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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

QUANTITATIVE ANALYSIS OF COUMARINS BY THIN LAYER CHROMATOGRAPHY, RELATED CHROMATOGRAPHIC STUDIES, AND THE PARTIAL IDENTIFICATION OF A SCOPOLETIN GLYCOSIDE PRESENT IN TOBACCO TISSUE CULTURE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

BRUCE CONRAD WINKLER

Norman, Oklahoma

1967

QUANTITATIVE ANALYSIS OF COUMARINS BY THIN LAYER CHROMATOGRAPHY, RELATED CHROMATOGRAPHIC STUDIES, AND THE PARTIAL IDENTIFICATION OF A SCOPOLETIN GLYCOSIDE PRESENT IN TOBACCO TISSUE CULTURE

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CHAPTER 1

INTRODUCTION

Scopolin and scopoletin (Fig. 1) are widely distributed in plants. Table I lists healthy plants from which scopolin and scopoletin have been isolated. Table II lists plants which under various stress conditions accumulated larger amounts of scopolin and scopoletin.

In addition to its occurrence in growing plants, Yang, Nakagawa and Wender (108, 109) point out the occurrence of scopoletin in cigarettes, cigars, snuff, chewing tobaccos, pipe tobaccos as well as in the smoke from a number of these products. Chr. F. van Sumere and Massant (98) and de Greef (35) have isolated scopoletin from the uredospores of Pucinnia graminis tritici.

Only a few studies have been made on the distribution of scopolin and scopoletin within a particular plant. Best (10) made a study of the distribution of scopoletin in a tobacco plant, and Watanabe and Wender (113) found that stamens, corollas, and calyxes or sepals but not the pistils of tobacco flowers contained scopolin. Gordon and Pollock (32) reported on the distribution of scopoletin in a growing Avena root.

TABLE I

OCCURRENCE OF SCOPOLIN AND SCOPOLETIN IN HEALTHY PLANTS

Plant	Compound Present	Reference
Nicotiana tabacum	Scopolin and scopoletin	48,50,108,113
Nicotiana sylvestris	Scopoletin	50
Nicotiana rustica	Scopoletin	50
Nicotiana glauca	Scopoletin	50
Nicotiana affinis, tissue culture	Scopoletin	101
Nicotiana tabacum, tissue culture	Scopolin and scopoletin	87
tobacco tissue culture	Scopolin and scopoletin	27
Datura Knightii	Scopolin	40
Datura arborea	Scopoletin	50
Datura inoxia	Scopoletin	50
Datura stramonium	Scopoletin	42,50
Datura tatula	Scopoletin	50
Sonchus spinosus	Scopoletin	7
Fouquiera splendens	Scopoletin	8
Skimmin laureola	Scopoletin	11
Impatiens	Scopoletin	12
Murraya exotica	Scopolin	13
Citrus	Scopoletin	24
Radix	Scopoletin	25
Hymenodyctoyon excelsum	Scopoletin	28
Helichrysum arenarium	Scopoletin	36
Mandragora	Scopoletin	43
Prunus domestica	Scopoletin	44
Viburum	Scopolin and scopoletin	45
Galium mollugo	Scopoletin	52,53
Anethum graveolens	Scopoletin	51
Coriandrum sativam	Scopoletin	51
Diospyros maritima BL	Scopoletin	69
Scammony	Scopoletin	79
Plumiera alba	Scopoletin	80
Melilotus albus and Trigonella Foenum-graecum grafts	Scopoletin	83
Nerium odorum	Scopolin and scopoletin	84
Ptaerolylon obliquum	Scopoletin	99
Dendrobium thyrsiflorum	Scopoletin	114
Artemisia	Scopoletin	30,85,89

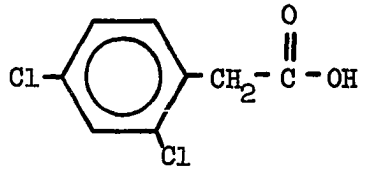
TABLE I--Continued

Plant	Compound Present	Reference
Gelsemium	Scopoletin	29,72,118
Althaeu officinalis L.	Scopoletin	42
Atropa belladonna L.	Scopoletin	29,42,50,56
Digitalis purpurea L.	Scopoletin	42
Hyoscyamus niger L.	Scopoletin	42
Malva silvestris L.	Scopoletin	42
Menyanthes trifoliata L.	Scopoletin	42
Tussilgo farafra L.	Scopoletin	42
Avena	Scopoletin	31,32
Brunfelsia	Scopoletin	74
Ipomoea	Scopoletin	29,78
Fabiana	Scopoletin	22,57
Convoluus scammonia	Scopoletin	29
Prunus serotina	Scopoletin	29
Angelica japonica	Scopoletin	41
Scopolia	Scopolin and Scopoletin	23,55,73,93,95
Fraxinus onnus	Scopolin and Scopoletin	55
Aesculus hippocustanum	Scopolin and Scopoletin	55,81
Jalap	Scopoletin	77
Capsicum annum	Scopoletin	50
Lycium barbarum	Scopoletin	50
Nierebergia frutescens	Scopoletin	50
Withania somnifera	Scopoletin	50

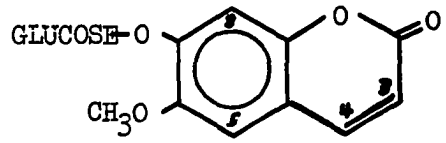
TABLE II

OCCURRENCE OF SCOPOLIN AND SCOPOLETIN IN PLANTS SUBJECTED TO STRESS

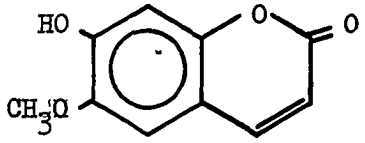
Plants	Stress	Compound Present	Reference
Tobacco	Interspecific tumors from hybrids	Scopolin and scopoletin	102
Tobacco	Decapitated and infected with spotted tomato wilt	Scopoletin	9
Tobacco	2,4-D	Scopolin and scopoletin	20
Tobacco	Boron deficiency	Scopolin	111
Tobacco	Infected with *Peronospora tabacina*	Scopoletin	67
Tobacco	Infected with *Pseudomonas solanacearum*	Scopoletin	92
Tobacco	Large excess of NaCl in nutrient medium	Scopoletin	26
Green mountain potato tubers *Solanum tuberosum*	Infected with leaf roll virus	Scopoletin	3,4
	Infected with Carla-y, Jubel-x, General yellow A and leaf roll virus	Scopoletin	82
Moskovski variety of potato tubers resistant to late blight fungus	Infected with *Phytophthora infestans*	Scopolin and scopoletin	96
Sweet potato roots	Infected with black rot	Scopolin	70
Sweet potato roots	Attacked by weevil *Cylas formicarius elegantulus*	Scopoletin	2
Sweet potato slices	Infected with *Ceratostomella fimbriata*	Scopoletin	104
Concolculus sepium	2,4-D	Scopoletin	100
Celery	Infected with *Septoria apii*	Scopolin and scopoletin	16,17
Helianthus annus	2,4-D	Scopolin and scopoletin	21



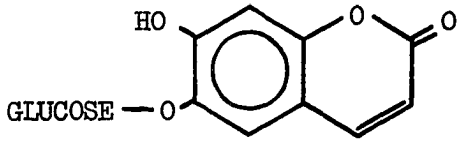
2,4-Dichlorophenoxyacetic acid



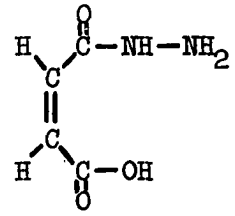
Scopolin



Scopoletin



Esculin



Maleic Hydrazide

Figure 1. Structural Formulas of Some Compounds Discussed in This Report

The role of scopoletin and scopolin in plant tissues has not been established. Scopoletin has been studied in greater detail than scopolin. Scopoletin appears to inhibit the growth of some plants (54) and roots (33, 76). Worsham, Klingman and Moreland (119) reported that scopoletin activated the germination of Striga asiatica seeds and in higher concentrations inhibited the elongation of the roots and caused branching.

