Changes in Chemical Composition of Burley Tobacco during Senescence and Curing. 3. Tobacco-Specific Nitrosamines

Harold R. Burton,* George H. Childs, Jr., Roger A. Andersen, and Pierce D. Fleming

Burley tobacco was harvested at three stages of maturity and cured in two curing environments (24 °C/70% RH, 32 °C/83% RH). Tobacco samples taken throughout the curing process were analyzed for *N'*-nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), nitrate nitrogen, and nitrite nitrogen. There was a positive correlation between nitrite nitrogen and nitrosamines when cured in a normal environment (24 °C/70% RH). Curing tobacco at a higher temperature and humidity (32 °C/83% RH) dramatically increased the accumulation of individual nitrosamines and nitrite. Again, a significant, positive correlation was observed between tobacco-specific nitrosamines (TSNA) and nitrite.

The detection of tobacco-specific nitrosamines (TSNA) in cured tobacco led to several studies to identify the precursors of these nitrosamines and conditions required for their accumulation. Brunnemann et al. (1983) indicated that a positive correlation existed between nitrate content of the leaf and TSNA. Studies by Djordjevic et al. (1985, 1987) and MacKown (1988) indicated that a positive correlation existed between *N'*-nitrosonornicotine (NNN) and nornicotine and *N'*-nitrosoanatabine (NAT) and anatabine. Parsons et al. (1986) indicated that on the homogenized leaf curing of burley tobacco (HLC) there was reduction of nitrate to nitrite. In an independent study on burley tobacco Andersen and Kemp (1985) showed that the increase of nitrite during HLC was accompanied by an increase of NNN. It was shown that TSNA increases during air-curing of burley tobacco (Djordjevic et al., 1985, 1987; Andersen et al., 1987); however, it is not known whether there is a specific time during curing when TSNA accumulation is optimal. Therefore, this study was initiated to determine if plant maturity and/or curing environment influenced the accumulation of TSNA and nitrite and when TSNA and nitrite accumulation occurred during curing.

EXPERIMENTAL SECTION

Nicotiana tabacum L. cv. Ky 14 was grown at the Kentucky Agricultural Experiment Station farm in 1985 with standard agronomic practices for burley tobacco production. Tobacco was harvested 1, 4, and 7 weeks after topping. The tobacco plants were cut and placed on sticks (6 plants/stick) and cured (18 sticks, 108 plants/chamber) in two controlled environmental chambers. The chambers were maintained at 24 °C/70% RH and 32 °C/83% RH, respectively. These conditions effected identical moisture losses from the tobacco lamina during curing (Walton et al., 1982). Three replicate samples (3 leaves/plant and 3 plants/replicate) were taken at 0, 1, 2, 3, 5, 7, 9, 12, 14, 16, 19, and 21 days after harvest from the top one-third of the plant. It should be noted each plant was sampled once. The samples were separated into lamina and midvein, weighed, freeze-dried, reweighed, ground to pass a 40-mesh screen, and stored at −40 °C until analyses.

Chemical Analyses. Nitrate was determined by *Escherichia coli* reduction as described by Lowe and Gillespie (1975). Nitrite was determined by the same procedure that was similar to that for nitrate except the *E. coli* was omitted and the procedure was modified as follows. One-gram samples were weighed into 25 × 200 mm screw-cap test tubes and extracted by shaking with 25 mL of water for 1 h on a reciprocal shaker. Samples were then filtered through Whatman No. 1 filter paper and a 10-mL aliquot was decolorized with 1.0 g of Norit A activated charcoal, by reciprocal shaking for 10 min. The samples were then filtered through Whatman No. 42 filter paper and analyzed colorimetrically for nitrite on a Technicon autoanalyzer system II. The manifold assembly described by Lowe and Gillespie (1975) was modified to achieve greater sensitivity by increasing the sample tube size (0.42 mL/min), decreasing the diluent tube size (0.60 mL/min), and substituting a 50-mm flow cell in the colorimeter. Also the delay coil in the heating bath was bypassed to decrease the system retention time.

Calcium was quantified by atomic absorption spectrometry of 8:1 perchloric acid–nitric acid digests of the tobacco samples.

