Journal of Chromatography, 595 (1992) 346–350
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 964

Short Communication

Determination of maleic hydrazide in tobacco by micellar liquid chromatography

S. S. Yang

Research Center, Philip Morris USA, P.O. Box 26583, Richmond, VA 23261-6583 (USA)

(First received November 13th, 1991; revised manuscript received December 18th, 1991)

ABSTRACT

A reversed-phase high-performance liquid chromatographic method with UV detection was developed to determine maleic hydrazide (MH) residues in tobacco. Sample preparation consisted of an initial hydrolysis of MH residues with 12 *M* hydrochloric acid by sonication at an elevated temperature, followed by neutralization with an aqueous sodium hydroxide solution. Chromatographic separation was performed on a C_{18} column with a mobile phase containing 4.0 m*M* cetyltrimethylammonium bromide in 40 m*M* phosphate buffer (pH = 7.0). Other sample preparation procedures were investigated including extraction/digestion with the aid of different energy sources (*e.g.*, conventional heater and microwave oven) and these results are also discussed.

INTRODUCTION

Maleic hydrazide (MH) is a synthetic plant growth regulator widely used in tobacco farming as a suckering control agent. The normal practice is to apply MH to the upper half of tobacco plants shortly after topping. The applied MH then gradually translocates to other parts of the plant. The fate and metabolism of MH in tobacco plants has been investigated using foliar-absorbed ^{14}C-labelled MH [1]. This study showed that MH and its metabolites translocated to actively growing tissues of the whole plant and some of these compounds are extractable with methanol. The major component in the methanol extract was identified as a β-D-glucoside of MH. A review article published in 1987 [2] discussed in detail the absorption, translocation and metabolism of MH in tobacco plants.

A number of analytical procedures for the determination of MH residues in tobacco or tobacco products have been published. The distillation–spectrophotometric method and its various modifications [3–6] have been widely used. In these procedures MH residues are reductively hydrolyzed to hydrazine by zinc in a concentrated alkali solution. The evolved hydrazine is subsequently derivatized with *p*-dimethylaminobenzaldehyde, and measured spectrophotometrically at 455 nm. A gas chromatographic method consisting of hydrolysis of MH residues in 2 *M* hydrochloric acid at 90–95°C and derivatization with N,O-bis(trimethylsilyl)acetamide has also been reported [7]. In addition, an immunoassay method using a monoclonal antibody-based enzyme for the analysis of MH was described in 1989 [8].

We describe here a reversed-phase high-performance liquid chromatographic (HPLC) procedure for the determination of MH residues in tobacco and tobacco products. The sample preparation involves hydrolysis of MH residues in 12 *M* hydrochloric acid, followed by neutralization with a sodium hydroxide solution after cooling the sample in a cold

0021-9673/92/$05.00

water bath. Several other sample preparation procedures were also evaluated.

EXPERIMENTAL

Reagents

Maleic hydrazide and cetyltrimethylammonium bromide (CTAB) were purchased from Aldrich and were used as received. β-D-Glucosidase was obtained from Sigma. All water used was treated by a Milli-Q system to provide organic-free, 18 mΩ grade water.

Apparatus

The instrument used consisted of a Hewlett-Packard 1090L high-performance liquid chromatograph equipped with a UV–VIS photodiode array detector, an autosampler, and a Hewlett-Packard 9000 LC workstation. The wavelength monitored for quantitation was 330 nm. The analytical column used was a Hewlett-Packard Hypersil ODS column (5 μm particle size, 20 cm × 4.6 mm I.D.). The injection volume was 5 μl. The column was maintained at ambient temperature. The mobile phase consisted of two solutions: solution A was 2.5 m*M* CTAB in 40 m*M* phosphate buffer (pH 7.0); solution B was 7.5 m*M* CTAB in 40 m*M* phosphate buffer (pH 7.0). The initial composition was 30% of solution B in A and was maintained for 4 min. Solution B was then raised to 90% in 0.5 min and kept for another 4 min before changing back to the initial 30% composition. The HPLC run was stopped after 20 min.

Sample preparation

A 0.25-g portion of ground tobacco was placed in a 30-ml glass vial with a PTFE-lined cap. After adding 2 ml of 12 *M* hydrochloric acid, the vial was capped, placed in an ultrasonic bath (Branson, Model 3200) containing hot water at a temperature of approximately 60°C or above and sonicated for 40 min (the sonication and the allowing neutralization steps should be performed under a working hood equipped with a sliding glass shield). The sample was then cooled in a cold water bath. Two 1-ml aliquots of 12 *M* sodium hydroxide were sequentially added to the sample which remained in the cold water bath with the vial cap, or a small watch glass, pressed on lightly (to vent pressure) during the whole neutralization process. After the addition of each 1-ml aliquot of sodium hydroxide solution, several minutes were allowed for heat dissipation. An aliquot of approximately 1 ml of the neutralized sample was then filtered through a 0.45-μm disposable filter into an autosampler vial for HPLC analysis.

