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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MAR 7 1990

MEMORANDUM

SUBJECT: Methyl Bromide Registration Standard Follow-Up. Plant Metabolism Interim Report LVW-89-267. (DEB No. 5834, RD Record No. 252191, HED No. 9-2196)

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Introduction

The Methyl Bromide Industry Panel submits an interim report on plant metabolism of methyl bromide in response to DEB's review (C. Deyrup, Ph.D., 2/9/89) of a plant metabolism protocol reflecting MeBr metabolism after postharvest fumigation.

Conclusions/Recommendation

The additional information which has been submitted has been noted (see "Detailed Considerations" below).

Raw data (i.e., chromatograms, etc.) should be submitted with the completed study to support conclusions.

DEB will evaluate the adequacy of the plant metabolism study when the completed study is submitted.

The data gaps cited in the Methyl Bromide Registration Standard remain outstanding.

DETAILED CONSIDERATIONS

DEB's comments from the review dated 2/9/89 (C. Deyrup, Ph.D.) will be reported below, followed by the response of the Methyl Bromide Industry Panel and by DEB's present conclusions.

DEB's Comments #1-4 (2/9/89)

1. The submitted study plan and the work completed thus far are aimed at characterizing chemically bound residues only. The contribution that MeBr makes to the total radioactive residue (TRR) will need to be taken into account.

2. DEB concludes that it is highly unlikely that volatile metabolites of concern would arise from the proposed use, as the methylation of polysaccharides, proteins, DNA, etc., should not yield volatile products. Therefore, the only volatile residue which needs to be determined is MeBr per se. The level of MeBr should be determined after a 1-2 hour aeration period.

3. Since the only volatile residue which needs to be quantitated is MeBr itself, it may be unnecessary to repeat all the radiolabeled metabolism studies. DEB suggests that the registrant fumigate the various commodities with MeBr (unlabeled), exactly as in the previously conducted metabolism studies and determine MeBr levels from replicate fumigations. If there is little variation in residue levels from replicate fumigations, the ppm MeBr could be added to the ppm of chemically bound MeBr equivalents (determined from radiolabeled studies) to yield an estimate of the TRR and the MeBr contribution to the TRR. The ppm in both cases should be based on the weight of the commodities before lyophilization and/or extraction.

4. If there is significant variation between replicate fumigations, the metabolism studies will need to be repeated in order to determine the contribution of the parent to the TRR."

MBIP's Response to DEB's Comments #1-4

1-4. "The first four comments concern the determination of the contribution of MeBr to the total radioactive residue. A protocol outlining a bridging study has been submitted but was not commented on by DEB. In discussion with the Methyl Bromide Industry Panel on April 18, 1989, it was decided to proceed using the King headspace method (suggested by DEB on Page 6) to determine levels of MeBr. Dr. Tom Duafala will supply details of the procedure which he has used as well as assemblies for the blending step. The commodities used for the study of chemically bound residues will be treated in triplicate with unlabeled MeBr as before (48 mg/l, 72 h) and the amount of MeBr determined after aeration periods of 1 h and 1,2,4 and 10 days."

DEB's Conclusion re: the MBIP's Response to Comments 1-4

DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #5 (2/9/89)

5. "Whenever ether extraction is used to defat the commodities before characterizing the chemically bound residues, the radioactivity in the ether extract should be measured. DEB could not distinguish whether this had been done in each case."

MBIP's Response to DEB's Comments #5

5. "Results of ether extractions of four commodities were presented in Table 3 of the October 1988 report. Radioactivity in the oil was determined after removal of the ether so it would not be expected to contain MeBr. The values presented do indicate, however, that the level of MeBr at the time of extraction must have been very low as essentially all the radioactivity was accounted for by that present in the oil and the extracted material. This is shown more clearly when values are all based on the weight of the commodity before extraction (see Table 1 which is appended). Comparison of data for other commodities (wheat, oatmeal, peanuts) before and after ether extraction indicated similarly that this step removed no significant radioactivity."

