A Rapid Quantitative Method for Maleic Hydrazide

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Maleic hydrazide (1,2-dihydro-3,6-pyridazinedione), a systemic plant growth regulator, is used extensively as a tobacco sucker inhibitor. A rapid analytical method for determining residual quantities of maleic hydrazide (MH) has been developed and involves direct extraction and derivatization to form the bis(trimethylsilyl) derivative, measurable by flame ionization gas chromatography. This method has been applied to tobacco leaf, midribs, reconstituted sheet, and cigarettes and should also be applicable to the analysis of MH in other plants or plant extracts. Various aspects of methodology are discussed and residue values for different tobaccos are presented.

Maleic hydrazide (1,2-dihydro-3,6-pyridazinedione), a systemic plant growth regulator, is used by tobacco farmers throughout the world as a sucker inhibitor. It is usually applied to the upper half or third of the tobacco plant within 24 hr after topping. However, it is subsequently found throughout the plant due to absorption and translocation. Maleic hydrazide (MH) is marketed under the names MH-30 and Royal MH-30 as solutions of the diethanolamine and potassium salts, respectively. An analysis of MH residues in tobacco was required that did not suffer from the interfering drawbacks of the earlier optical method of Wood (1953). The optical method, modified by Anglin and Mahon (1958), Lane *et al.* (1958), and Hoffman (1961), is based on the hydrolytic reduction of maleic hydrazide to hydrazine by zinc in aqueous sodium hydroxide solution. The hydrazine is subsequently steam distilled into an acidic solution of *p*-dimethylaminobenzaldehyde, which produces the acid salt of an azine, with an absorption maximum at 455 nm. Although solutions of these water-soluble salts do obey Beer's law, this method, when applied to tobacco, is plagued by interferences from pyrrole, resorcinol, tryptophan, and possibly other leaf constituents. Furthermore, any hydrazine remaining in the MH preparation would be analyzed and not be distinguishable from the MH. Since the inadequacies of the colorimetric method appeared difficult to overcome, a method based upon the direct unambiguous analysis of MH was developed. This paper describes a procedure, using silylation and flame ionization gas chromatography, for the rapid and direct quantitative determination of maleic hydrazide in tobacco. The method is quantitative, with a precision of 0.1 ppm, and can easily determine 1 ppm of MH residue in tobacco plants. It should prove applicable to MH residue determinations in other plants or plant extracts.

EXPERIMENTAL SECTION

Apparatus. A gas chromatograph equipped with a flame ionization detector was employed (Varian Aerograph Model 2800). A 3 m × 2 mm stainless steel column was packed with 20% OV-11 on 80–100 mesh Chromosorb W-HP. Helium (zero gas) flow was maintained at 25–35 ml/min. The column, injection port, and detector temperatures were 160, 220, and 240°, respectively.

Reagents. MH was recrystallized twice from distilled water. *N,O*-Bis(trimethylsilyl)acetamide (BSA) was obtained from Pierce Chemical Co. as the specially purified grade.

Sample Preparation. Tobacco samples were first desiccated for 24 hr and then pulverized in a jar mill (Norton Co., Akron, Ohio). The tobacco was removed and redesiccated to constant weight.

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Sample Extraction and Derivatization. A 20-mg portion of the dry tobacco powder was placed into a 0.3-ml microreaction vessel equipped with a Teflon-lined cap. BSA reagent (0.1 ml) was added, and the vial was sealed tightly and shaken vigorously to disperse the tobacco powder throughout the BSA. The sample was then placed on a hotplate, set to low heat (100°) for approximately 30 min, and repeatedly shaken. Calculations on standard samples of MH indicated 100% conversion to the TMS derivative, which was stable for at least a week.

Gas Chromatography and Quantitation. Glass tubing was installed in the steel injection port of the gas chromatograph to prevent possible degradation of TMS derivatives. The OV-11 column was partially conditioned by programming the temperature from ambient to 300° at 6°/min. Column conditioning was completed by injecting 15-μl portions of pure BSA at 20-min intervals until a stable base line was attained. The analysis was carried out isothermally at 160°.

