Determination of Arsenical Herbicide Residues in Plant Tissues[1]

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Abstract. Paper chromatographic separation of hydroxydimethylarsine oxide (cacodylic acid), monosodium methanearsonate (MSMA), sodium arsenate, and sodium arsenite was achieved with the aid of four solvent systems. Aqueous extracts of plant tissues removed essentially all the arsenicals applied, but methanolic fractionation was required before the extracts could be analyzed by paper chromatographic procedures. A standard nitric-sulfuric acid digestion procedure was employed for arsenic analyses, but great care was taken to avoid sulfuric-acid-induced charring by first adding relatively large amounts of nitric acid to drive off chlorides present. Depending upon the amount of chloride present, substantial losses of arsenic as arsine chlorides were observed if the samples charred. Five minutes in fuming sulfuric acid to completely break the carbon-arsenic bonds was another critical requirement for the quantitative determination of arsenic from cacodylic acid and MSMA. The silver diethyldithiocarbamate colorimetric method was useful for detecting as little as 0.6 μg or as much as 20 μg of arsenic per sample.

INTRODUCTION

THROUGH the long history of use of arsenicals as trypanocides, insecticides, and herbicides, several hypotheses concerning the toxic effect of arsenic-containing compounds have been proposed. Many investigations have indicated differences in absorption or metabolic conversion of the applied arsenical to other compounds, the toxicity of which may be greater than the parent arsenical (11). Comparative phytotoxicity studies in our laboratories of foliar and root applications of hydroxydimethylarsine oxide (cacodylic acid), monosodium methanearsonate (MSMA), sodium arsenate, and sodium arsenite revealed that cacodylic acid was the most potent of the four when all were foliarly-applied, whereas sodium arsenite was by far the most phytotoxic when all were root-applied (11). Hence, the need for an examination of problems of relative absorption, transport, and metabolism was indicated. New methods of analysis were developed and others modified to investigate these problems. The methods are described in this paper. Studies of comparative phytotoxicity, absorption, and metabolism will appear separately (11).

MATERIALS

Seedlings of bean (*Phaseolus vulgaris* L., var. Black Valentine), with the first trifoliolate leaves about half expanded, served as the plant tissue in all experiments.

Cacodylic acid samples were about 96% pure in crystaline form, and MSMA was obtained as a 52% solution. Reagent grade sodium arsenate[5] and sodium arsenite[5] were used to make herbicidal solutions.

Silver diethyldithiocarbamate (herein after referred to as AgDDC) dissolved in reagent grade pyridine was used for colorimetric determination of arsenic.

Reagent grade nitric and sulfuric acids were used for digestion of plant tissues, and reagent grade solvents were used for chromatographic purposes. Whatman 3MM paper, in sheets or 3.8-cm strips, was used for paper chromatography; Eastman Chromagram Type 6060 cell-

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[5]Obtained from the Fisher Chemical Company.

ulose and Type 6061 silica gel sheets were used for thin layer chromatography. A Bausch and Lomb Spectronic 20 was used for optical density measurements at 540 mμ.

Methods and Results

Arsenic determination. The AgDDC colorimetric method for determining arsenic was used essentially as described by Powers *et al.* (10) but with modified apparatus and elimination of the silica gel adsorber. A stable red complex is formed between arsine and AgDDC that can be measured quantitatively at one of the absorption maxima; most often it is measured at 5400 mμ. The arsine generator designed for this study consisted of a 100-ml Kjehldahl digestion flask, fitted by a serum rubber cap stopper to a filter tube (used as a "scrubber"), which in turn was fitted to a reflexed capillary tube by rubber stopper (Figure 1). The down-turned end of the capillary