TABLE 1

SUMMARY OF RESULTS FOR ETHER EXTRACTION OF [^{14}C]METHYL BROMIDE TREATED COMMODITIES[a]

Commodity	Radioactivity (dpm/g)[b] Unextracted (1)	Extracted (2)	Oil (3)	Total (2 +3)	% Accounted For After Extraction
Almonds	230,630	229,712	442	230,154	99.8
	217,715	227,748	252	228,000	104.7
Corn	148,412	142,359	654	143,013	96.4
Alfalfa	393,299	368,541	2,555	371,096	94.4
Orange peels	109,342	120,462	130	120,592	110.3

[a]Based on Table 3, October 1988 Report.

[b]Results are expressed relative to the weight of the commodity before extraction.

DEB's Discussion re: the MBIP's Response to Comment #5

Table 1 (above) compares radioactivity in unextracted almonds, corn, alfalfa, and orange peels with the sum of the radioactivity in the extracted material and oil. The percentage accounted for after extraction (in the extracted material and oil, excluding ether extracts) ranged from 94.4 to 110.3%.

DEB's Conclusion re: the MBIP's Response to Comment #5

DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be evaluated when the completed study is submitted.

DEB's Comment #6 (2/9/89)

6. "The decline studies do not enable DEB to estimate the initial MeBr levels in the metabolism studies, because MeBr *per se* was not determined, the treatment rates in the storage studies differed from that used in the metabolism studies, and it is not possible to determine how much of the activity remaining after 10 days was due to physically bound MeBr."

MBIP's Response to DEB's Comments #6

6. "A number of analyses were performed during the limited decline studies reported in October 1988 (Ref. 1, Table 2). The results are presented in Table 2 and the procedures employed are

outlined below.

Determination of Methyl Bromide in Treated Commodities

After an aeration period of 15 min, 2 g of each treated commodity were added to a flask (total volume 39 ml), fitted with an adapter and septum, containing 5 ml acetone - water (5:1). After 24 h at 20 - 23°, 1 µl aliquots were analyzed by gas chromatography using a 2 m x 3 mm (i.d.) nickel column packed with 60 - 80 mesh Tenax in a Hewlett-Packard 5890 gas chromatograph equipped with an electron capture detector. Desorption studies were performed by transferring 2 g of the treated commodity after the same aeration period to a similar flask. After 24 h, 50 µl aliquots of the headspace gas was analyzed on a 2 m x 3 mm (i.d.) nickel column packed with 35 - 60 mesh Tenax in the same instrument using a flame ionization detector.

As noted before (1), the number of analyses carried out was low and the results showed considerable variability. Nevertheless, it is clear that methyl bromide is present in the commodities after an aeration period of 15 min and that a major portion of it is desorbed during the first day. The observed variability probably accounts in large part for the differences in the amounts of methyl bromide desorbed and the equivalents of [^{14}C]MeBr lost during the first day following fumigation. Results for initial content (Table 2) would not include those reaction products of MeBr which are extracted by acetone-water (5:1). The level of methyl bromide in the treated commodities which were examined was found upon extraction and GC analysis to be below 1 ppm after aeration for nine days at 20 - 23° (Table 2)."

TABLE 2

CONTENT OF METHYL BROMIDE IN FUMIGATED COMMODITIES
AND QUANTITIES DESORBED DURING DAY 1 POST-TREATMENT, μG/G (± SD)[a]

Commodity	Initial Content[b] MeBr Extracted	Initial Content[b] [^{14}C]MeBr Equivalents Remaining	MeBr Desorbed 0 to 1 Day	[^{14}C]MeBr Equivalents Lost 0 to 1 Day[c]	MeBr Extracted after 9 Days Aeration
Corn	25.4 (3.8)	29.8 (13.8)	16.6 (3.4)	4.5	
Corn meal	14.8 (1.7)	37.6 (3.2)	14.5 (1.7)	15.5	ND[d]
Wheat	12.6 (8.5)	64.0 (6.8)	7.4 (3.5)	8.2	ND
Whole wheat flour	15.6 (3.5)	72.9 (1.5)	10.0 (0.6)	11.4	ND
White flour	58.9 (13.3)	93.3 (10.4)	20.8 (2.0)	46.3	ND
Oatmeal	25.2 (1.6)		8.2 (0.6)	15.8	
Peanuts	39.4 (8.6)	72.2 (25.0)	21.7 (8.8)	40.9	0.5 (0.1)

[a]Average of 3 determinations for corn and oatmeal and 2 determinations in the remainder.