Analyses of Tobacco-Specific Nitrosamines. *N'*-Nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT), and 4-(*N*-methyl-*N*-nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) were analyzed by capillary GC using a nitrogen-specific detector as previously described (Andersen and Kemp, 1985). Their identities were confirmed by GC–MS using a Finnigan 700 ion trap detector interfaced with a Varian 3700 GC equipped with an on-column injector. Recovery–response factors were determined for each authentic nitrosamine, and azobenzene was used as the internal standard.

RESULTS AND DISCUSSION

Influence of Plant Maturity and 24 °C/70% RH Curing Conditions on Accumulation of TSNA. Data for the accumulation of tobacco-specific nitrosamines (TSNA) during air-curing at 24 °C/70% RH are presented in Table I. Results from three harvest dates are presented in order to show that plant maturity influenced the accumulation of nitrosamines. Since these tobaccos were cured in an environmentally controlled curing chamber, differences in nitrosamine accumulation should be due to plant maturity and not due to differences of the curing environments.

Immature tobacco, harvested 1 week after topping, contained low concentrations of NNN (Table I): however, there was a 5-fold increase in NNN between harvest and final sampling (21 days). The most significant increase of NNN occurred between the 14th and 16th day after harvest. This increase occurred at the end of yellowing,

Department of Agronomy (H.R.B., G.H.C.) and USDA—ARS, MSA (R.A.A., P.D.F.), University of Kentucky, Lexington, Kentucky 40546.

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Table I. Changes of Tobacco-Specific Nitrosamines, Nitrate, and Nitrite during Air-Curing (24 °C/70% RH) of Burley Tobacco Harvested at Three Stages of Maturity

day	NNN/μg/g	NAT/μg/g	NNK/μg/g	total/μg/g	NO_3 N/mg/g	NO_2 N/μg/g
			Harvest 1[a]			
0	0.40	1.69	0.80	2.16	4.33	0.64
1	1.33	1.37	1.17	3.87	6.14	0.72
2	0.83	1.17	0.07	2.07	5.73	0.63
3	0.72	0.48	0.08	1.28	6.19	0.43
5	0.54	6.34	0.18	7.06	5.93	0.60
7	0.94	1.61	0.16	2.71	6.78	0.54
9	0.54	0.69	0.06	1.29	6.34	0.37
12	0.75	2.40	0.40	3.55	7.01	0.86
14	0.65	5.79	0.13	6.58	7.29	0.43
16	1.99	4.56	0.37	6.92	8.43	1.11
19	1.32	3.35	0.32	4.99	9.21	2.35
21	2.06	3.31	0.47	5.84	8.61	7.38
LSD (05)	0.40	1.19	0.18	1.84	1.36	1.92
			Harvest 2[b]			
0	0.43	1.98	0.13	2.54	4.42	0.62
1	1.40	1.77	0.10	3.27	3.13	0.73
2	1.12	0.86	0.20	2.18	4.26	0.55
3	1.62	1.17		2.79	4.23	0.38
5	1.58	3.65	0.20	5.44	3.78	2.50
7	2.10	4.98	0.45	7.53	4.39	0.83
9	2.17	3.50	0.25	5.92	4.61	1.05
12	2.63	8.76	0.91	12.30	3.87	3.49
14	3.21	8.39	0.49	12.09	5.21	3.42
16	2.25	3.55	0.13	5.93	3.29	1.68
19	5.45	9.82	0.54	15.81	4.12	5.08
21	3.51	5.63	0.17	9.31	3.99	1.39
LSD (05)	2.05	2.79	0.43	4.30	0.86	1.49
			Harvest 3[c]			
0	0.56	2.14	0.10	2.80	1.15	0.47
1	3.03	4.35	0.50	7.88	2.67	2.16
2	2.39	3.67	0.21	6.27	2.96	0.74
3	2.75	3.48	0.13	2.88	3.61	0.71
5	2.80	4.18	0.42	7.40	3.81	1.53
7	2.58	3.71	0.62	6.73	4.54	1.19
9	1.91	2.28	0.37	4.56	4.14	1.11
12	2.89	3.29	0.49	6.66	3.71	1.47
14	4.74	4.31	0.61	9.66	4.03	2.63
16	4.64	6.83	0.31	11.79	4.58	3.85
19	3.79	7.00	0.54	11.33	3.74	4.73
21	3.38	5.69	0.41	9.47	4.26	2.43
LSD (05)	2.45	4.15	0.46	6.38	1.19	3.17