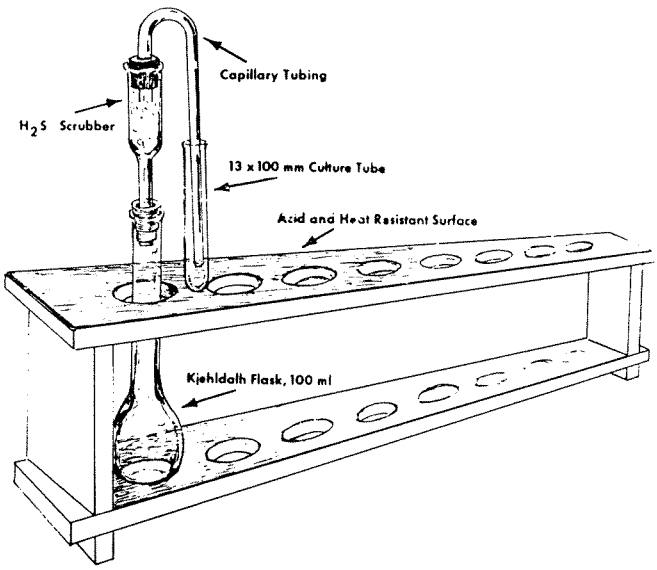

Figure 1. Arsine generator and rack for Kjehldahl digestion flasks. Diagram drawn to scale.

tube is immersed in 5 ml of a 0.5% (w/v) AgDDC-pyridine solution. The generation mixture consisted of the digested sample in 2 ml of H_2SO_4 diluted to 40 ml with distilled water, 2 ml of 15% potassium iodide, and 12 drops of 40% stannous chloride in 12.5-N HCl. Fifteen minutes were allowed for reduction of pentavalent to trivalent arsenic. Then 10 ml of concentrated HCl and approximately 4 g of granular zinc (20 mesh) were added to form nascent hydrogen, which reduced trivalent arsenic to arsine, a highly volatile compound that is trapped by the AgDDC-pyridine solution.

The "scrubber", which is a glass wool plug saturated with 10% lead acetate, is used as a precautionary step to trap hydrogen sulfide that may be produced by reduction of sulfuric acid in the generator. Hydrogen sulfide will react with AgDDC and interfere with the detection of arsenic. However, reduction of sulfuric acid can be largely avoided by making certain that its concentration is 5% or less before the reductants are added (6).

When arsenic determinations were made on undigested samples containing plant tissue, 2-propanol was added to the generation mixture to prevent excessive foaming and overflow from the arsine generator. Between 0.5 and 1 ml of 2-propanol was sufficient to reduce foaming in samples containing 1 g of dried plant tissue. The addition of 2-propanol reduced the rate of evolution of nascent hydrogen and, hence, the rate of formation of arsine, and prolonged the generation time. Determinations of arsenic in samples may not be quantitative and reproducible until hydrogen generation has ceased completely, usually about 30 to 35 min after the zinc is added.

We were able to detect as little as 0.6 μg and as much as 20 μg of arsenic per sample with a 5 to 15% variation of the mean. Over this range there was a linear relationship between optical density (absorbance) at 540 mμ and the concentration of the arsine-AgDDC complex (Figure 2). Owing to operational errors in spectrophotometers,

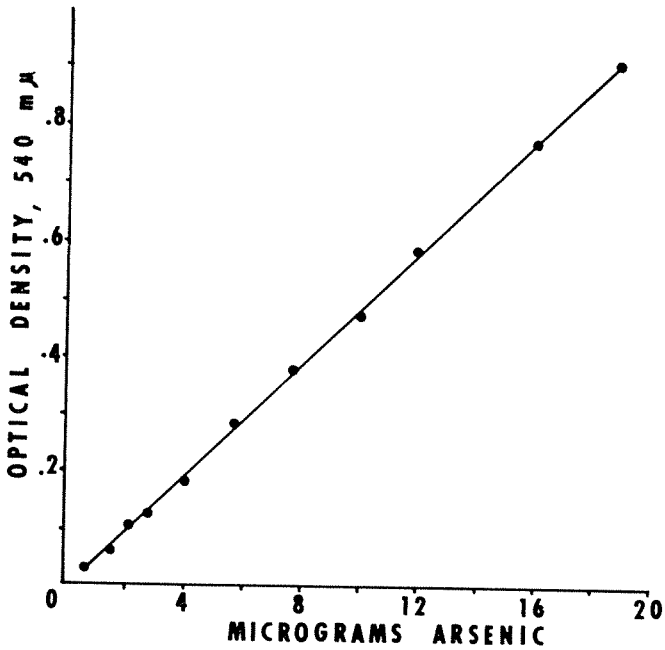

Figure 2. Typical standard curve for arsenic determination as the arsine-AgDDC complex at 540 mμ. Although the line passes through the origin, the most reproducible readings of optical density are obtained between 0.200 and 0.300 optical density units (4).