[b]Samples placed in acetone-water (5:1) after 15 min. aeration.

[c]Determined by subtracting [^{14}C]MeBr equivalents on day 1 from that determined after 15 min. aeration (see data previously submitted (reference 1)).

[d]ND = none detected. Limits of detection = 0.1 μg/g.

DEB's Discussion re: the MBIP's Response to Comment #6

This DEB comment also concerns initial MeBr levels in the metabolism studies. This subject is addressed elsewhere (see MBIP's Response and DEB's Conclusions re: Comments 1-4).

DEB's Conclusion re: the MBIP's Response to Comment #6

DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #7 (2/9/89)

7. "The decline studies did not specify the temperature used in conducting the investigation. For the sake of completeness, the authors should include the storage temperatures in the final report."

MBIP's Response to DEB's Comment #7

7. "The decline studies and all other studies indicated to be at room temperature were conducted at 20-23°."

DEB's Conclusion re: the MBIP's Response to Comment #7

DEB concludes that issue #7 is resolved by submission of the additional information regarding temperature.

DEB's Comment #8 (2/9/89)

8. "In the studies aimed at determining the extent of O-, S-, or N- methylation, the specific activity of the MeBr was not given so that DEB could not calculate the ppm MeBr equivalents. DEB needs this information in order to judge whether the nature of the residue has been adequately delineated."

MBIP's Response to DEB's Comment #8

8. "The specific activity of the [^{14}C]MeBr used in the study of chemically bound residues is presented in Table 3 for each of the commodities. This table also shows the calculated quantities of residues expressed in MeBr equivalents found in each of the fumigated commodities. A higher amount of chemically bound residue was found in this study than in that for which results are presented in Table 2 because of the higher dosage of methyl bromide and the longer treatment time. The more extreme conditions were chosen deliberately in order to ensure sufficient reaction to permit easy investigation of reaction products."

TABLE 3

EXTENT OF REACTION OF METHYL BROMIDE (MeBr) WITH COMMODITIES DURING FUMIGATION

Commodity	Specific Activity of MeBr Used for Treatment, dpm/μg	Level of Chemically Bound Residues of MeBr Before Fat and Oil Extraction			Level of Chemically Bound Residues of MeBr After Fat and Oil Extractions	
		Radioactivity dpm/g[b]	MeBr Equivalents μg/g	MeBr Equivalents before Lyophilization μg/g[c]	Radioactivity dpm/g[d]	MeBr Equivalents μg/g
Wheat	1,133	106,575	94.1		102,303	90.3
Oatmeal	1,500	549,909	366.6		566,467	377.6
Peanuts	2,136	319,657	149.7		575,494	269.4
Almonds	1,206	230,630	191.2		470,047	389.8
Corn	2,104	123,161	58.5		134,538	63.9
Alfalfa	1,499	393,299	262.4		380,528	253.9
Apples	1,472	81,808	55.6	7.7		
Orange peel[e]	771	109,342	141.8	98.7	133,075	172.6
Orange pulp[e]	771	143,075	185.6	27.1		
Potato skins[e]	1,574	842,262	535.1	113.1		
Potato pulp[e]	1,574	553,500	351.7	74.2		
Potatoes	2,442	1,059,000	433.7	91.4		

[a]Treated with 48 mg/L [^{14}C]MeBr for 72 h at 20-23°.
[b]Determined after combustion of ground wheat, oatmeal, peanuts, almonds and alfalfa, dried and ground orange peel and potato skins and homogenized and lyophilized apples, whole potatoes and orange and potato pulp (see Ref. 1).
[c]Calculated using weights of commodities before and after drying.
[d]Determined by combustion of the ground, ether-extracted commodity and scintillation counting.
[e]Treated whole with MeBr and peeled after aeration.