Libbert and Lübke (59, 60, 61, 62) in a series of articles discussed the effect produced by scopoletin, indoleacetic acid and a combination of these two compounds on various plants.

Skoog and Montaldi (94) and Sargent and Skoog (86) looked at the effect of various levels of auxin and kinetin (6-furfurylamino-purine) in regard to the scopolin and scopoletin levels in tobacco tissue cultures. Their evidence suggested that indoleacetic acid caused a conversion of scopolin to scopoletin and that kinetin prevented this release until its toxic levels were reached. The authors suggested that auxin-kinetin levels therefore regulate the scopoletin-scopolin equilibrium in tissue. Fritig, Hirth and Ourisson (27) correlated the amount of scopolin and scopoletin present in tobacco cultures with the protein content.

Andreae and Andreae (6) and Andreae (5) indicated that scopoletin inhibited the oxidation of indoleacetic acid and suggested that scopoletin was a competitive inhibitor. In an earlier paper, Andreae and Andreae (3) had correlated the fluorescence with abnormal starch content in the leaves of potatoes infected with leaf roll virus.

Sequeira (91) and Sequeira and Kelman (92) also discovered that scopoletin inhibited the destruction of indoleacetic acid in plants infected with Pseudomonas solanaceanum. Both indoleacetic acid and scopoletin increased continually until the plant died. The inhibition was correlated with the increase in the scopoletin within diseased tissue. However, their data indicated that the inhibition was non-competitive but followed complex kinetics.

Schaeffer, Buta and Sharpe (88) investigated the rate of indoleacetic acid oxidation with horseradish peroxidase in the first five minutes after the addition of various phenolic compounds. Scopolin and scopoletin both inhibited the rate of indoleacetic acid destruction although scopoletin is more than ten times as effective as scopolin in this respect.

Dewey and Stepka (19) indicated that scopolin became radioactive during post-harvest conversion of nicotine-CH_3-C^{14} to nornicotine in the leaves of a strain of cigar tobacco. All of the radioactivity was found in the -OCH_3 group of scopolin.

Martin (66) quantitatively determined the amount of scopoletin present in root water samples by a direct measurement of the fluorescence at different pH values. Hughes and Swain (46) used a similar procedure to determine the scopolin present in potato tubers infected with Phytophthora infestans.

Sequeira (92) determined scopoletin by chromatographing a tissue extract on Whatman #1 paper and cutting the scopoletin area out and weighing it.

Skoog and co-workers (86, 94) determined scopoletin quantitatively using a combination of paper chromatography or column chromatography followed by a fluorescence analysis at a pH of 10. Skoog determined scopolin by acid hydrolysis to scopoletin and then calculation back to the amount of scopolin represented by the amount of scopoletin determined.

Waltz, Haeuserman and Krull (110) developed a polyamide column chromatographic procedure to determine the amount of scopoletin present in cigarette-smoke condensate.

Yang, Nakagawa and Wender (107) reported a method for the quantitative determination of scopoletin in cigarette-smoke condensate. This method involved extended paper chromatography in five solvents followed by elution of the scopoletin from the paper and measurement of its absorbance at 344 mμ.

Watanabe et al. (111) devised a fluorimetric method for the determination of scopolin. An aliquot of the tissue extract was first subjected to extended paper chromatography in two solvents. The scopolin was eluted and hydrolyzed and the pH adjusted to 10. The fluorescence of this solution was then measured.

Dieterman et al. (20) reported on the quantitative analysis of scopolin and scopoletin. Scopolin was determined by paper chromatography of an aliquot of the tissue extract in three solvents, followed by elution and measurement of the fluorescence of the eluted solution. Scopoletin was determined in a similar manner except that a different solvent was used for the last development of the paper chromatogram than was used for scopolin.

Akazawa and Uritani (1) used a thin layer method to determine scopoletin quantitatively. The tissue extract was chromatographed on a silica gel plate and the scopoletin eluted and further purified by paper chromatography. The scopoletin was then eluted from the paper and determined fluorimetrically.

This paper deals with the development of a rapid, quantitative method for the estimation of scopolin and scopoletin and the subsequent use of this method to determine quantitatively the amount of change in the concentration of scopolin and scopoletin in 2,4-D treated and maleic hydrazide treated tobacco plants as a function of time. The method developed used thin layer chromatography and fluorescence. A partial identification of a scopoletin glycoside from tobacco tissue culture was also undertaken.

CHAPTER II

EXPERIMENTAL PROCEDURES AND RESULTS

Chromatographic Studies

To explore the usefulness of thin layer chromatography for analysis of phenolic compounds, a number of compounds has been tested in various solvent systems. The results of these studies are listed in Tables III-VIII. The following comments apply to Tables III-V.

For analysis, the samples were dissolved in methanol at an approximate concentration of 1 μg/μl and 5 μl of this solution was applied onto the adsorbent. The solvent was allowed to ascend approximately 15 cm. The polyamide layers were spread with a Desaga spreader to an approximate thickness of 250 microns. Each chamber was lined with filter paper and was saturated with solvents before insertion of the plate. All of the R_f values were obtained by measuring to the top of the spot. The temperature of the chromatography room was approximately 70°F. The following recipes were used to make five plates. Twelve g of Macherey and Nagel polyamide (distributed by Alupharm Chemicals, New Orleans, La.) was added to 45 ml of a benzene:methanol (2:3; v/v) solution or 45 ml of