greatest reproducibility, i.e. the lowest variation of the mean, was obtained at absorbance values of 0.200 to 0.300, which was equivalent to 4 to 6 μg arsenic (4). Whenever a new AgDDC-pyridine solution was made, a new standard curve was derived; this precaution eliminated errors due to preparation and variations in different batches of AgDDC.

Recovery of arsenic from generation flasks was close to 100% when the samples contained 20 μg of arsenic or less, but recovery decreased rapidly as the arsenic content increased (Figure 3). The decline in recovery may have been due to very rapid arsine evolution beyond the complexing capacity of the AgDDC-pyridine solution or to incomplete release of arsine from the generation

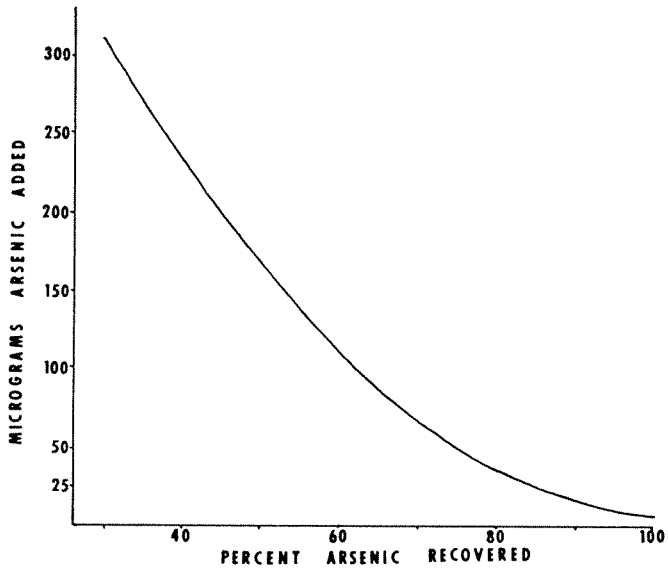

Figure 3. Percent recovery of arsenic as a function of the amount of arsenic added to the arsine generator.

flask. A recovery correction factor derived from Figure 3 was applied when the arsenic content was in excess of 20 μg, or a portion of the sample containing less than 20 μg of arsenic was used for analysis.

Organic arsenicals, such as cacodylic acid and MSMA, form volatile arsine derivatives that react with AgDDC to form red to brown complexes with absorption spectra different from arsine-AgDDC. Chromatographic analysis of these complexes on thin layer sheets of silica gel, with a pyridine-methanol (50:50) solvent system, revealed at least two compounds different from arsine-AgDDC. Observations suggest that much smaller amounts of these arsine derivatives are released from cacodylic acid and MSMA than from inorganic arsenic, or the extinction coefficients for the AgDDC complexes are much lower than that for arsine-AgDDC. Digestion of the organic arsenicals, which ruptures the carbon-arsenic bonds before reduction to arsine, increases the optical density at 540 mμ more than eightfold for cacodylic acid and twofold for MSMA.

Digestion. Plant tissues, extracts, and chromatographic paper were digested by a nitric acid-sulfuric oxidation procedure to convert all arsenicals to the inorganic pentavalent form. In subsequent experiments large numbers of samples had to be digested; hence, the procedures described by Powers *et al.* (10) were modified to reduce the time required for each digestion. Since the digestion procedure proved to be a very critical phase of the investigation where substantial losses of arsenicals could occur, it will be described in detail.