Aliquots of standard MH solution were placed into microreaction vessels and desiccated, at atmospheric pressure, to constant weight. Pure *N,O*-bis(trimethylsilyl)acetamide was added and the sealed vessels were warmed briefly on a hotplate (100°). (In order to eliminate volumetric errors, *n*-tetradecane may be added as an internal standard to both standards and tobacco samples.) Five-microliter portions of standard or sample solution were injected into the gas chromatograph. The MH-TMS peak eluted with a retention time of 22.5 min (Kovats retention index 1462).

The following conditions were used for a confirmatory procedure. A 2.5 m × 2 mm stainless steel column packed with 5% OV-101 on 80–100 mesh Chromosorb G was maintained at 160°, after conditioning similar to that of the OV-11 column. Injector, detector, carrier gas, and flame gas parameters were identical to those described above. The retention time for the MH-TMS was 10.2 min (Kovats retention index 1342).

The values obtained *via* the above procedures were verified by the use of an alkali flame ionization detector. All sample preparation, extraction, and derivatization were carried out as previously described. The flow rates of helium, air, and hydrogen were adjusted to maximize the signal-to-noise ratio; typical values were about 35, 290, and 35 ml/min, respectively. Since the use of excessive amounts of silylating agents can destroy the response of both flame ionization and alkali flame ionization detectors, the reagent must either be vented or the ionization detector collector cleaned periodically to remove accumulated silica. It was found to be more convenient to clean the ionization detector collector.

MH values were calculated by comparison of the area of the MH-TMS peak either with areas produced by standards utilizing an electronic digital integrator (Infotronics Model CRS-204) or with the log–log standard curve determined separately for each detector. (Since the alkali

flame ionization detector does exhibit variability, a standard should be injected periodically for calibration purposes.)

DISCUSSION AND RESULTS

MH is one of the agricultural chemical residues found in tobacco and in other crops. The exact content of MH in both domestically used and exported tobacco should be determined. This is especially important for foreign export, since some foreign governments have placed the MH content of cigarette smoke under food law restrictions. The currently used colorimetric method is cumbersome and does not actually measure MH. This paper details a rapid quantitative method for MH, based upon the gas chromatographic separation and determination of the bis(trimethylsilyl) derivative of MH. Inspection of Figure 1 shows the presence of two aromatic hydroxyl groups available for silylation. The MH-TMS derivative was readily prepared not only from pure MH but also from the sodium salt of MH and from dried residue of commercial MH-30, using either Tri-Sil DMF, Tri-Sil Z, or BSA as silylating agents.

Positive proof of the identity of the gc peak for the MH-TMS derivative was obtained by the combined gas chromatograph–mass spectrometry (gc–ms) technique. The mass spectrum resulted in parent and base peaks at m/e 256 and 241, respectively. The fragmentation pattern is listed in Table I. The parent peak corresponds to the molecular weight of the bis(trimethylsilyl) derivative of maleic hydrazide. The base peak at m/e 241 results from the loss of a methyl group by the parent molecule.

The reported MH method was not an immediately devised procedure but evolved over a period of time along two avenues of approach. One approach was to perfect the gas chromatographic separation of the TMS derivative from other interfering tobacco extract constituents, while the other objective was to develop a good extraction procedure that would be rapid and selective. It may be of benefit to detail briefly the development of the method.

Initially, cigarettes with added MH were extracted with hot distilled water. The extract was concentrated and subjected to gel filtration on various substances, such as Bio-Gel P-10, P-100, Sephadex G-25, and LH-20, in efforts to fractionate the MH from other interfering substances. Fractions that exhibited uv absorbance in the 305–325 nm region, characteristic of MH, were combined, taken to dryness, and silylated. Gas chromatography (gc) on OV-11 coated columns yielded a poorly resolved peak for the MH derivative. Gc separation of the extract was improved by using Sephadex G-15 for gel filtration, and now the determination was good to about 10 ppm of MH in tobacco. Concurrently, it was shown that MH could be directly determined (down to 50 ppm) by separation of the gel filtration fractions by high-speed, high-pressure liquid chromatography (lc) on a strong anion exchange column, using a pH 8.50 borate buffer. However, this method was not further pursued due to the fact that we wanted to develop a universally applicable method and not all laboratories possess an lc instrument.