TABLE III

R_f x 100 OF VARIOUS DEPSIDES, COUMARINS AND PHENOLIC ACIDS

Compound	KFW	MW	NM	BzM	IFW-1	NFM	NIFBz	TFEF
2-hydroxy-5-glucoxy benzoic acid	63	5	7	7	60	33	22	28
5-hydroxy-2-glucoxy benzoic acid	50	0	4	3	70	38	20	19
1-guntisoyl-β-D-quinic acid	65	55	32	28	72	51	30	29!
1-feruloyl-D-quinic acid	65	9:	16	17:	77	57	-	49!
3-feruloyl-D-quinic acid	65	7:	22	20:	64	58	-	33!
4-feruloyl-D-quinic acid	64	7:	16	16:	63	55	-	32!
5-feruloyl-D-quinic acid	63	10:	11	13:	63	52	-	30!
chlorogenic acid	58	6	10	11:	58	28	19	14
1-feruloyl-β-D-glucose	74	70	42	42	85	66	50	36!
scopolin	67	85	72	49:	89	81	73!	43:
scopoletin	62	28	71	57	55!	78	79	46
4-hydroxycoumarin	52	6	25:	37:	59	45	-	32
7-hydroxycoumarin	60	17	37	50	47	66	-	40
esculetin	58	14	34	34	57!	50	-	32:
esculin	67	6!	26	26	84	56	-	23
6,7-dimethoxycoumarin	71	46	80	80	79	93	-	82:
o-hydroxycinnamic acid	61	6	26	34	44	36	-	44!
o-methoxycinnamic acid	71	5	57	60	58	71	-	71
m-hydroxycinnamic acid	59	6	26	37	50	42	-	44
m-methoxycinnamic acid	-	6	57	62	58	71	-	73
p-hydroxycinnamic acid	58	7	27	32	48	37	-	42
p-methoxycinnamic acid	58	7	27	32	48	70	-	42
caffeic acid	53	9	18	19	47:	23	19	28
hydrocaffeic acid	-	30	35	31	65	49	-	37
ferulic acid	64	14	46	45	61	57	53	58
isoferulic acid	62	12	42	42	57	59	50	53
methyl caffeate	-	-	-	-	52	58	-	51

Adsorbent: Macherey and Nagel Polyamide (250 Microns)

KFW: methylisobutylketone:formic acid(98%):water; 14:3:2(v/v/v)
MW: methanol:water; 1:1(v/v)
NM: nitromethane:methanol; 5:2(v/v)
BzM: benzene:methanol; 3:1(v/v)
IFW-1: 2-propanol:formic acid(98%):water; 10:0.3:5(v/v/v)
NFM: nitromethane:formic acid(98%):methanol; 5:0.15:2(v/v/v)
NIFBz: nitromethane:2-propanol:formic acid(98%):benzene; 5:2:0.2:4(v/v/v/v)
TFEF: toluene:formic acid(98%):ethyl formate; 5:1:4(v/v/v)

! = spot partially streaked : = spot badly streaked

TABLE IV

R_f × 100 OF VARIOUS DEPSIDES, COUMARINS, PHENOLIC ACIDS AND GROWTH REGULATORS

Compound Solvent:	KFW	NIFBz	MW	NFM	NM	BzM	BzMF-2	IFW-1	BzMF-1
2-hydroxy-5-glucoxy benzoic acid	69	-	25:	-	8	5	-	80	-
5-hydroxy-2-glucoxy benzoic acid	81	-	0	-	9	6	-	83	-
1-gentisoyl-β-D-glucose	74	-	73	-	43	32	-	86	-
1-feruloyl-D-quinic acid	71	29	26:	25	11	8	62	87	17
3-feruloyl-D-quinic acid	77	49	22:	37	15	11	79	82	56
4-feruloyl-D-quinic acid	73	30	19:	25	11	8	56	78	48
5-feruloyl-D-quinic acid	72	30	20:	21	11	7	52	80	48
chlorogenic acid	63	10	16:	20	10:	6	49	74	28
isochlorogenic acid	52	4	9	5	4	3	25,11	41	11
1-feruloyl-β-D-glucose	75	34	61:	36	52	39	84	90	52
scopolin	76	73	89	68	85	65	92	96	67
scopoletin	88	84	49	84	87	76	92	80	80
4-hydroxycoumarin	-	-	19:	-	27	-	-	73	-
7-hydroxycoumarin	89	-	42	-	78	73	-	78	-
esculetin	78	49	34	50	52	44	79	72!	58
esculin	67	30	63	37	42	27	79	90	50
6,7-dimethoxycoumarin	93	-	66:	-	100	95	-	92!	-
o-hydroxycinnamic acid	91	-	14	-	26:	27:	-	75	-
o-methoxycinnamic acid	94	-	20:	-	55:	64:	-	88	-
m-hydroxycinnamic acid	85	-	15	-	25:	25:	-	77	-
m-methoxycinnamic acid	96	-	16	-	50:	56:	-	86	-
p-hydroxycinnamic acid	84	-	16:	-	29:	29:	-	74	-
p-methoxycinnamic acid	91	-	18:	-	56:	59:	-	85	-
caffeic acid	74	22	21:	28	21:	20:	65	65!	40
ferulic acid	86	71	21:	61	41:	35:	88	80	75
isoferulic acid	85	-	20:	-	35:	27:	-	80	-
hydrocaffeic acid	78	-	24:	-	29:	22:	-	74	-
methyl caffeate	-	-	-	-	-	-	-	76	-
vanillic acid	85	68	-	58	-	-	83	84	64
indoleacetic acid	83	71	-	63	-	-	76	77	60
kinetin	86	87	-	81	-	-	94	92	76

Adsorbent: Woelm Polyamide (250 Microns)

KFW: methylisobutylketone:formic acid(98%):water; 14:3:2(v/v/v)
NIFBz: nitromethane:2-propanol:formic acid(98%):benzene; 5:2:0.2:4(v/v/v/v)
MW: methanol:water; 1:1(v/v)
NFM: nitromethane:formic acid(98%):methanol; 5:0.15:2(v/v/v)
NM: nitromethane:methanol; 5:2(v/v)
BzM: benzene:methanol; 3:1(v/v)
BzMF-2: benzene:methanol:formic acid(98%); 3:2:0.1(v/v/v)
IFW-1: 2-propanol:formic acid(98%):water; 10:0.3:5(v/v/v)
BzMF-1: benzene:methanol:formic acid(98%); 3:1:0.1(v/v/v)

! = spot partially streaked : = spot badly streaked

TABLE V

R_f x 100 OF VARIOUS FLAVONOIDS

Compound Solvent:	KFW	IFW-1	BzMF-1	NFM	NIFBz	BzMF-2
isosakuranetin-7-β-rutinoside	81	94	56	65	59	91
quarcitrin	64	76	18!	7	7	64
kaempferide	80!	59!	63,59	46	62!	67
isorhamnetin	70:	52:	46:	30!	37:	64!
kaempferol	68:	45:	33:	16	22	58
rhamnetin	68:	52:	51:	44,66:	44:	68:
quercetin	58!	44!	20!	12	12	45:
rhoifolin	61	89	24	12	10	72
luteolin	62!	57:	28:	17	18!	61!
diosmetin	71:	63:	54:	46:	51:	74!

Adsorbent: Woelm polyamide (250 microns thick)

KFW: methylisobutylketone:formic acid(98%):water; 14:3:2(v/v/v)
IFW-1: 2-propanol:formic acid (98%):water; 10:0.3:5(v/v/v)
BzMF-1: benzene:methanol:formic acid(98%); 3:1:0.1(v/v/v)
NFM: nitromethane:formic acid(98%):methanol; 5:1.15:2(v/v/v)
NIFBz: nitromethane:2-propanol:formic acid(98%):benzene; 5:2:0.2:4(v/v/v/v)
BzMF-2: benzene:methanol:formic acid(98%); 3:2:0.1(v/v/v)

! - spot partially streaked : - spot badly streaked

methanol. This was shaken vigorously for approximately one minute, spread immediately and air-dried before use.