Other sample preparation procedures

Water extraction. Ground tobacco (1.0 g) was placed in a 50-ml flask and 10 ml of water were added. The mixture was shaken on an orbital shaker for 1 h. An aliquot of approximately 1 ml of the extract was filtered and sealed in a sample vial.

Water extraction/enzyme hydrolysis. Ground tobacco was extracted and filtered as under *Water extraction*. Approximately 2 ml of the filtrate were transferred into a test tube and 10 mg of β-D-glucosidase were added. The test tube was immersed in a 37°C water bath for 4 h with occasional shaking by hand. A portion of the sample was then filtered and used for HPLC analysis.

NaOH digestion/reflux. A 5-g amount of ground tobacco were mixed with 25 ml of 10 *M* sodium hydroxide solution in a 150-ml round-bottom flask and the mixture was refluxed for 3 h. After refluxing, the condensor was removed and the round-bottom flask was placed in a cold water bath under a working hood equipped with a glass shield. A total volume of 50 ml of 5 *M* hydrochloric acid solution was slowly added to the sample flask, which remained in the cold water bath and was covered with a watch glass during the neutralization process. Approximately 1 ml of the neutralized sample solution was filtered and sealed into a sample vial.

NaOH digestion/microwave irradiation. A ground tobacco sample of 0.1 g was weighed into a PTFE-lined microwave digestion vessel equipped with a safety disk for high pressure venting (CEM, Matthews, NC, USA) and 3 ml of 10 *M* sodium hydroxide solution were added. After the vessel cap was hand-tightened, the whole assembly was placed in a MDS-81 microwave oven. The sample mixture was subjected to three 6-min cycles, each consisting of 3 min of microwave irradiation at 15% power followed by 3 min fan-cooling, for a total treatment time of 18 min. After the sample cooled and the internal pressure was released, the vessel was carefully opened and 3 ml of 10 *M* hydrochloric acid

solution were slowly added following the procedures described under *Sample preparation*. An aliquot of neutralized sample solution was filtered and sealed into an HPLC autosampler vial.

Standards and addition plot

A stock solution of MH was prepared by weighing 100 mg of MH into a 100-ml volumetric flask and diluting to volume with water (nominally 1 mg MH/g of solution). This stock solution was used for both preparation of calibration standards and recovery studies.

Spiked samples were prepared by weighing 0.25 g of ground, MH-free tobacco into each of five 25-ml glass vials and weighing 50, 37.5, 25, 12.5 and 5 mg of the stock solution into each vial (nominally 200, 150, 100, 50 and 20 μg of MH per g of tobacco), respectively. After waiting for 1 h, each spiked sample was treated by the procedure described under *Sample preparation*. Data obtained from HPLC analysis were used to establish an addition plot using peak area *vs.* amounts of MH in tobacco (ppm). Calculation was based on external standards using the addition plot.

RESULTS AND DISCUSSION

Due to the high solubility of MH in water and alcohol, an initial attempt using water and/or methanol as an extracting solution was made. However, only a small amount of MH, *e.g.*, 40–50 ppm, was observed in a sample (No. 9), which contained 210 ppm of MH as previously determined by a method [6] from the International Organization for Standardization (ISO) (see Table I). The MH determined from a water/methanol extraction would account only for the free form of MH and not for any conjugated MH. It has been known that MH, after being sprayed on crops, can be metabolized to glucosides, *e.g.*, mostly β-D-MH-glucoside in potato [9]. As such, an enzyme-catalyzed hydrolysis using β-D-glucosidase was performed on the extract after the tobacco sample was extracted with water. The amount of MH measured increased to 80–90 ppm, which obviously was a combined contribution from free MH and hydrolyzed β-D-MH-glucoside.

In the published methods, total MH was obtained using strong acid or base to hydrolyze the bound or conjugate form of MH. MH residues were first hydrolyzed in concentrated sodium hydroxide or hydrochloric acid solutions with heating. MH was then either converted into hydrazine [3–6] in the presence of a catalyst or derivatized for subsequent chromatographic analysis [7], except in one case where MH was directly quantitated using ion chromatography [10]. In our study, a 3-h reflux with 10 *M* sodium hydroxide was required for the hydrolysis of MH residues. Although such a procedure produced data comparable to the ISO method, other less time-consuming sample preparation techniques were investigated.