The first critical step was the addition of relatively large quantities of nitric acid and heating before sulfuric acid was added. Milton and Waters (6) emphasized that nitric acid alone should be used initially to avoid reducing conditions that may prevail locally, and, in the presence of chloride, cause the formation of volatile arsenic-chloride compounds. If sulfuric acid is added initially, charring usually occurs as the nitric acid disappears; at this time the volatile chlorides may form. We found that, depending upon the amount of chloride present, as much as 50% of the arsenic present was lost if sulfuric-acid-induced charring occurred before sufficient time was permitted for nitric acid digestion. However, it was not necessary to continue adding nitric acid until a clear solution was obtained, as suggested by Milton and Waters (6). Usually 80 to 100 ml of nitric acid were sufficient to remove all chloride normally present in 1 g of dried plant tissues. When this volume was reduced to a few milliliters, the flask was allowed to cool and 3 ml of sulfuric acid were added.

Heating was continued until copious fumes were produced for 5 min; this was the second critical stage in the digestion procedure, particularly for MSMA and cacodylic acid recovery. Apparently the carbon-arsenic bonds in these compounds were not ruptured in hot nitric acid (120 C), but only in fuming sulfuric acid (170 to 190 C). No more than 5 min in fuming sulfuric acid was required, but it was a critical requirement. Several tests were run with cacodylic acid in which the fuming stage was terminated in something less than a minute and recovery was reduced to 1 to 30%. The duration of the fuming sulfuric stage was less critical for MSMA, but quantitatively reproducible results were obtained only when fuming was continued for 5 min.

Extraction of plant tissues for chromatographic analyses. Bean plants weighing approximately 15 g were treated with 150 μg of arsenic as cacodylic acid and 27 μg of arsenic as sodium arsenate. The arsenicals were added dropwise to the leaves, the solutions were allowed to dry, and the tissues then were extracted fresh or dried in an oven at 70 C for 24 hr. Fresh plants were ground in a blender with distilled water to make a puree; the puree was heated to boiling for 5 min and then vacuum-filtered through Whatman #1 paper. The filtrate was evaporated to dryness under reduced pressure. Dried plants were ground to a powder in a Wiley mill through a 20-mesh screen. Thirty milliliters of distilled water were added per gram of tissue. The mixture was stirred on a hot plate, boiled for 5 min, and then vacuum-filtered through Whatman #1 paper; the filtrate was treated in the same manner as the fresh tissues. In two experiments, using three plants per arsenical treatment, the procedures described were adequate to give 70 to 99% recovery of applied cacodylic acid and sodium arsenate (Table 1).

Table 1. Recovery of cacodylic acid and inorganic arsenate from fresh dried bean seedlings.

Experiment	Cacodylic acid		Inorganic arsenate	
	Fresh	Dried	Fresh	Dried
	(%)	(%)	(%)	(%)
1	85	64	108	102
2	64	73	84	98
Average	74	69	96	100

Losses occurred during transfer of the extracts and in the filtration process.

Two tests with ^{14}C-MSMA on fresh tissues yielded a 56% average recovery based on ^{14}C applied. Since negligible $^{14}CO_2$ was trapped, substantial losses occurred in preparation of the extract. The causes of such losses are unknown, but ion exchange of arsenicals on glassware and paper would be expected.

The data in Table 2 show that, although slightly more cacodylic acid and sodium arsenate were bound to the hot water insoluble fraction in dried tissues, the amount bound was never more than 3% of the total recovered. Thus, the drying process did not fix large amounts of arsenicals to proteins or other hot water insoluble tissue fractions. For experiments in which analyses were made 7 days after treatment with arsenicals, up to 25% of the arsenic from sodium arsenite and arsenate was bound to the hot water insoluble fraction (11).

Table 2. Arsenic recovered in the extract and plant residue.

Fraction	Fresh tissue		Dried tissue	
	Cacodylic acid	Arsenate	Cacodylic acid	Arsenate
Extract	99[a]	99	97	98.5
Residue	1	1	3	1.5

[a]Data derived from experiments reported in Table 1. Averages of both experiments are given. The data are the percent of total arsenic recovered; e.g. $\frac{\text{Arsenic in extract}}{\text{Total arsenic}} \times 100 = \%$ arsenic in extract.