It can be seen that the addition of acid to the solvent system employed usually increased the R_f value and helped to decrease spreading or streaking of the material. It was also discovered that formic acid was more effective in this respect than acetic acid. With the polyamides on thin layer plates, very rapid migration times were obtained when compared to those by paper chromatography. In the solvent systems studied, it was noted that with Woelm polyamide migration times were approximately 2-3 times faster than with the corresponding Macherey and Nagel polyamide. Woelm polyamide also produced essentially no background fluorescence when viewed under ultraviolet light whereas the Macherey and Nagel polyamide did have a slight purple fluorescence. The purple background made it difficult to locate some of the compounds studied. The polyamides, however, were not so useful for flavonoid separation as the data in Table III indicated. Many of the compounds exhibited partial or pronounced streaking even in the solvents to which acid had been added. A disadvantage of the polyamide layers was their fragility. Woelm polyamide was particularly bad in this respect.

Later in the course of research a microcrystalline cellulose called Avicel-SF (distributed by FMC Corporation, American Viscose Division, Marcus Hook, Pa.) became generally available. This material gave separations similar to those of paper chromatography but the resolution was far superior. In addition, the material adhered very

firmly to the plate, thus overcoming the pronounced fragility of the polyamides. Phenolic compounds of interest were tested on Avicel-SF, and the results are tabulated in Tables VI-VIII.

The Avicel-SF layers were made by taking approximately 3.5 g of Avicel-SF/plate and adding 4.5 ml of water/g of Avicel-SF and beating at high speed in a Waring blender for approximately 45 seconds. This procedure eliminated any stringiness in the material. The material was spread with a Desaga spreader to an approximate thickness of 375 microns and allowed to air-dry before use. For a plate approximately 250 microns thick, 2.5 g of Avicel-SF was used/plate. For a plate approximately 500 microns thick, 5 g of Avicel-SF was used/plate. Over-beating of the material must be avoided as the material becomes very thick and difficult to spread. It can be seen from the data in Table VI that a good separation of the Chlorogenic acid isomers was obtained.

A number of sugars and amino acids was also tested using this medium. These data are presented in Tables VII and VIII. A study of the recent literature indicated that although some work with sugars has been done with thin layer chromatography on cellulose powder plates, not very much has been done with Avicel-SF. (106, 117). No work has been done in which the solutions were streaked or banded rather than spotted at the origin. The sugar samples used in Table VII were dissolved in 10% 2-propanol:water at an approximate concentration of 3 μg/μl, and 5 or 10 μl were spotted. The chambers were not lined with filter paper but the chamber was saturated before the plate was

TABLE VI

R_f x 100 OF VARIOUS COMPOUNDS

Compound	IFW-2
chlorogenic acid	57
neochlorogenic acid	63
band 510	51
isochlorogenic acid	25,16,9
scopoletin	25
scopolin	67
esculetin	30!
esculin	53
caffeic acid	28!
ferulic acid	28
1-feruloyl-D-quinic acid	67!
3-feruloyl-D-quinic acid	58!
4-feruloyl-D-quinic acid	52!
5-feruloyl-D-quinic acid	67
rutin	38
kaempferol-3-rhamnoglucoside	38
kaempferol	3
methyl-p-coumarate	49:
apigenin-7-β-rutinoside	15
o-hydroxycinnamic acid	47:
protocatechuic acid	47
rhamnetin	3
robinetin	3
morin	9
o-methoxycinnamic acid	43:
p-methoxycinnamic acid	49:
anisic acid	60:
1-gentisoyl-β-D-glucose	79,73,50
rhoifolin	17
isoferulic acid	26:
quercetin	3
apigenin	3

Adsorbent: Avicel-SF (375 microns thick)

IFW-2: 2-propanol:formic acid(98%):water; 5:4:95(v/v/v)

! - spot partially streaked : - spot badly streaked

TABLE VII

R_f x 100 OF SUGARS AND RELATED COMPOUNDS

Compound	Solvent: I	II	III	IV(developed once)
ribose	51	32	62	39
xylose	46	25	58	34
arabinose	40	23	51	27
mannose	39	-	54	-
glucose	35	16	48	25
galactose	30	15	42	22
fructose	37	21	50	30
rhamnose	56	37	67	45
cellobiose	23	5	35	18
maltose	24	7	37	18
inositol	17	7	18	10
galacturonic acid	16	15	17	8
galacturonolactone	73	32	75	58
glucuronic acid	18	17	20	10
glucuronolactone	75	40	73	66
quinic acid	28	30	33	13
shikimic acid	51	41	60	17

Compound	II	III	IV(developed twice)
ribose	48	83	60
xylose	40	78	53
arabinose	37	73	46
mannose	-	75	-
glucose	27	71	41
galactose	25	65	36
fructose	35	74	45
rhamnose	56	88	65
cellobiose	8	56	28
maltose	10	60	30

Adsorbent: Avicel-SF (375 microns thick)

Solvent I: pyridine:ethyl acetate:acetic acid:water; 5:5:1:3 (v/v/v/v)

Solvent II: formic acid(98%):methylethylketone:t-butanol:water; 15:30:40:15(v/v/v/v)

Solvent III: 2-propanol:pyridine:acetic acid:water; 8:8:1:4 (v/v/v/v)

Solvent IV: ethyl acetate:pyridine:water; 2:1:2, upper layer (v/v/v)

TABLE VIII

R_f x 100 OF AMINO ACIDS

Compound	Solvent: V	VI	VII	VIII	IX	X
alanine	40	41	59	32	53	22
aspartic acid	23	20	29	30!	35	15
arginine	20	9	24	2	28	11
cystine	0	5	-	5	0	5
cysteine	7	39	-	80	10	25
glutamic acid	35	30	37	40!	43	24
glycine	25	24	51	18	36	16
histidine	16	16	60	16	24!	11
hydroxyproline	25	39	51	26	36	16
isoleucine	68	68	68	57	76	51
leucine	71	67	70	58	79	57
lysine	19	8	59	3	28!	9
methionine	-	57	66	48	66	40
phenylalanine	66	64	68	53	70	50
proline	42!	64	59!	39	52	25
serine	24	23	65	25	35	14
threonine	36	35	73	42	42	17
tryptophane	63	42	67	35	58	40
tyrosine	56	51	60	42	55	35
valine	57	60	67	49	68	40

Adsorbent: Avicel-SF (375 microns thick)

Solvent V: sec-butanol:acetone:formic acid(98%):water; 40:40:8:20(v/v/v/v)
Solvent VI: phenol:methanol:water; 35:15:50(v/v/v)
Solvent VII: methanol:acetone:ammonia:water; 40:40:8:20(v/v/v/v)
Solvent VIII: methanol:acetone:collidine:water; 40:40:8:20 (v/v/v/v)
Solvent IX: 2-propanol:formic acid(98%):water; 20:2:5(v/v/v)
Solvent X: n-butanol:acetic acid:water; 6:1:2(v/v/v)

! - spot partially streaked

inserted. The sugars were detected with an aniline hydrogen oxalate spray (115) or a $AgNO_3$ spray (97). The quinic acid and shikimic acid were detected with a $KMnO_4$ spray (34). Solvent IV seemed to be the most sensitive to varying loads and therefore it produced the greatest variation in R_f values from plate to plate. This may have been due, in part, to the fact that solvent IV is a two layer solvent and small changes in temperature may cause a variation in the proportion of the solvents in the upper layer. Solvent II was the most consistent solvent under varying loads although the bands produced were larger than in the other solvents. All the R_f values were measured to the top of the band. Banding produced very compact zones, most of them being less than 1/4 of an inch wide. Thus, even closely related sugars could quite easily be distinguished. Solvent I, II, and IV take approximately 3-4 hours to ascend and solvent III takes approximately 6-8 hours. Three of the solvents were developed a second time to see if any further separation could be achieved as in the case of solvent II as employed by Vomhof and Tucker (106). Although a better separation did occur, it was felt that the advantage of the separation was offset in most cases by the extra time it would take to develop the plate twice.