One means for reducing sample preparation time involved the use of microwave digestion with closed vessels. Microwave irradiation has been used in elemental analysis to aid acid digestion. In our study, however, digestion of organic material (*e.g.*, ground tobacco) mixed with a concentrated sodium hydroxide solution was cautiously attempted. Small samples, generally less than 0.1 g tobacco mixed with 3 ml of 10 *M* sodium hydroxide solution, were irradiated at low power levels. The results obtained using this method were similar to those obtained from the ISO method. However, sparks and arcing were observed during the irradiation process and as a result, corrosion-like, damaged areas were found

TABLE I

COMPARISON OF DIFFERENT METHODS FOR DETERMINING MH RESIDUES

Samples are Bright tobacco.

Sample	MH residues (ppm)		
	ISO[a]	LC-1[b]	LC-2[c]
1	110	118	105
2	78	75	90
3	97	105	96
4	158	144	145
5	112	107	117
6	49	54	38
7	104	97	97
8	97	93	87
9	210	220	209
10	29	22	18

[a] ISO = International Organization for Standardization (ISO) official method.
[b] LC-1 = NaOH digestion with 3 h reflux + HPLC.
[c] LC-2 = HCl digestion with sonication + HPLC.

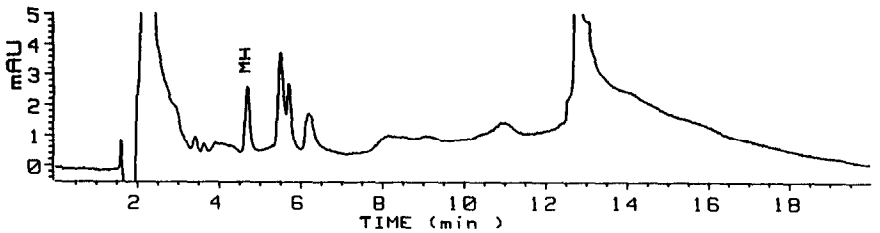

Fig. 1. Chromatogram of a Bright tobacco sample at a 80 ppm level of maleic hydrazide. Column, Hewlett-Packard Hypersil ODS, 200 mm × 4.6 mm I.D.; detection, UV 330 nm; injection volume, 5 μl; column temperature, ambient; mobile phase, cetyltrimethylammonium bromide in phosphate buffer (pH 7.0); flow-rate, 0.8 ml/min. Details in text.

on the inner wall of the PTFE-lined vessels. A possible explanation is that the large quantity of energy from microwave irradiation causes rapid evaporation of water molecules and leads to the desolvation of some sodium hydroxide, especially those around the edge of solution surface. These sodium hydroxide crystals interacted with microwave energy and created "hot spots", where damage occurred.

Another approach to reduce sample preparation time was the use of sonication as an external energy source. Ground tobacco samples mixed with concentrated hydrochloric acid were sonicated in a water bath at or above 60°C. Although more than 70% of the MH residues can be extracted in the first 20 min, a sonication time of 40 min is necessary to obtain a satisfactory recovery. After sonication, the yellow-brown tobacco sample had turned into a dark slurry. The slurry solution was neutralized by slow addition of sodium hydroxide solution. Vapor loss during acid–base reaction was minimized by using a cold water bath and a watch glass to enhance vapor condensation. The total sample loss from vaporization was usually less than 1% (w/w). As shown in Table I, the results obtained using this sample preparation procedure were consistent with those from the ISO method and the more rigorous basic reflux sample preparation.

Fig. 1 is a representative chromatogram obtained from a tobacco sample containing 80 ppm MH. Chromatographic separation was performed on a C_{18} column. MH was eluted isocratically from the column at 4.7 min by a mobile phase containing (CTAB) in phosphate buffer (pH 7.0). The concentration of CTAB was initially 4.0 m*M* for the elution of MH and was then increased to 7.0 m*M* to speed the elution of strongly retained compounds. As the concentration of the surfactant in the mobile phase is well above its critical micellar concentration (cmc = 1.3 m*M* for CTAB), there is no change in the amount of surfactants adsorbed on the stationary phase regardless of the change in the total surfactant concentration [11]. This makes it possible to change mobile phase composition or even perform gradient elutions without time-consuming column re-equilibration between injections.