[a] Harvested 1 week after topping. [b] Harvested 4 weeks after topping. [c] Harvested 7 weeks after topping.

an event corresponding to the time when 98% of the chlorophyll was degraded in lamina and lamina leaf color had changed from yellow to brown (Burton et al., 1983, 1985; Tso, 1972). For tobacco harvested at 4 weeks after topping (harvest 2, recommended harvest time), the increase of NNN was significant by the 12th day after harvest. There was a gradual increase of NNN from harvest until the 9th day; however, this increase was not statistically significant. Again, the higher levels of NNN that accumulated by the 14th day occurred after yellowing and during browning of the lamina.

There was a significant increase of NNN 1 day after harvest for the overmature tobacco (harvest 3). Concentrations of NNN remained high throughout the air-curing process, indicating the accumulation of NNN occurred at earlier stages of curing in comparison to the other harvests. The level of NNN increased during the latter stages of curing, but when adjusted for dry-matter loss (Burton and Crutchfield, 1988), there was no increase in the concentration of the TSNA. Results from this study showed that plant maturity influenced accumulation of *N'*-nitrosonornicotine and the increase in NNN occurred within 1 day after harvest for the overmature tobacco. This latter observation would indicate that a significant increase occurred in the tobacco before desiccation of the lamina (Burton and Kasperbauer, 1985) (Figure 1).

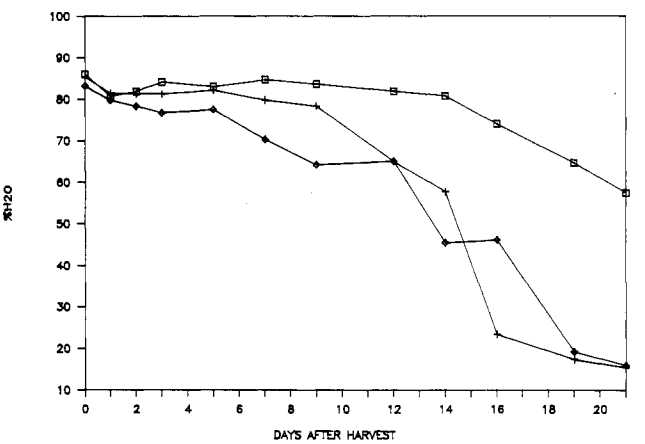

Figure 1. Moisture content in lamina during curing at 24 °C/70% RH: □, harvested 1 week after topping; +, harvested 4 weeks after topping; ◇, harvested 7 weeks after topping.

The accumulation of *N'*-nitrosoanatabine (NAT) during air-curing paralleled the accumulation of NNN. This would be predicted since anatabine, the probable precursor for NAT, is present in these tobacco samples. However, for almost every sampling date, NAT concentration was greater than the NNN level. This is of interest since initial

Table II. Changes of Tobacco-Specific Nitrosamines, Nitrate, and Nitrite during Air-Curing (32 °C/83% RH) of Burley Tobacco Harvested at Three Stages of Maturity

day	NNN/μg/g	NAT/μg/g	NNK/μg/g	total/μg/g	NO_3 N/mg/g	NO_2 N/mg/g
			Harvest 1[a]			
0	0.40	1.69	0.08	2.16	4.33	<0.01
1	1.33	1.37	1.17	3.87	6.14	<0.01
7	0.33	12.72	0.14	13.19	6.71	0.02
12	1.72	3.13	0.77	5.63	6.07	0.03
14	4.09	18.48	1.77	24.33	7.01	0.03
16	12.19	36.65	3.85	52.69	7.77	0.05
19	54.17	333.18	115.69	503.04	7.90	0.93
21	88.33	298.48	118.90	505.72	8.18	1.00
LSD (0.5)	51.94	123.96	61.38	345.94	1.44	0.37
			Harvest 2[b]			
0	0.43	1.98	0.13	2.54	4.42	<0.01
1	1.40	1.77	0.10	3.27	3.13	<0.01
7	4.79	34.30	3.78	42.88	4.71	0.03
12	3.52	18.60	2.70	24.81	4.50	0.05
14	22.40	66.33	11.91	100.64	5.55	0.11
16	30.98	100.75	21.33	153.06	5.98	0.63
19	221.96	545.49	145.46	912.91	5.03	1.00
21	21.57	153.48	28.77	203.81	3.96	0.51
LSD (05)	70.58	79.60	24.78	105.74	1.38	0.35
			Harvest 3[c]			
0	0.56	2.14	0.10	2.80	1.15	<0.01
1	3.03	4.35	0.50	7.88	2.67	<0.01
7	2.94	20.32	0.90	24.16	3.97	0.02
12	32.28	90.75	1.01	133.77	4.63	0.03
14	12.21	32.83	4.40	49.44	4.98	0.07
16	41.75	78.09	6.33	133.58	5.88	0.36
19	20.41	95.52	10.79	126.71	5.93	0.29
21	71.04	145.74	18.24	235.02	7.25	0.25
LSD (05)	42.43	63.57	11.86	107.88	1.41	0.18