An interest in proline prompted an investigation into the separation of amino acids on Avicel-SF. A survey of the recent literature indicated that a number of solvents had been tried with cellulose powder but not with Avicel-SF. Therefore, six solvents were chosen and the amino acids were run and their R_f values determined as

listed in Table VIII. This work was done in cooperation with Carroll Smith.

Banding was not employed since most of the spots remained compact and no appreciable increase in separation was noted by banding. The compounds were dissolved in water at an approximate concentration of 1 μg/μl and 5 μl of solution was spotted. The plates were put into chambers which had been loaded with solvent immediately before use, so there was no chamber saturation. This seemed to improve the separation and compactness of the spots. The amino acids were detected by spraying the plate with a solution of 0.2% solution of ninhydrin in acetone. For proline, an isatin spray was used. This consisted of 300 ml of a 0.2% solution of isatin to which had been added 200 ml of 4% acetic acid. The R_f was measured in the middle of the spot since most amino acids are reported in this manner. A number of two-dimensional plates was also tried with 20 μl of a solution combining all of the amino acids. The following combinations were tried in an effort to find a combination that would satisfactorily separate proline from the rest of the amino acids as well as allow a possible determination of other amino acids: a) solvent V, solvent VI; b) solvent VII, solvent VI; c) solvent VIII, solvent VI; d) solvent IX, solvent VI. Solvent VII followed by solvent VI gave the best overall results. When this system was tried on a number of crude extracts the results indicated that the crude extracts would have to undergo some purification before attempting to determine in this manner the amino acids present. Solvents V-VIII were taken from

an article by Chiari, et al. (18) and solvent IX was a modification of a solvent employed by Jones and Heathcote (49). Solvent X is a very common solvent employed in this laboratory for many years.

Growth, Treatment and Harvesting of Plants

2,4-D-Treated-Tobacco (Code: Tob-3-2,4-D-). Nicotiana tabacum, One-Sucker variety, seeds were spread on the surface of sterile quartz sand on October 11, 1965. These seeds were watered once a day with approximately 400 ml of complete solution (47, 65). The crock of sand with the seeds was placed in a large growth chamber under the following conditions: 16 hours of light/day, temperature - 80°F in the light period and 50°F in the dark period. The light intensity was approximately 700 ft-candles. Seedlings were transplanted at intervals beginning on November 12, 1965, until December 1, 1965, to #29 crocks of sterile quartz sand. One seedling was placed in each crock. The plants started to flower on January 3, 1966 (age = 84 days). The plants were sprayed on January 14, 1966, with a solution of 2,4-D until the solution ran off the leaves. The 2,4-D solution consisted of 1 g of 2,4-D dissolved in approximately one liter of water with 5 g of Carbowax 1500 added. The pH was adjusted to 7.0 with ammonium hydroxide. Two drops of tween-20 were added, and the solution was adjusted to exactly one liter with additional water. The controls were sprayed with a control solution which contained all of the above materials just listed except the 2,4-D. The plants were harvested

at weekly intervals beginning on January 21, 1966 (age = 102 days), and continuing until February 18, 1966 (age = 130 days). Single plants were harvested in duplicates. The plants were harvested in the morning after the plants had been under illumination for approximately two hours. The plants were divided into four fractions: flowers, stems, roots, and leaves. The parts were weighed immediately after separation and plunged into boiling methanol for 5 minutes. This was done as rapidly as possible to avoid the possibility of any major changes in the phenolic content.

Maleic Hydrazide-Treated Tobacco (Code: 1510-JMI-11,12-MH-). Seeds of Nicotiana tabacum, One-Sucker variety, were planted on sterile quartz sand on October 11, 1965, and watered with 400 ml of complete solution every day. These seeds were germinated under the same conditions as stated on page 21 for 2,4-D-Treated-Tobacco. Seedlings were transplanted to large crocks of soil in the greenhouse on November 12, 1965. The soil mixture consisted of approximately 2 parts soil, 1 part dried manure and 1 part sand. Ninety plants were used.

On December 8, 1965 (age = 56 days), the plants were divided into equal groups, and one group was sprayed with a 1% maleic hydrazide solution and the other with a control solution. The plants were sprayed on the upper surface of the leaves until the solution ran off freely. The maleic hydrazide solution was made up as follows: 11.8 ml of triethylamine was added to approximately one liter of water. Ten g of maleic hydrazide was dissolved in this solution, heating if

necessary. The pH was then adjusted to approximately 7.0 with glacial acetic acid, and 5 g of Carbowax 1500 was added. The solution was then adjusted to exactly one liter. The control solution contained all of the above except the maleic hydrazide.

Control and treated plants (9 in each group) were harvested 3, 7, 15, 29 and 47 days after treatment. The plants were divided into roots, stems and leaves and plunged into boiling methanol, after weighing, for approximately 15 minutes.

About 1-1$\frac{1}{2}$ weeks after spraying, the plants of the two groups began showing a marked difference. It appeared that very little or no terminal growth was taking place in the maleic hydrazide treated plants, whereas the controls appeared normal. Some lateral growth may have taken place in the maleic hydrazide treated plants but this appeared slight. At the last harvest the treated plants were either dead or very severely chlorotic. This work on growth and treatment of tobacco plants with maleic hydrazide was done by J. W. Mizelle.

Nitrogen Dioxide-Treated Beans (Code: 1510-BWIII-15,16-B-1-NO_2-,). Red kidney bush beans were planted in four inch clay pots filled with a sterile soil mixture which consisted of 4 parts soil, 2 parts sand, 2 parts of dried manure and 1-2 parts of peat moss. Fifty pots were planted. Five seeds were planted at a depth of approximately 3/4 of an inch on January 20, 1967, and the contents of the pots were watered thoroughly with distilled water. The pots

were covered with a piece of slitted filter paper to help prevent rapid drying of the soil. After the seeds had germinated, they were placed in a small growth chamber. They were given 16 hours of light/day. The light intensity was approximately 1500 ft-candles. The temperature was 85°F during the light period and 65°F during the dark period. The plants were gradually thinned until one plant was left in each pot. The plants received water every day and complete solution twice a week.

The plants, approximately 15 cm in height, were treated with NO_2 gas. The treatment was begun after the plants had been illuminated three hours to insure that the stomates were open. The plants were treated on February 6, 1967 (age = 17 days). They were placed in a modified paper chromatography tank of approximately 80 liters and covered with a large piece of glass to let the light into the chamber during treatment. The NO_2 gas, at a concentration of 50 ppm (Matheson, Coleman, and Bell, Rutherford, N. J.), was released into the chamber continuously. A slight, positive pressure gradient was maintained. The gas was placed into the chamber at the rate of 600 ml/minute for two hours and then for an additional two hours at a rate of 300 ml/minute. Controls were placed in the chamber for four hours with compressed air flowing through the chamber.