Adequate buffer strength is important for peak shape and sensitivity. Initially a buffer of 10 m*M* phosphate was used. This buffer gave reasonable peak shape for a small volume of standard compound (Fig. 2a), but distorted peaks and hence poor detection limits were observed for tobacco samples

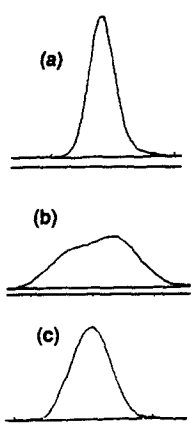

Fig. 2. Peak shape of MH in chromatograms. Chromatographic conditions as in Fig. 1 except buffer strength in the mobile phase. (a) Standard solution of MH, 10 m*M* phosphate buffer; (b) tobacco sample, 10 m*M* phosphate buffer; (c) tobacco sample, 40 m*M* phosphate buffer.

TABLE II

REPLICATE ANALYSES OF TWO BRIGHT TOBACCO SAMPLES

Run No.	MH residues (ppm)	
	Sample 4	Sample 9
1	148	210
2	150	203
3	150	215
4	141	207
5	149	210
Average	144	209
R.S.D. (%)	4	2

TABLE III

RECOVERIES OF MALEIC HYDRAZIDE SPIKED INTO TOBACCO SAMPLES

Amount added (μg/g)	Amount found (μg/g)	Recovery (%)
29	27	92
66	68	103
103	98	95
194	180	93
241	228	95
249	238	95
Average recovery		95

as shown in Fig. 2b. A possible source for this peak distortion could be the presence of a high concentration of sodium chloride (*e.g.*, approximately 5–6 *M*), produced from the acid–base neutralization, which caused slower solute diffusion in the injected sample solution than in the bulk mobile phase. This was further confirmed by obtaining a similarly distorted peak from a standard solution of MH which was 6 *M* in NaCl. The peak shape of MH in tobacco samples was improved by using a stronger buffer of 40 m*M* phosphate solution (Fig. 2c). This stronger buffer capacity also greatly reduced other possible causes of peak distortion, one of which may be that the pH of the 'neutralized' tobacco solution may not be neutral, pH 7 ± 0.5.

This procedure proved to provide the degree of precision and accuracy needed for the routine determination of MH in tobacco. The relative standard deviation from five replicate analyses of two samples, Nos. 4 and 9, were 4% and 2%, respectively (Table II). Recoveries of MH from tobacco samples are listed in Table III and the average recovery was 95%. The detection limit was 20 μg/g for tobacco samples. The standard addition plot in the range 29–249 ppm gives a slope of 0.189 with an intercept of 0.827 and a correlation coefficient of 0.998.

CONCLUSIONS

The reported procedure provides a method for the determination of MH in tobacco. Sample preparation is straightforward and the use of a micellar mobile phase for the HPLC analysis gives good reproducibility. This method has been tested on a variety of tobacco samples including Burley, Bright and Oriental tobaccos and their products, with satisfactory results.

ACKNOWLEDGEMENTS

The author would like to thank his senior colleagues, Mr. D. Davis, Dr. D. Ingraham, Mr. W. Ryan and Mr. D. Watson for their helpful discussion.

REFERENCES

1 D. S. Frear and H. R. Swanson, *J. Agric. Food Chem*, 26 (1978) 660.
2 S. A. Meyer, T. J. Sheets and H. Seltmann, *Rev. Environ. Contam. Toxicol.*, 98 (1987) 43.
3 J. R. Lane, D. K. Gullstrom and J. E. Newell, *J. Agric. Food Chem.*, 6 (1958) 671.
4 M. Ihnat, R. J. Westerby and I. Hoffmann, *J. Assoc. Off. Anal. Chem.*, 56 (1973) 1164.
5 *Official Methods of Analysis*, Vol. 13, Association of Official Analytical Chemists, Arlington, VA, 1980, p. 1018.
6 *ISO Method No. 4867*, Internation Organization for Standardization, Paris, 1980.
7 A. F. Haeberer and O. T. Chortyk, *J. Assoc. Off. Anal. Chem.*, 62 (1979) 171.
8 R. O. Harrison, A. A. Brimfield and J. O. Nelson, *J. Agric. Food Chem.*, 37 (1989) 958.
9 W. H. Newsome, *J. Agric. Food Chem.*, 28 (1980) 1312–1313.
10 W. H. Newsome, *J. Agric. Food Chem.*, 28 (1980) 270–272.
11 J. G. Dorsey, M. G. Khaledi, J. S. Landy and J. L. Lin, *J. Chromatogr.*, 316 (1984) 183–191.