DEB's Conclusion re: the MBIP's Response to Comment #8

DEB concludes that the specific activity of the MeBr has been submitted as requested.

DEB also concludes that the adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #9 (2/9/89)

9. "Although DEB can conclude that methylmethionylsulfonium derivatives are the source of some of the dimethyl sulfide, it is not clear if they are the only source. As planned, the authors should determine whether S-methyl cysteine is the source of methyl mercaptan. They should also consider whether there are other sources of methyl mercaptan and dimethyl sulfide besides S-methyl cysteine and methylmethionylsulfonium derivatives."

MBIP's Response to DEB's Comment #9

9. "Both [^{14}C]-labeled S-methylcysteine and methylmethioninesulfonium bromide have been prepared and treated with 1 N sodium hydroxide in the same manner as the fumigated commodities.

S-[^{14}C]Methylcysteine

L-Cysteine was methylated using a procedure based on that described by Rochat et al. (5). Labeled and unlabeled methyl bromide were introduced through a septum into a flask containing cysteine in ethanol-water (1:4) after adjusting the pH to 6.5. After one week at room temperature, excess methyl bromide and the solvent were removed. The product was purified as described (8) followed by chromatography over Dowex 50W-X8 (H^+ form) and then over a 150 x 19 mm μBondapack C_{18} column with 10 mM heptafluorobutyric acid at 4 ml/min. In each case the radioactive peak corresponded to the elution volume of S-methylcysteine.

When S-[^{14}C]methylcysteine (135,000 dpm) was treated with 1N sodium hydroxide at reflux for 5 h with trapping of volatiles as described earlier (1), 11.3 percent of the radioactivity was trapped with 9.1 percent in the aqueous mercuric cyanide trap. Replacement of the traps and treatment for another 5 h period yielded an additional 4.2 percent of the radioactivity in the trap for methyl mercaptan. These results are consistent with the proposal (1) that this volatile may be formed from S-methylcysteine when the fumigated commodities are treated with alkali. Since the decomposition was only partial, S-methylcysteine would also be expected to remain in the hydrolysate of the residue from 1 N sodium hydroxide treatment.

This has been confirmed by rechromatography of the residue, after neutralization, over the Dowex 50W-X8 and μBondapak C_{18} columns.

S-[^{14}C]Methylmethioninesulfonium Bromide

Methylation of methionine with [^{14}C]methyl bromide as described by Toennies and Kolb (6) yielded S-[^{14}C]methylmethioninesulfonium bromide. When a sample (23,400 dpm) was treated with 1 N sodium hydroxide as usual, 95 percent of the radioactivity recovered was found in the mercuric chloride trap which is consistent with the formation of dimethyl sulfide.

While it is felt that methylmethioninesulfonium salts are the main source of dimethyl sulfide and S-methylcysteine is the main source of methyl mercaptan, other precursors may also exist. For example, further methylation of S-methylcysteine, analogous to the methylation of methionine, would afford the dimethylsulfonium derivative which would also yield dimethyl sulfide when heated with alkali. Challenger and Hayward (7) proposed that S-methylsulfonium salts decompose when treated with hot, aqueous, alkaline solution by two pathways yielding either dimethyl sulfide and homoserine or methionine and methanol. Later, Ramirez et al. (8) confirmed the first pathway but found no evidence for the formation of methanol. Instead, they discovered that methionine production was accompanied by the formation of a trimethylsulfonium salt. This apparently arose from nucleophilic attack of dimethyl sulfide on the S-methylsulfonium salt. In our case, this reaction would probably be minimized since dimethyl sulfide was constantly being swept away by nitrogen. Although not tested, it is possible that S-methylmethioninesulfonium salts formed in the commodities may subsequently alkylate other constituents leading to the regeneration of methionine. In this regard, Naider and Bohak (9) have reported that methioninesulfonium salts are especially susceptible to attack by sulfur nucleophiles. Such a regeneration step would lead to radiolabeled methionine which would increase the complexity of the mixture resulting from methyl bromide treatment and subsequent hydrolysis. Any attempt to define minor sources of dimethyl sulfide and methyl mercaptan in the fumigated commodities would be very time consuming."