Ten plants were harvested on February 10, 1967, four days after treatment, and every three days thereafter. These ten plants were divided into leaves, stems, and roots and plunged into boiling 2-propanol-water azeotrope for five minutes after weighing. Since

the treated plants not yet harvested did not appear to show any severe damage they were treated again on February 15, 1967, for two hours at a flow rate of 300 ml/minute. Thirteen days after treatment the plants began to bud and by the last harvest (16 days after treatment) they were flowering.

Nitrogen Dioxide-Treated Tomatoes (Code: 1510-BWIII-17,18-T-1-NO_2-,). Tomatoes, Lycopersicon esculentum Mill., Marglobe variety, were planted on January 9, 1967, following a procedure similar to that described on pages 23 and 24 for Nitrogen Dioxide-Treated Beans. Fifty pots were planted. The soil mixture was modified slightly, due to problems incurred when peat moss was present, to the following proportions: 3 parts of soil, 2 parts of sand and 1 part dried manure. The seeds were only inbedded slightly into the soil. Seven-ten seeds were placed in each pot. After germination, the seedlings were placed in small growth chambers under conditions similar to those described under the section entitled Nitrogen Dioxide-Treated Beans. The pots were thinned to one plant of uniform size/pot and treated on February 6, 1967 (age = 28 days). The plants were approximately 12-15 cm in height at the time of treatment. They were treated with NO_2 gas as described on page 24 for beans. Ten plants were harvested four days after treatment and every three days thereafter, using the procedure described for beans on page 24.

The tomatoes, as the beans, did not show any significant damage at the first harvest so that plants not yet harvested were

treated again on January 15, 1967, with NO_2 gas for two hours at a rate of 300 ml/minute.

Sulfur Dioxide-Treated Beans (Code: 1510-BWIII-19,20-B-2-SO_2-,). Red kidney bush beans were planted and treated as described under Nitrogen Dioxide Treated Beans on January 3, 1967, and January 22, 1967, respectively. In this case, however, SO_2 gas at a concentration of 10 ppm (Matheson, Coleman, and Bell, Rutherford, N. J.) was used. The plants harvested three days after treatment showed little, if any damage. On January 27, 1967, the plants not yet harvested were subjected to an atmosphere of 10% SO_2 for 25 minutes to note the effect of a short exposure to a severe overdose. The leaves became a dull brown after treatment. Ten plants were harvested on January 28, 1967, one day after treatment. The stem tissue was still green, although the leaves had become severely damaged. An additional ten plants were harvested on February 3, 1967, four days after treatment.

Since 50 ppm NO_2 and 10 ppm SO_2 caused no apparent damage, the remaining bean plants were subjected to a series of varying concentrations of NO_2 and SO_2 for a period of one hour in an attempt to define more closely the limits of permissible treatment, which would cause physical damage to the plant but not kill it outright. The amount of gas needed to produce a given concentration was generated by use of an appropriate reaction and the amount needed calculated, using a chamber volume of 80 liters. In the case of the NO_2 the reaction of copper with concentrated nitric acid was

used, and in the case of SO_2 the reaction of sodium bisulfite with sulfuric acid was used.

Three bean plants were subjected to 1000 ppm NO_2 (Code: B-2-10^3NO_2-,) for one hour on March 3, 1967, and harvested on March 6, 1967. The leaves were severely damaged with a large number of yellow-brown spots, but the stems appeared to have sustained little damage.

Three bean plants were then subjected to 750 ppm NO_2 (Code: B-2-750NO_2-,) for one hour on March 6, 1967. These plants were harvested on March 13, 1967. The leaves were severely damaged with many of the leaves bleached and withered.

On March 6, 1967, three bean plants were treated with 300 ppm SO_2 (Code: B-2-300SO_2-,) for one hour. They were harvested on March 13, 1967. Only minor damage was noted with a little spotting on some leaves.

Sulfur Dioxide-Treated Tomatoes (Code: 1510-BWIII-21, 22-T-2-SO_2-,). Tomatoes, Lycopersicon esculentum Mill., Marglobe variety, were planted, using conditions described previously under the section entitled Nitrogen Dioxide-Treated Tomatoes on January 23, 1967, and treated on February 22, 1967 (age = 30 days), with SO_2 gas at a concentration of 10 ppm (Matheson, Coleman, and Bell, Rutherford, N. J.). Ten plants were harvested on February 25, 1967. There being little, if any, noticeable damage to the plants, they were subjected to a large overdose of SO_2 as described on page 26 under the section entitled Sulfur Dioxide-Treated Beans on February 27, 1967, for a

period of one hour. The leaves turned a dark brown and the petioles lost most of their green color and became almost translucent. Seven plants were harvested on February 28, 1967, one day after treatment. All of the treated plants were completely bleached and appeared to be dead.

The remaining tomato plants were treated with varying concentrations of NO_2 and SO_2 to determine the permissible limits of treatment which would allow severe damage but not kill them outright. NO_2 and SO_2 were generated as described under the section entitled Sulfur Dioxide-Treated Beans, and on March 1, 1967, three plants were treated with 500 ppm SO_2 (Code: T-2-500SO_2-,). They were harvested on March 3, 1967. There was extensive leaf damage with curling and bleaching. The stem tissue appeared to be healthy. On March 1, 1967, three plants were treated with 500 ppm NO_2 (Code: T-2-500NO_2-,) and harvested on March 6, 1967. The leaves showed only minor damage with some spotting of the lower leaves.

On March 7, 1967, three plants were treated with 100 ppm SO_2 (Code: T-2-100SO_2-,). Since there did not appear to be any damage to the plants one day after treatment, the plants were left for 11 days before harvesting. There was still no apparent damage to the leaves but the stems appeared to be thickened and had some tumor-like growths present.

Because of the minor damage to the tomatoes at 500 ppm NO_2, a higher level was tried. On March 3, 1967, three plants were treated with 1000 ppm NO_2 (Code: T-2-10^3NO_2-,) and harvested three

days after treatment. The plants showed fairly severe damage with a general browning of the leaves.

It appeared, from these studies, that tomatoes were more readily affected by SO_2 and beans more readily affected by NO_2. For tomatoes, the concentration needed to produce damage, with a one hour treatment period, would be approximately 100-500 ppm for SO_2 and 750-1500 ppm for NO_2. For beans the appropriate levels would be approximately 500-1000 ppm for SO_2 and NO_2.

Extraction of Plant Material

It was discovered that a single extraction of the macerated material with 2-propanol:water azeotrope was not sufficient to remove all the fluorescing material from the plant tissues. The following procedure was, therefore, designed for a quantitative extraction of the tobacco tissues.

The leaves, stems, or flowers were transferred to a Waring blender, with the aid of methanol. The material was then ground at high speed for 30 seconds or one minute. This suspension was poured into a thimble which had been placed in a glass extraction thimble funnel. This allowed the liquid to filter through the thimble directly into a round-bottom flask. Methanol was used to aid in the transfer of the material from the blender to the thimble. The tissue was then extracted by pouring the following solvents over the tissue.