The final step in preparation for chromatographic analysis was addition of 50% methanol to the vacuum-dried extract. This procedure eliminated large quantities of water-soluble compounds that retarded the movement of the arsenicals on paper and at times interfered with solvent flow across the origin. Usually, three 1-ml washes of 50% methanol were sufficient to suspend the residue from 15 g of plant tissue. This suspension was centrifuged at 10,000 × g for 30 min; the supernatant was decanted and streaked on chromatographic paper. Losses of sodium arsenite, sodium arsenate, cacodylic acid, and MSMA due to methanolic fractionation were negligible (Table 3) and most probably due entirely to occlusion in the pellet, which was not washed.

Table 3. Recovery of arsenicals in 50% methanol from plant extracts.[a]

Fraction	Cacodylic acid	MSMA	Arsenate	Arsenite
	(%)	(%)	(%)	(%)
Supernatant	93	98.5	98.5	99
Precipitate	7	1.5	1.5	1

[a]Eleven μg of cacodylic acid and 154 μg of the other arsenicals were added to filtered, heated plant extracts; absolute methanol was added to bring the concentration to 50%. The solutions were centrifuged and both the pellet and supernatant were analyzed for arsenic.

Analysis by paper chromatography. The extract from 1 g of dried plant tissue, containing as much as 160 μg of arsenic, could be applied to a 15-cm-wide sheet. Consistent R_f values were obtained only in controlled temperature conditions; the values quoted in Table 4 were obtained at 23 to 26 C. Depending upon the solvent system, solvent fronts moved 25 to 30 cm beyond the origin in 4 to 8 hr.

A stepwise procedure of chromatographic analysis of plant extracts was developed for each arsenical (Table 4). Solvent systems, henceforward, will be referred to by Roman numerals: I = 1-propanol:NH_4OH (7:3, v/v); II = 2-propanol:H_2O:acetic acid (10:3:0.1, v/v); III = 2-propanol:H_2O (7:3, v/v) (9); and IV = methanol: 10^{-3} MNH_4OH (8:2, v/v) (5). Solvent system I was used first to separate cacodylic acid from arsenate, arsenite, and MSMA, which are considered as potential metabolites of cacodylic acid (11). The cacodylic acid fraction was eluted with 50% methanol and rechromatographed by solvent system III or IV. If residual arsenic at R_f 0 to 0.1 was of sufficient magnitude to warrant further analyses, the paper at these regions was eluted and rechromatographed in solvent II, III, or IV.

Table 4. R_f values for arsenicals developed on 3MM paper.

Solvent system number[a]	Plant extract	Arsenate R_f	Arsenite R_f	MSMA R_f	Cacodylic acid R_f
I	−	0.0–0.1	0.2–0.3	0.0 –0.0	0.4 –0.5
I	+	0.0–0.1	0.0–0.1	0.0 –0.1	0.1 –0.3
II	−	0.3–0.4	0.5–0.6	0.6 –0.7	0.4 –0.5
II	+			0.27	
III	−	0.2–0.24	0.6–0.7	0.6 –0.7	0.6 –0.7
III	+			0.25–0.35	0.25–0.35
IV	−	0.5–0.7	0.6–0.8	0.6 –0.7	0.8 –0.9

[a]Solvent systems:
I 1-propanol:NH_4OH (7:3, v/v)
II 2-propanol:H_2O-acetic acid (10:3:0.1, v/v)
III 2-propanol-H_2O (7:3, v/v)
IV methanol:10^{-3}M NH_4OH (8:2, v/v)

Extracts from MSMA-treated plants were chromatographed first in solvent systems II, III, or IV and, following elution of the major arsenical peak, then in solvent system I. The reason for this procedure was to insure initial separation from arsenate and then arsenite. Note that arsenite runs ahead of MSMA in solvent I (Table 4). In one experiment with ^{14}C-MSMA, the broad MSMA peak from solvent II was eluted and rerun in the same solvent (11). Two peaks of ^{14}C were clearly resolved by this procedure; this result suggests that both MSMA and the unknown compounds, presumably a histidyl-MSMA complex (12), were sufficiently retarded by materials at the origin to impede resolution of the two peaks. The MSMA peak was retarded from R_f 0.67 to 0.27 in system II.