DEB's Discussion re: the MBIP's Response to Comment #9

In summary, the MBIP indicates the following: S-methylcysteine is a source of methyl mercaptan. S-[^{14}C]methylmethioninesulfonium salts are a source of dimethyl sulfide. Other sources of methyl mercaptan and dimethyl sulfide are possible.

DEB's Conclusion re: the MBIP's Response to Comment #9

DEB has no further comment on this protocol issue at this

time. The adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #10 (2/9/89)

10. "DEB has discussed with TOX (D. Ritter, 1/24/89) the degradative approach taken by the authors in determining the extent of O- and S-methylation. TOX said that as much information as possible should be generated on the original site of methylation. This approach was taken for studying the extent of methylation of DNA and for the determination of the methylated histidines. The authors should provide any information available on the original sites of methylation (polysaccharides, phenols, etc.)."

MBIP's Response to DEB's Comment #10

10. "In general, an indirect approach has been taken in the determination of the sites of methylation because of the difficulty in investigating "original sites of methylation". One problem is the instability of the products during isolation and the hydrolytic steps. No attempt has been made to look at methylation of polysaccharides or phenols. This would be very time consuming as procedures would need to be developed for each of the commodities. It should be noted that both our studies and those published by others indicate that the major site of methylation is the protein."

DEB's Conclusion re: the MBIP's Response to Comment #10

DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #11 (2/9/89)

11. "TOX is especially concerned with the extent of methylation that occurs in proteins and whether methylated glutathione is formed (D. Ritter, 1/24/89). Do the authors have any information on the presence of methylated glutathione?"

MBIP's Response to DEB's Comment #11

11. "In the manuscript (1) submitted as part of the October 1988 report, it was noted that [^{14}C]MeBr fumigated apples, oranges and potatoes yielded higher levels of radiolabeled methyl mercaptan than did the other commodities studied. Since this product may originate from S-methylcysteine (2), it was proposed that higher levels in the fleshy commodities may be a reflection of the ability of these to metabolize methyl bromide by enzyme-catalyzed conjugation with glutathione. In addition, it was observed that following 6 N HCl hydrolysis of material remaining after 1 N

sodium hydroxide treatment, the cation-exchange fraction (peak 1) in which S-methylcysteine would be expected was higher for these commodities (see October 1988 report) as would be expected since alkali-induced decomposition of S-methylcysteine would not be complete (see above).

To specifically investigate the possibility that glutathione was being methylated, extracts were prepared using 60 percent ethanol as was employed previously in a study of glutathione-dependent degradation of methyl bromide by the granary weevil (3). Table 4 presents results for the extraction of homogenized and lyophilized oranges (pulp) and potatoes (whole) with 60 percent ethanol. For comparison, a similar extract of ground corn has been prepared. Consistent with the earlier supposition, a greater proportion of the radioactivity was extracted from the potatoes and oranges than from the corn. In addition, the corn extract probably contained zein (see Ref. 10) which would account for the portion of the extract which could not be redissolved in water (see footnote, Table 6). To relate the extracts to the whole commodities, the extracted materials and the residues from the extraction were separately treated at 100°C with 1 N NaOH and the volatiles trapped as before (1). Results are presented in Table 5. Similar extracts were studied to determine if S-methylglutathione and S-methylcysteine were components. Using procedures of the earlier study (3), the extracts were chromatographed over a strong cation exchange column (H^+ form) and the peak containing neutral and acidic amino acids and related substances (Peak 1; Table 6) was rechromatographed over an anion exchange resin (Bio-Rad AG 1-X2). In the case of extracts from the potatoes and oranges, the latter afforded two major radioactive peaks (Peaks 1A and 1B; Table 6). These corresponded in elution volumes to authentic S-methylcysteine and S-methylglutathione when chromatographed on both the anion exchange column and on a 15 cm x 19 mm μBondapak C_{18} column eluted at 4 ml/min with 10 mM heptafluorobutyric acid. Identity of the first peak as S-methylcysteine was confirmed by derivatization with 1-fluoro-2, 4-dinitrobenzene (4) and comparison of the product with the corresponding derivative of authentic S-methylcysteine by HPLC on the μBondapak C_{18} column with acetonitrile-water (30:70) containing 10 mM trifluoroacetic acid at 4 ml/min. Similar derivatization of material in the second peak (peak 1B) and chromatography on the same column with acetonitrile-water (20:80) containing 10 mM trifluoroacetic acid at 4 ml/min. yielded two radioactive peaks, one of which corresponded to the retention volume of the 2,4- dinitrophenyl derivative of S-methylglutathione. At this time, it is not known if the unidentified peak is an artifact formed from S-methylglutathione or the derivative of another methylation product which was not separated from S-methylglutathione during reversed-phase chromatography. Hydrolysis of material from peak 1B with 6 N hydrochloric acid yielded a single radioactive peak upon reversed-phase HPLC which eluted at the retention volume of