1) hot 2-propanol-water (1:1; v/v)

2) hot IBMW (2-propanol:benzene:methanol:water; 2:1:1:1; v/v/v/v)

3) hot 2-propanol-water azeotrope.

The amount of solvent used in the case of 1) and 3) was three times the fresh tissue weight of the sample if it was a control sample and four times if it was a treated sample. The amount of IBMW used was 4 times the fresh tissue weight for a control sample and 5 times for a treated sample.

The thimble was then placed in a Soxhlet extraction apparatus and extracted for 24 hours with 2-propanol-water azeotrope. The tissue was then extracted a second time with fresh 2-propanol for another 24 hours. All of these extracts were combined and the solvent removed *in vacuo*. The dry or resinous material was then redissolved in a solvent mixture which approximated IBMW as follows:

1) The total volume necessary was calculated by multiplying the fresh tissue weight by 2.5 (Example: A 100 g sample would require a volume of 250 ml).

2) Approximately 70% of the total volume of benzene and water necessary was then added, and the mixture was shaken until the material was in suspension. (Example: With a 250 ml final volume, a total of 50 ml of benzene and water would be necessary. Therefore, 35 ml (70%) of benzene and water would be added.)

3) When the material had been suspended, the required amount of 2-propanol was added. (100 ml of 2-propanol would be necessary in the example.) This usually resulted in a clear solution.

4) This solution was then transferred to an appropriate volumetric flask and the round-bottom flask was rinsed with IBMW

to complete the transfer of all the material to the volumetric flask. The solution was then diluted to the mark with IBMW and 2-propanol or 2-propanol-water (1:1; v/v). These solutions were then put in containers and placed in the cold room. A small vial of the sample was poured out for general use and placed in the freezer.

The root samples were chopped or cut rather than ground in the Waring blender since grinding produced a suspension which did not allow solvents to filter through the extraction thimble easily.

Thin layer chromatography of some of the initial tomato and bean plant extracts indicated that the extraction procedure could be shortened. The extraction procedure was modified as follows:

1) The hot 2-propanol-water azeotrope extraction was eliminated for all of the tomato tissue samples and for the root and stem tissue samples of the beans.

2) The length of the first Soxhlet extraction with 2-propanol azeotrope was shortened to 6-12 hours for all of the tomato tissue samples, the bean stem, and root tissue samples.

3) The second Soxhlet extraction with 2-propanol was eliminated for all tomato and bean tissue samples.

4) The total volume necessary was calculated by multiplying the fresh tissue weight by 2 instead of 2.5

Chromatographic Studies on the Separation of Scopolin and Scopoletin

Development of a Multi-dimensional Separation of Scopolin and Scopoletin on Macherey and Nagel Polyamide. Macherey and Nagel polyamide was first used in these thin layer chromatography experiments since it gave a firmer plate than did Woelm polyamide. The following solvents were tried on a treated tobacco leaf and root sample as well as on standard scopolin and scopoletin solutions in order to observe the clarity of separation of scopolin and scopoletin from other compounds, particularly from the chlorophyll in the leaves: 1) water, 2) 2% acetic acid (v/v), 3) 2% formic acid (v/v), 4) MW (1:1; v/v), 5) MW (2:8; v/v), 6) benzene, 7) BzMF-1, 8) MAW (methanol: acetic acid:water; 5:0.2:5; v/v/v), followed by benzene in the same direction, 9) NIF (nitromethane:2-propanol:formic acid (98%); 5:2:0.3; v/v/v), 10) IFBz (2-propanol:formic acid (98%):benzene; 5:0.15:2; v/v/v), 11) BAW, 12) IFW-1, and 13) NFM. The results indicated that a two-dimensional development would be necessary to achieve the desired separation. Therefore, the following solvent combinations were tested on a treated leaf sample: 1) NFM, IFW-1; 2) IFW-1, NFM; 3) KFW, IFBz; 4) IFBz, KFW; 5) NIF, IFBz; 6) NIF, KFW; and 7) IFBz, NFM. The results of the one dimensional studies indicated that a different set of solvents should be necessary for use on the root samples. The following two-dimensional combinations were tested on a treated root sample: 1) IFW-1, NFM; 2) NFM, IFW-1; 3) IFW-1, NIF; 4) NIF, IFW-1; 5) NIF, NFM; 6) NFM, NIF; and 7) IFW-1, IFBz.

The results indicated that the scopolin and scopoletin in the root samples were satisfactorily separated by IFW-1, NFM. The leaf samples, however, did not yield the desired separation even in two dimensions, in the combinations studied. Therefore, a number of three-dimensional solvent combinations were tried. In these cases, the third solvent was run in the same direction as the first solvent. The hope was that the third solvent would make the scopolin and scopoletin spots more compact. After a review of the data the following six solvent combinations were tested: 1) NIFBz, NFM, IFW-1; 2) NIFBz, IFW-1, NFM; 3) benzene, NFM, IFW-1; 4) benzene, IFW-1, NFM; 5) NIFBz, NFM, IFW-1; and 6) NMFBz (nitromethane:methanol: formic acid (98%):benzene; 5:2:0.25:5; v/v/v/v), IFW-1, NFM.

A review of all the data collected indicated that the best solvent combinations of those tried were IFW-1, NFM for the root samples and NIFBz, IFW-1, NFM for the leaf samples.

Since a rapid method was being sought, it was decided not to use Macherey and Nagel polyamide for the following reasons: Two different solvent combinations would be needed to analyze the samples and one solvent combination was preferable. A rapid method was being sought and the best solvent combinations found included IFW-1 which had a migration time of approximately $5\frac{1}{2}$ hours. The length of this development plus the drying time and development in one or two other solvent combinations appeared to make this approach rather lengthy. Also, it was felt that the slight purple fluorescent background might interfere in the direct readout method contemplated.

Development of a Two-Dimensional Separation of Scopolin and Scopoletin on Woelm Polyamide. Woelm polyamide was used in the rest of the studies since it did not have a fluorescent background and it usually yielded better separations than did Macherey and Nagel polyamide. Also, with it, faster migration times were obtained than with the corresponding solvents developed with Macherey and Nagel polyamide. The following multi-dimensional solveng combinations were tested on Woelm polyamide which had been spotted with a treated leaf sample plus scopolin and scopoletin: 1) BzMF-1, KFW; 2) KFW, BzMF-1; 3) BzMF-2, KFW; 4) KFW, BzMF-2; 5) BzMF-1, IFW-1; 6) IFW-1, BzMF-1; 7) BzMF-2, IFW-1; 8) IFW-1, BzMF-2; 9) IFW-1, KFW, BzMF-2; 10) IFW-1, KFW, BzMF-1; 11) IFW-1, BzMF-2, KFW; 12) BzMF-2, IFW-1, KFW; 13) NIFBz, IFW-1, KFW; 14) IFW-1, KFW; 15) IFW-1, BzMF-2, BzMF-2; 16) BzMF-1, IFW-1, BzMF-2; 17) KFW, IFW-1; 18) NIFBz, KFW, IFW-1; 19) IFW-1, IFW-1; 20) KFW, KFW; 21) BzMF-2, KFW, BzMF-1; 22) KFW, BzMF-2, BzMF-1; 23) KFW, BzMF-2, BzMF-2; 24) BzMF-2, KFW, BzMF-2; 25) IFW-1, BzMF-2, BzMF-1; 26) BzMF-1, MW; 27) BzMF-1, NIFBz; 28) MW, BzMF-1; 29) NFM, IFW-1; 30) NFM, KFW; 31) NFM, BzMF-1; 32) NIFBz, IFW-1; and 33) NIFBz, NFM.