Subsequently, other extraction solvents and silylating agents were examined. Use of BSA reagent partially improved the gc separation, yielding fewer peaks. Extraction of tobacco with dimethylformamide or dioxane simplified the extraction procedure and also improved the gc separation because not as much material was being removed from the tobacco, while still extracting all of the MH. By this procedure, 1-g samples of tobacco could be analyzed for MH to a limit of 10 ppm.

The problem of overlapping gc peaks was subsequently solved by the use of larger percentages of liquid phases in the gc columns. Previously, columns with liquid phase contents of 5% or less were employed. However, the use of 20% OV-11 on Chromosorb W immensely improved the separation, with precisions of resultant MH determinations good to 0.1 ppm. It should be mentioned that numerous other liquid phases had been tried previously but with inadequate resolutions of peaks.

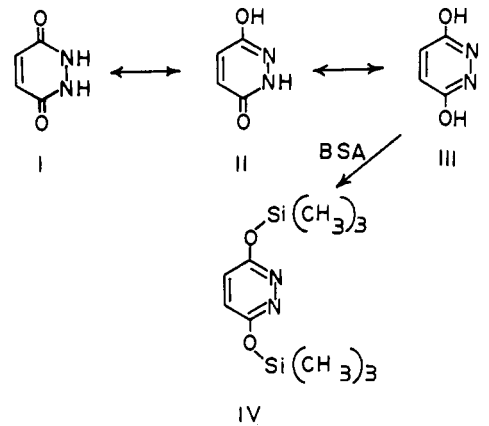

Figure 1. Conversion of MH to its TMS derivative.

Final improvement in the method was the use of the BSA reagent for the direct extraction and derivatization of MH from powdered tobacco. Using this direct BSA procedure, we performed a series of recovery studies to evaluate the accuracy of the method. Standard deviations indicated that the results (Table II) were satisfactory and that extraction by the BSA reagent was as good as with previous solvents. Optimum recoveries were obtained when the tobacco was pulverized. Recoveries of MH from hand-suckered tobacco, never treated with MH in the field, when fortified with 0.5, 1.0, 2.0, 5.0, and 10.0 ppm of MH, were 94% or better. This batch of hand-suckered nonfortified tobacco still gave a value of 23.8 ppm of MH residue. Another sample of domestic hand-suckered tobacco, obtained from a different source, gave a value of 35 ppm of MH. Repeated collection of this small gc peak through a thermal conductivity detector yielded a sample that was again confirmed by gc–ms as the bis(trimethylsilyl) deriv-

Table I. Mass Spectral Data for the MH-TMS Derivative[a]

m/e	%
256	22
255	24
243	10
242	22
241	100
169	38
147	13
113	27
100	13
99	11
98	17
73	16

[a] Only fragments over 10% of base peak are reported.

Table II. Recovery of Maleic Hydrazide Added to "Untreated" Tobacco

Added, ppm	Found, ppm	% recovery, avg
0.0 (untreated)	23.8	
0.5	24.4	108
1.0	24.9	104
2.0	24.7	94
5.0	28.8	99
10.0	33.8	99