[a] Harvested 1 week after topping. [b] Harvested 4 weeks after topping. [c] Harvested 7 weeks after topping.

results indicated that there was the same specificity for the accumulation of NAT as there was NNN. Plant maturity also influenced the accumulation of this nitrosamine. The immature cured tobacco contained the lowest level of NAT whereas the mature (4 weeks) and overmature (7 weeks) cured tobacco contained the highest levels of NAT. For tobacco harvested 4 weeks after topping, there was a significant increase of NAT between days 9 and 12 after harvest. This increase corresponded to the moisture in the lamina (Figure 1). This moisture loss suggested loss of cell integrity (Burton et al., 1983), and loss of cell integrity would allow for invasion of exogenous microbes into the dying tobacco cells. The microbes may be indirectly responsible for the formation of the nitrosamines, since some of the identified microbes on tobacco are nitrate-reducing organisms (Parsons et al., 1986; Douglass et al., 1978).

When tobacco was harvested at 7 weeks after topping, NAT increased 1 day after harvest but the increase was not significant until 16 days after harvest, in comparison to the harvest date.

The mean values for NNK throughout curing, except for one case, were less than 1.0 μg/g. Even though there were some statistically significant values, there were no trends between the NNK content and the different stages of curing. It was evident that, under controlled curing at 24 °C/70% RH, there was no significant increase in the concentration of this biologically active nitrosamine.

Summation of the concentration of each individual tobacco-specific nitrosamine (NNN, NAT, NNK) reflected the net accumulation of these nitrosamines during curing. Over 90% of the TSNA accumulation was due to increases of the NNN and NAT content during the curing process (Table I). For the first two harvests, increases occurred during the third week of curing. TSNA was also highest during the third week of curing in the overmature tobacco (harvest 3). Using a general linear model for comparing data from these three harvests (data not shown) indicates that NNN, NAT, and total are significantly lower for the immature tobacco. This shows that time of harvest influenced the concentration of nitrosamines that accumulated in the lamina.

Data from these curing studies showed there was an increase of TSNA during curing, but there is no explanation for these observed increases. It was assumed that as nitrosamines increase, there also should be an increase of nitrite levels in the lamina during the curing process. This is based on the premise that nitrite is required for nitrosamine formation (Mirvish, 1975; Douglass et al., 1978). Because of low concentrations of nitrite in air-cured burley tobacco (<10 μg/g), an analytical procedure was developed to determine nitrite concentration between 0.040 and 10 μg/g (Burton and Crutchfield, 1988). By using this procedure, it was possible to determine more precisely the concentration of low levels of nitrite in tobacco from harvest through air-curing.

Values for nitrite concentration are presented in Table I along with the nitrate concentrations. Nitrate values were included to show that they were not correlated with nitrite concentrations or the tobacco-specific nitrosamines. The mean nitrite concentrations from all harvest dates range from 0.4 to 7.38 μg/g, which is an over 18-fold difference in nitrite concentration in the tobacco lamina. For all three harvests, the nitrite concentrations were highest during the third week of curing. Generally when there were higher concentrations of nitrite, there were correspondingly higher concentrations of the nitrosamines. Correlation coefficients between nitrite and the individual nitrosamines in Table I show that there were significant and positive correlations between nitrite and all individual nitrosamines at the 99% confidence level. Correlation coefficients between nitrite and NNN, NAT, NNK, and total TSNA were 0.60, 0.56, 0.44, and 0.49, respectively.