S-methylcysteine. These results indicate clearly that cysteine in oranges and potatoes is a major site of methylation during fumigation with methyl bromide. Although not demonstrated, the S-methylcysteine is probably derived from S-methylglutathione, the initial methylation product. Saetre and Rabenstein (11) have found that levels of glutathione are significantly higher than cysteine in orange juice. The nearly equal quantities of S-methylglutathione and S-methylcysteine found in these studies may be indicative of fairly rapid hydrolysis of S-methylglutathione.

A similar investigation of peak 1 from cation exchange chromatography of the corn extract did not show peaks at the elution volumes of S-methylcysteine and S-methylglutathione when chromatographed on the anion exchange column.

In the case of the [^{14}C]MeBr fumigated potatoes, S-methylcysteine was shown to be the major radiolabeled substance remaining after 1 N NaOH treatment. Acid hydrolysis (6 N HCl) of non-volatile residue from 1 g of potatoes afforded soluble material with 352930 dpm and a black residue (55446 dpm) accounting for 33.3 and 5.2 percent of the original radioactivity, respectively. A peak (181110 dpm) with the retention volume of S-methylcysteine was obtained from the anion exchange column. Its identity was further established by chromatography over the 15 cm x 19 mm μBondapak C_{18} column as above. Derivatization with fluorodinitrobenzene and chromatography over the same column with acetonitrile-water containing 10 mM trifluoroacetic acid at 4 ml/min yielded a radiolabeled peak (138420 dpm) with the same elution volume as the corresponding derivative of S-methylcysteine."

TABLE 4

RESULTS OF EXTRACTING [^{14}C]METHYL BROMIDE TREATED COMMODITIES WITH 60 PERCENT ETHANOL

Commodity	Weight Extracted, g	Radioactivity dpm/g	% of Original[a] Extract Wt.	Extract dpm	Residue Wt.	Residue dpm
Potatoes (1)	1	1,007,952	23.4	74.1	84.0	16.7
(2)	1	1,007,952	22.7	75.3	79.8	16.1
Oranges (1)	4	143,075	75.1[b]	63.7	24.7	30.0
(2)	4	143,075	78.4[b]	65.9	21.6	24.2
Corn (1)	1	144,748	4.6	15.9	99.5	69.7
(2)	10	171,110	7.3	14.5	92.9	66.4

[a]Variation in moisture content is felt to be the main reason that values do not total 100 percent.

[b]Weight of extract estimated by subtraction.

TABLE 5

RADIOANALYTICAL RESULTS OF TREATING THE 60 PERCENT ETHANOL EXTRACTS AND RESIDUES OF [^{14}C]METHYL BROMIDE TREATED COMMODITIES WITH 1 N NaOH AT 100°C AND TRAPPING OF VOLATILES PRODUCED

Commodity	Radioactivity dpm	Radioactivity, % of Chemically Bound Residue					
		Neutralized Reaction Product	Trap 1 (CH_3OH)	Trap 2 (CH_3SH)	Trap 4 $(CH_3)_2S$	Total Trapped Volatiles	Recovery
Potatoes	1,007,952						
Extract[a]	792,350	31.8	6.9	10.8	13.9	33.5	
Residue	215,602[b]	5.4	1.1	1.4	7.8	10.7	81.4
Oranges	572,300						
Extract	315,200	12.3	11.5	18.4	10.5	41.3	80.8
Residue	172,125[b]	6.2	4.8	3.4	12.6	21.0	
Corn	144,748						
Extract	23,014	8.3	4.6	0.0	1.3	5.9	74.2
Residue	100,921	22.9	18.5	1.6	16.6	37.1	

[a]Mixture treated with 1 N NaOH for 10 h.

