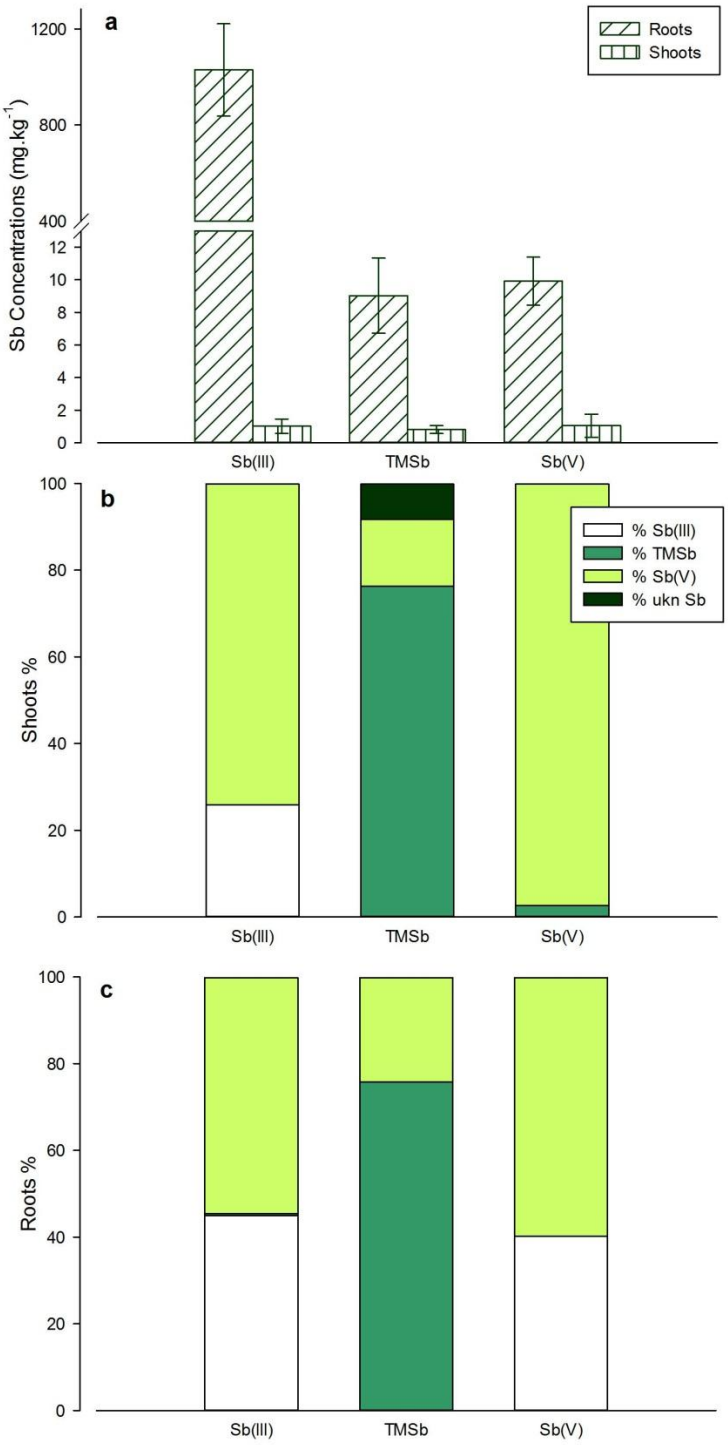



Figure 3: a) Recovery and speciation change for Sb(V), TMSb and Sb(III) spiked in a blank extracting solution (50 µg.kg$^{-1}$), extracted and measured by ICP-MS and HPLC-ICP-MS (mean ± SD, n=3) b) Recovery and speciation change for Sb(V), TMSb and Sb(III) spiked in a solution of already extracted CRM Citrus Leaves (50 µg.kg$^{-1}$) and measured by HPLC-ICP-MS (mean ± SD, n=3)




491

492 Figure 4: HPLC-ICP-MS chromatograms of extracts of shoots and roots for each treatment. a) Sb species
493 present in roots of plants exposed to Sb(III), b) Sb species present in roots of plants exposed to Sb(V), c)
494 Sb species present in roots of plants exposed to TMSb, d) Sb species present in shoots of plants exposed
495 to Sb(III), e) Sb species present in shoots of plants exposed to Sb(V), f) Sb species present in shoots of
496 plants exposed to TMSb. Chromatograms of Sb(III), TMSb and Sb(V) standards have been overlaid in
497 graphs a, c and d respectively. The Sb concentrations in all treatments was 1 $mg.L^{-1}$




498

499 Figure 5: a) Concentration of Sb in shoots and roots of rye grass (*L. perenne*) measured by ICP-MS for
500 each treatment (mean ± SD, n=2, except for Sb(V) treatment: n=3). Percentage of the different Sb
501 species extracted from b) shoots and c) roots of rye grass (*L. perenne*) exposed to 1 $mg.L^{-1}$ of Sb(III),
502 TMSb or Sb(V) (n=3).

503 Table:

504 **Table 1: Concentrations of the different Sb species in shoots and roots of rye grass for each treatment (mg.kg$^{-1}$, mean ± SD,**
505 **n=3) compared with total concentrations obtained by acid digestion (mean ± SD, n=2, except for Sb(V) treatment: n=3)**

| | Treatment | Ukn Sb | Sb(V) | Sb(III) | TMSb | Sum | Total (ICP-MS) |
|---|---|---|---|---|---|---|---|
| Shoots | Sb(V) | | 0.72 ± 0.41 | <LOD | 0.02 ± 0.01 | 0.74 ± 0.42 | 1.05 ± 0.71 |
| | Sb(III) | | 0.46 ± 0.26 | 0.17 ± 0.22 | <LOD | 0.64 ± 0.19 | 1.01 ± 0.44 |
| | TMSb | 0.05 ± 0.005 | 0.09 ± 0.01 | <LOD | 0.44 ± 0.04 | 0.58 ± 0.06 | 0.81 ± 0.24 |
| Roots | Sb(V) | | 3.1 ± 0.5 | 2.1 ± 0.2 | <LOD | 5.1 ± 0.7 | 9.91 ± 1.48 |
| | Sb(III) | | 261 ± 118 | 224 ± 123 | 2.3 ± 1.4 | 488 ± 242 | 1029 ± 192 |
| | TMSb | <LOD | 0.7 ± 0.8 | <LOD | 1.6 ± 0.2 | 2.7 ±1.6 | 9.02 ± 2.31 |

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507