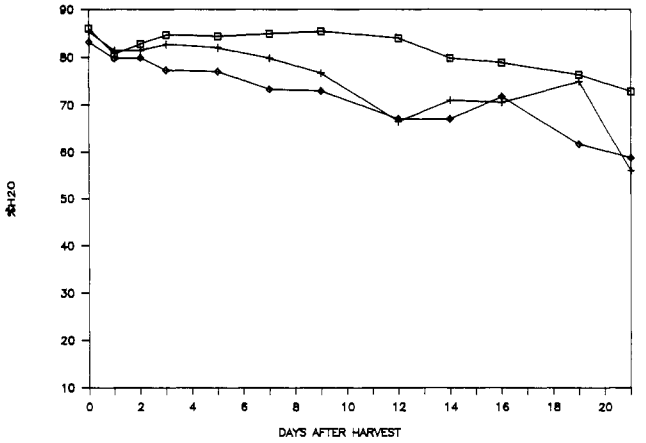

Figure 2. Moisture content in lamina during curing at 32 °C/83% RH: □, harvested 1 week after topping; +, harvested 4 weeks after topping; ◇, harvested 7 weeks after topping.

These data showed that, under an environment considered ideal for curing burley tobacco, nitrite concentration was important for the accumulation of tobacco-specific nitrosamines. It should be noted again there was no significant correlation between nitrate and the individual as total nitrosamine levels.

Influence of Plant Maturity and 32 °C/83% RH Curing Conditions on Accumulation of TSNA. Another aspect of this study was to determine whether increased temperature and relative humidity influenced the accumulation of tobacco-specific nitrosamines and nitrite. Results for TSNA, nitrite, and nitrate from burley tobacco harvested at three stages of maturity and cured at 32 °C/83% RH are presented in Table II. Individual TSNAs were low at harvest and increased significantly during the later stages of curing. During curing of tobacco at the higher temperatures and relative humidities, the total TSNA concentration approached 1 mg/g. This was approximately a 400-fold increase of the nitrosamine concentration during the curing process. Even though these high TSNA values did not occur for conventionally air-cured burley tobacco, these data indicated that if higher temperature and relative humidities were maintained during the latter stages of curing, the resulting tobacco could contain significantly higher levels of nitrosamines. Multivariate analyses of the data in Table I and II showed (data not presented) there is a statistically significant influence of temperature/relative humidity on the accumulation of all individual nitrosamines.

The increase of nitrosamines in tobacco was most likely due to the high levels of nitrite that accumulated during the curing process. For the first and second harvests, there was 100-fold increase of nitrite concentration. This increase of nitrite occurred between the second and third weeks after harvest for all three harvests and corresponded to the increases of the individual nitrosamines and total TSNAs. Correlations between nitrite and NNN, NAT, NNK, and total TSNA were 0.77, 0.91, 0.93, and 0.90, respectively. These correlations were significant at the 99.9% confidence level. It should be noted there was no significant correlation between nitrate concentrations and the individual nitrosamines or the total tobacco-specific nitrosamines. The absence of correlation between nitrate and nitrite would be expected since the nitrate concentration was constant except for changes that occurred during the curing process.

Accumulation of high levels of nitrite during the latter stages of curing at the higher temperature and relative humidity may be due to enhanced activity of exogenous nitrite-reducing organisms. Even though the color of tobacco lamina changed from green to yellow to brown, indicating a normal curing process, the moisture content only decreased to 56% (Figure 2). After 21 days, this high moisture content in the cured lamina should enhance microbial activity in the cured lamina. The influence of moisture content in cured lamina on the accumulation of nitrite in cured tobacco lamina is presently being investigated.

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Registry No. NNN, 16543-55-8; NAT, 71267-22-6; NNK, 64091-91-4; NO_3^-, 14797-55-8; NO_2^-, 14797-65-0.