[b]Dpm in residue estimated by subtracting extract value from that for original.

TABLE 6

ION-EXCHANGE SEPARATIONS OF 60 PERCENT ETHANOL EXTRACTS OF [^{14}C]METHYL BROMIDE TREATED COMMODITIES

			Radioactivity, % of Initial				
			Dowex 50W x 8			Bio-Rad AG1 x 2	
60% Ethanol Extract[a]	WT., g	dpm	Peak 1	Peak 2	Peak 3	Peak 1A	Peak 1B
Potatoes (1)	0.2344	743,400	51.0	3.0	8.1	19.3	19.0
(2)	0.2272	758,774	43.1	4.8	8.0	18.7	19.2
Oranges (1)	3.0056	364,300	37.6	3.4		7.3	15.2
(2)	3.1342[b]	377,300	32.5	9.0	2.0	8.9	11.6
Corn (1)	0.7299	247,550[c]	5.4	0.4	0.5		

[a]Extracts prepared from 1 g batches of homogenized and lyophilized potatoes (whole), 4 g batches of homogenized and lyophilized oranges (pulp) and 10 g of ground, ether-extracted corn.

[b]Weight of extract estimated by subtraction.

[c]Of this, 201,600 dpm was contained in the soluble portion when dried extract was redissolved in H_2O before applying to the Dowex 50W x 8 column.

DEB's Discussion re: the MBIP's Response to Comment #11

In summary, the MBIP indicates that methyl glutathione may be formed.

DEB's Conclusion re: the MBIP's Response to Comment #11

DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #12 (2/9/89)

12. "It may be necessary to study the volatiles which are derived from the hydrolysis of protein fractions instead of from the whole commodities. At the very least, DEB needs to know what proportion of the TRR is contained in the protein fractions, as was done with corn."

MBIP's Response to DEB's Comment #12

12. "Protein concentrations have been estimated by a number of procedures (See Ref. 12, 13) but most do not involve the prior separation of the protein. Indeed, Marks et al. (12) reported that proteins are not quantitatively extracted from dried plant samples. It would also be expected that the content of methylmethioninesulfonium in the protein would be diminished during an extraction process such as that used by Jones et al. (13). For this reason, we chose an indirect approach to determining the content and identity of the methylated amino acids occurring free and in the proteins. This approach was used by Marks et al. (12) to measure total protein in plant samples. To isolate proteins from each of the commodities would require a great deal of work and, for reasons outlined above, would not be expected to provide a more accurate measurement of the proportion of the total radioactive residue which is contained in the proteins than can be derived by the procedures we have employed."

DEB's Discussion re: the MBIP's Response to Comment #12

The MBIP indicates that separation of proteins before measurement of radioactivity is not practical. No further information is given on the proportion of the TRR which is contained in the protein fractions.

DEB's Conclusion re: the MBIP's Response to Comment #12

DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #13 (2/9/89)

13. "If a significant portion of the TRR is contained in non-protein fractions, attempts should be made to identify the sites of reaction in these fractions as well."

MBIP's Response to DEB's Comment #13

13. "Non-protein associated residue would not be retained on the cation-exchange column unless it were basic. Previously (Table 2, October 1988 report), data was presented showing that only a small portion of the radioactivity was non-retained (neutral and acidic). Values were given in the same table for the insoluble residue formed upon HCl hydrolysis. This material consists of protein-carbohydrate decomposition products called humin (14).