Extracts from plants treated with arsenite or arsenate were chromatographed in solvent I initially to check for the presence of arsenite or organic arsenicals. The arsenical at the origin was eluted, the eluate applied to paper, and the paper developed in system III or IV. In some cases arsenite did not move from the origin in system I; and, hence, other solvents were required to separate the two inorganic forms.

Discussion

Several methods for the quantitative analysis of arsenic have been devised (1, 2, 6, 10, 13), but in recent years silver diethyldithiocarbamate (AgDDC) colorimetric detection has been widely recommended (1, 12) where relatively expensive atomic absorption or neutron activation equipment is not available. Powers *et al.* (10) were able to detect as little as 0.1 ppb of arsenic in organic compounds by combining chromatographic adsorption of arsines to silica gel with the AgDDC method. However, this method required special glassware and was too time-consuming for the large number of analyses required in our investigations (11); hence, we simplified the apparatus and eliminated the silica gel adsorption step. We were able to detect as little as 0.6 μg of arsenic per 10-g sample, or 0.06 ppm.

Colorimetric procedures proved to be less troublesome

than the nitric-sulfuric acid digestion step. Considerable difficulties were encountered initially in obtaining quantitative recoveries of arsenic from arsenical standards because of inadequate or variable digestion in nitric acid and avoidance of sulfuric-acid-induced charring. Up to 50% of the arsenicals added were lost during digestion as volatile arsine chlorides if the chloride was not first removed as nitrosyl chloride.

Variable or inadequate time in fuming sulfuric acid was another source of error, particularly in quantitative determination of cacodylic acid and MSMA. In both cases the carbon-arsenic bonds were not ruptured in hot nitric acid; in fact they were not severed until the fuming sulfuric acid stage was maintained for 5 min. If the carbon-arsenic bonds remained intact, then arsines were generated that formed complexes with AgDDC of different absorption maxima and extinction coefficients; thus, inadequate digestion may cause an apparent loss of 50 to 90% of the arsenic added as MSMA or cacodylic acid.

Paper chromatographic procedures appeared to be well adapted for fractionating cacodylic acid, MSMA, arsenate, and arsenite in plant extracts. Four solvent systems, 1-propanol:NH_4OH (7:3, v/v), 2-propanol:H_2O: acetic acid (10:3:0.1, v/v), 2-propanol:H_2O (7:3, v/v) and methanol:10^{-3}M NH_4OH (8:2, v/v), permitted complete separation of the arsenicals from one another. Oguma (8) used acidic acetone, butanol, and ethanol solvent systems in separating arsenate from arsenite on silica gel thin layers. These systems have not been tested on paper, although the relative movement of arsenicals appears essentially the same on silica gel, cellulose layers, and paper. The chief virtue in Oguma's solvent systems is that arsenate moves considerably in advance of arsenite and, hence, in combination with any of our four systems, would, provide optimal separation of the two.

Anion and cation exchange resins have been used for fractionation of arsenicals (3, 7, 9). Our studies with one anion exchange resin, Rexyn 201 (OH), indicated that all four arsenicals could be removed almost completely from plant extracts adjusted to pH 11; however, elution with weak or strong hydrochloric acid or several other solutions failed to remove more than 20 to 50% of the MSMA and cacodylic acid and did not elute completely arsenate and arsenite. The arsenic-free effluent that passed through the column appeared to contain a large portion of the methanol-precipitable and pigmented compounds. Thus, once the elution problem is solved, anion exchange resins should be the most useful, and by far the fastest, means for separating parent arsenical herbicides and their metabolites from interfering materials in plant extracts.

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