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Dissipation of Isomalathion on Solid Pesticide Carriers, Container Surfaces, and Leaves and Some Degradation Products of Isomalathion on Carriers[1]

S. Rengasamy* and Balraj S. Parmar

Isomalathion undergoes degradation on solid pesticide formulation carriers, container surfaces, and *Vigna radiata* L. leaves. Nearly 40–50% of the applied isomalathion was lost on carriers bentonite, kaolinite, hydrated calcium silicate, and silica gel H on 12-day incubation at 55 ± 1 °C. The loss was 8–18% on different container surfaces under the same conditions. On *V. radiata* L. leaves, nearly 99% of the applied isomalathion was lost within 5 days of application, the loss being initiated within 30 min of application. Of the several degradation products formed on carriers, *O,S,S*-trimethyl phosphorodithioate, diethyl mercaptosuccinate, and diethyl methylmercaptosuccinate were identified.

Since the 1976 malathion poisoning episode in Pakistan (Baker et al., 1978), several studies on the formation of isomalathion in malathion products have been reported (Miles et al., 1979, 1980; Verschoyle et al., 1982; Halder, 1982; Halder and Parmar, 1984; Rengasamy and Parmar, 1988). Some of these studies indicated the instability of isomalathion (Halder, 1982; Halder and Parmar, 1984). However, no systematic information on its fate has been reported. In the present study, the loss of isomalathion on different carriers, container surfaces, and *Vigna radiata* L. leaves has been reported. An effort has been made to identify some of the transformation products of isomalathion on solid carriers.

MATERIALS AND METHODS

Isomalathion was prepared from technical malathion as per details reported earlier (Rengasamy and Parmar, 1988). Bentonite, kaolinite, hydrated calcium silicate, and silica gel H used in this study were also detailed earlier (Rengasamy and Parmar, 1988).

Isomalathion Loss on Carriers. A 10-mg portion of isomalathion in chloroform was applied to 1 g of each carrier, worked into a paste, dried for 24 h under ambient conditions (31 °C, RH 52%), and ground to a fine powder and 100 mg of powder (0 day sample) withdrawn from each. The powders were then incubated in bakelite screw-capped sample vials at 55 ± 1 °C for 12 days and sampled periodically. The samples were extracted in 5 mL of chloroform and filtered (Whatman No. 42), a suitable aliquot of the filtrate was withdrawn, and solvent was removed on a rotary evaporator. The residue was dissolved in 2 mL of 2-propanol and analyzed for isomalathion content. The percent loss of isomalathion was calculated on the basis of quantity applied and recovered.

Isomalathion Loss on Container Surface. A borosilicate glass Petri dish (diameter 5 cm, height 1 cm), aluminum box (diameter 5 cm, height 1 cm), and high-density polyethylene bottles (diameter 5 cm, height 5 cm) provided the test surfaces. Chloroform solution containing 500 μg of isomalathion was spread in each of the seven containers per test surface. Chloroform was evaporated under a ceiling fan to obtain a thin film. One sample per test surface was worked up immediately (0 day), and the rest of the containers were incubated at 55 ± 1 °C for 12 days. One container under each test surface was periodically withdrawn, the contents were dissolved in 2 mL of 2-propanol, and isomalathion content was analyzed. The percent loss of isomalathion was calculated as above.

Isomalathion Loss on Leaf Surface. Seven 40-day-old green gram (*V. radiata* L.) plants were selected in field, and three trifoliated leaves per plant were treated with 1000 μg of isomalathion in 2:1 methanol–water, using a pipet. One plant (three trifoliated leaves) was plucked per sampling at 30 min (0 day) and at 1, 2, 3, 4, 5, and 6 days after treatment. The treated leaves were macerated with 5 g of anhydrous Na_2SO_4 and extracted in 75 mL of chloroform. The extract was concentrated to 10 mL on a rotary evaporator and passed through a column of charcoal–anhydrous Na_2SO_4 (5 + 5 g) admixture sandwiched between anhydrous Na_2SO_4 layers. The column was eluted with 50 mL of chloroform, the solvent removed in a rotary evaporator, residue dissolved in 2 mL of 2-propanol, and isomalathion content analyzed. The recovery of isomalathion when applied to cleanup column was 99%. It was 35% from leaves after 30 min of application.

A Kontron Analytic liquid chromatograph, Series 640, from Kontron Electrolab, London, England, equipped with

Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi 110012, India.
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