Table III. MH Contents of Flue-Cured Tobacco and Assorted Cigarettes

Sample	MH content, ppm
Untreated, hand-suckered flue-cured tobaccos	24, 35
MH-treated flue-cured tobaccos	136, 176
Experimental cigarettes, University of Kentucky, type 1-R-1	103
Experimental cigarettes, University of Kentucky, type 1-A-4	120
Havana cigars, 1959	5
Commercial cigarettes:	
85 mm, filter, Japan	88
85 mm, filter, U. S.	98
85 mm, filter, U. S.	113
85 mm, filter, U. S.	79
85 mm, filter, U. S.	68
85 mm, filter, U. S.	133
85 mm, nonfilter, U. S.	61
100 mm, filter, U. S.	119

ative of maleic hydrazide. Apparently, MH from treatments in previous years remains in the soil and is absorbed by the tobacco plant. Large quantities of MH may enter the soil. Generally, MH is applied to tobacco in the field at a rate of about 6 pints of 30% MH per acre. Some overzealous farmers apply much more. For flue-cured tobacco the average yield is 2000 lb/acre. If all of the MH were to remain on the tobacco and not be degraded, this would amount to about 900 ppm of MH. Obviously, a significant portion of the MH will find its way into the soil and even with some degradation there still remains a sufficient amount in the soil to be absorbed by subsequent crops. There is no literature to substantiate this point, but it is certainly in need of investigation.

This method was applied to the determination of MH in various tobaccos and in commercial and experimental cigarettes. The results are summarized in Table III. MH contents of commercial cigarettes and experimental cigarettes, designed to simulate commercial cigarettes, fall in the range of 60–120 ppm. The value for flue-cured tobacco was quite representative. Other batches of hand-suckered tobacco gave values similar to the domestic types. The lowest value found was for a brand of 1959 Havana cigars, a surprisingly low 5 ppm.

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COMMUNICATIONS

The Lipids of Mono Lake, California, Brine Shrimp (*Artemia salina*)

Lipids were extracted from Mono Lake freeze-dried brine shrimp (*Artemia salina*) by chloroform–methanol (2:1) and examined by standard procedures for fatty acid composition and other parameters.

Mono Lake is alkaline (pH 9–10) and contains about twice as much dissolved solids as the ocean. The 7.4% total salts are accounted for mostly as ions of sodium, chloride, and bicarbonate (Mason, 1967). A species of brine shrimp that flourishes seasonally in this lake is harvested in ton lots mostly for aquaria feed (Whitney, 1967). Brine shrimp are of interest not only because they seem to be uniquely useful for feeding all types of aquaria fish but, in addition, they show promise in the feeding of lobsters (*Homarus americanus*) (Shleser and Gallegher, 1974) and other species (Wickins, 1972) that might be adapted to aquaculture. They also furnish an example of how the many underutilized salt lakes of the world might be used for the production of high quality protein for food and feed.

Previous workers (Bowen, 1964; Mason, 1967) have indicated that the brine shrimp of Mono Lake are physiologically and morphologically distinct from those that occur in Great Salt Lake or San Francisco Bay. We report some observations on the composition of the lipids of Mono Lake brine shrimp.

EXPERIMENTAL SECTION

Freeze-dried brine shrimp were received from the California Koi Co. at ambient temperature and stored at −18° pending analysis. Protein, fat, fiber, moisture, and ash were assayed by Ralston Purina Laboratories, St. Louis, Mo.

In this laboratory, moisture was determined by a standard AOAC method (1970a) with the following modification: the drying period was interrupted after 30 min in order to break up the caked powder with a glass rod, the powder adhering to the rod was brushed back into the container used for drying and weighing, and the drying was resumed.

Lipids were extracted from weighed portions of the dried brine shrimp in large Soxhlet extractors with chloroform–methanol 2:1 (Medwadowski *et al.*, 1971). A slow stream of nitrogen was passed through the solvent during the extraction periods to minimize oxidation. The lipid extracts were purified by Sephadex G-25 chromatography, and yields of extractable lipid were determined by drying and weighing aliquots (Smith *et al.*, 1973).

Activated Mallinckrodt Silica AR CC-7, 100–200 mesh columns were used to separate fractions containing (a) mainly neutral lipids and free fatty acids, (b) sphingolipids, and (c) phospholipids (Medwadowski *et al.*, 1967).

Preliminary results for unsaponifiables determined with a standard AOAC method (1970b) were high and irreproducible. Thin-layer chromatography indicated the presence of significant amounts of esters that had escaped sa-