[^{14}C]Methanol, formed when [^{14}C]methyl bromide treated commodities are reacted with 1 N sodium hydroxide at 100°C, may be protein derived as must be the situation with that originating from the fumigated sample of bovine serum albumin (Ref. 1, Table 2). Some evidence also exists that it may be derived from non-protein precursors in treated potato. When the 60 percent ethanol extract of potatoes was chromatographed over Dowex 50, 9.4 percent (95,000 dpm from 1 g lyophilized potato) of the radioactivity in the sample was not retained. Upon treatment with 1 N sodium hydroxide at 100°C with trapping of the volatiles in the usual manner, 43 percent of the recovered radioactivity (72 percent of that trapped) was in the traps expected to contain methanol. In this experiment, the presence of smaller amounts of radioactivity in the mercuric cyanide and mercuric chloride traps used to trap methyl mercaptan and dimethyl sulfide, respectively, indicates that the ion-exchange column may not have removed all the amino acid related material from the extract."

DEB's Conclusion re: the MBIP's Response to Comment #13

This issue remains outstanding. DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #14 (2/9/89)

14. "At the meeting of 11/10/88, Dr. Starrat said that it might be possible to look for 5-BrU with HPLC with electrochemical detection. DEB recommends that this route be pursued."

MBIP's Response to DEB's Comment #14

14. "Problems with HPLC analysis of 5-bromouracil with electrochemical detection were outlined in the proposed study plan which was submitted in December 1988. A recent paper (15)

describing the use of HPLC with UV detection for the quantification of 5-bromouracil in plasma lends support to the proposal to use this technique in our study."

DEB's Conclusion re: the MBIP's Response to Comment #14

DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be determined when the completed study is submitted.

DEB's Comment #15 (2/9/89)

15. "The authors reported the presence of 3-methylguanine in wheat DNA hydrolysates. The authors should indicate on the chromatograms where this residue would elute, as they did with the other methylated DNA bases."

MBIP's Response to DEB's Comment #15

15. "The elution position of 3-methylguanine was not shown on the chromatogram which was presented in Figure 5 of the publication reporting methylation of DNA of corn and wheat (16) since it could not be used as an internal marker due to co-elution with large quantities of cytosine and adenine (see Figure 1 where its elution position is shown). The peak at fraction 22 did, however, correspond to the elution time of 3-methylguanine run separately. The following sentence appears in the Results and Discussion section: "Peaks corresponding to the elution times of 7-methylguanine, 1-methyladenine, 3-methylcytosine, 3-methyladenine, and 3-methylguanine in a ratio of 6.7:4.2:1.2:1.1 were observed."

DEB's Conclusion re: the MBIP's Response to Comment #15

DEB has no further comment on this protocol issue at this time. The adequacy of the metabolism study will be determined when the completed study is submitted.

Other Considerations

The MBIP indicates the following future plans:

"Future study plans concerning plant metabolism studies (from October 1988 Report)

An attempt has been made to identify the site of methylation of DNA from potatoes fumigated with methyl bromide. Homogenized and lyophilized potato (24 g) yielded a DNA pellet (1.15 mg) having only 810 dpm. Chromatography of the hydrolysate over a Partisil 10 SCX column as described earlier (16) yielded 2 peaks of radioactivity (260 and 107 dpm respectively) with elution volumes corresponding to the retention time of 7-methylguanine

and 1-methyladenine. This pattern is in agreement with that observed earlier for corn and wheat (16)."

"Plans for Period of June 1, 1989 to March 31, 1990

Attempts will be made within this period to complete the following studies:

(1) Investigation of the possible formation of 5-bromouracil.

(2) Measurement of the quantity of physically bound residues of methyl bromide present in fumigated commodities after 1 h aeration to supplement data concerning chemically bound residues.

(3) Finish various aspects of incomplete investigations discussed in this and earlier reports."

cc: N. Dodd (DEB), RF, SF, Circulation (7), PP#5F3300, Methyl Bromide Registration Standard - W. Boodee, E. Eldredge (ISB/PMSD)

RDI:D.Edwards:2/27/90:R.Loranger:2/27/90
H7509C:DEB:CM#2:Rm800D:x1681:N.Dodd:mb:2/21/90:edited:2/28/90