An analysis of the data indicated that solvent combinations 1) and 2) gave excellent separations of scopolin from scopoletin as well as from the chlorophyll. The scopolin and scopoletin spots were also compact. BzMF-1 appeared to be a good system for a one-dimensional separation of scopolin from scopoletin. The major disadvantage of the two-dimensional system using solvent

combinations 1) and 2) was that KFW increased the fragility of the plate.

Some additional studies were done at a later time in order to see if the separation could be improved further since BzMF-1 seemed to be very temperature dependent. Small changes in temperature sometimes caused a double solvent front to form. This led to irregular spots for scopolin and scopoletin and a smaller than desired separation of scopolin from scopoletin.

BzMF-1 was modified by the addition of more benzene to the mixture. Two different modifications were tested: a) BzMF-4 (benzene:methanol:formic acid(98%); 4:1:0.1; v/v/v) and b) BzMF-5 (benzene:methanol:formic acid(98%); 5:1:0.1; v/v/v). These two modified solvents were tested on a treated leaf sample plus scopoletin in the following two-dimensional solvent combinations: 1) BzMF-4, MW; 2) BzMF-5, MW; 3) BzMF-4, KFW; 4) BzMF-5, KFW; and 5) BzMF-1, BzMF-4. Although excellent separations of scopolin and scopoletin occurred in all of the above systems, only solvent combination 3) yielded spots which were compact enough. BzMF-4 also yielded excellent separations in one-dimension.

It was thought that the crude mixture probably contained water or MW (1:1) soluble compounds, such as sugars and amino acids, which contributed to the elongation of the scopolin and scopoletin spots, with a concomitant loss in separation. With this in mind, a new three-dimensional technique was tried. The procedure involved allowing water or MW (1:1) to migrate up the plate for various

distances, drying the plate and running a second solvent in the same direction, followed by a third solvent at ninety degrees to the first two solvents. The following combinations were tested on a treated leaf sample plus scopoletin.

1) Water migrated approximately 3 cm, BzMF-4, BzMF-5;

2) Water migrated approximately 15 cm, BzMF-4, BzMF-5;

3) MW (1:1) migrated approximately 3 cm, BzMF-4, BzMF-5;

4) MW (1:1) migrated approximately 3 inches, BzMF-4, BzMF-5;

5) MW (1:1) migrated approximately 15 cm, BzMF-4, BzMF-5; and

6) MW (1:1) migrated approximately 3 inches, BzMF-5, BzMF-4.

Analysis of the data indicated that only solvent combinations 2) and 4) yielded spots which were significantly more compact. Examination of the plates developed with Woelm polyamide (in one-, two-, or three-dimensions) indicated that there were no major blue-fluorescent contaminants in the scopolin and scopoletin areas of a plate developed in BzMF-1 and BzMF-4. Since a rapid method of analysis was being sought, a one-dimensional approach was used since it appeared to be as efficient as a multi-dimensional method and had the advantage of speed.

Development of a New, Rapid Quantitative Method for the Analysis of Scopolin and Scopoletin

Attempted Development of a Direct Readout Procedure. The initial series of experiments were devised with a one-dimensional approach using BzMF-1 for the following reasons:

It was more rapid than a two-dimensional method both from the point-of-view of the time required to run the second solvent and because up to nine samples could be placed on a plate using a one-dimensional technique and only one sample could be spotted in a two-dimensional technique.

Analysis of the chromatographic data indicated that the scopolin and scopoletin areas contained no other blue-fluorescing spots which could interfere in the analysis.

The polyamide layer tended to be less fragile when only one solvent was used rather than two or three.

The best separating solvent for the second dimension was KFW which tended to increase the fragility of the plate greatly.

In the first series of experiments, small amounts of standard scopolin solutions in methanol were spotted on a Woelm polyamide plate with a Kirk-design micropipet. This plate was developed in BzMF-1 (approximately one hour migration time). The plate was placed in a drying chamber equipped with exhaust fans for one hour or more to remove all traces of formic acid from the polyamide layer. The plate was then placed, with the polyamide layer face down, on the stage of a Photovolt TLC Densitometer Model 530, equipped with a Photovolt Multiplier photometer Model 520-M, Varicord variable response recorder Model 42-B, and Integraph integrator Model 49. This instrument had been modified for fluorimetry. The instrument contained a means for changing the secondary filter but not the primary filter. No information could be obtained about the

excitation wavelength of this filter. The polyamide plates were placed on the stage so that the developed spots of scopolin and scopoletin were scanned in a direction which was ninety degrees to the direction in which the chromatogram had been developed. Appropriate settings were made on the instrument, and the area of each spot on the plate was determined at least twice. Three plates were spotted with 5-9 samples of different concentration of scopolin ranging from 0.125-10.80 μg, and analyzed as described above. The instrument was set at 100 x and R 1. R is a response setting on the recorder. This can be varied from 1-12, but 1 was suggested by the Photovolt company for fluorimetry. The photomultiplier could be varied from 1 x to 1000 x in multiples of ten. 100 x or 10 x were used in all cases. A #110-816, sharp cut filter, color spectrum #2A, which passes all wavelengths longer than 415 mμ, was used as the secondary filter. In the early experiments the fluorescent lights were turned off since they interfered in the analysis. In later experiments a wooden frame covered with several layers of a dull black cloth was used to eliminate the interference due to the fluorescent lights.

This first series of experiments indicated several problems. There seemed to be a great deal of photodecomposition as indicated by a large decrease in the area when determined a second time. A straight line could not be drawn through all of the points due to the variability of the data. A straight line drawn through as many of the points as possible did not go through the origin.

The photodecomposition was thought to be partly due to the primary filter. Therefore the instrument was modified so that the primary filter could be changed as well as the secondary filter. The modified instrument was then equipped with a #110-811, narrow pass filter, color spectrum # 7-60, which peaked at 360 mμ.

In the next series of experiments, three plates were spotted with nine samples on each plate ranging in concentration from 0.125-10.80 μg and determined as described above. Although the results indicated a smaller photodecomposition, the data were still extremely variable and a line drawn through as many points as possible did not pass through the origin. The data also indicated that the linear range may be above one μg or that there were two linear ranges (0-1 and 1-10 μg) with different slopes. Scopolin at concentrations above 10 μg seemed to undergo quenching so 10 μg appeared to be the upper limit.

In an attempt to eliminate some of the variability in the data, the samples were spotted in triplicate on each plate. Three more plates were analyzed as indicated previously using scopolin in a concentration range of 1.15-8.85 μg. Although the variability of the data decreased, a straight line could not be drawn which would include all of the points and the line did not pass through the origin. A plate was analyzed with a series of different secondary filter combinations and photomultiplier settings in an attempt to improve the sensitivity. The following conditions were tested:

Secondary filter	Settings
#2A	100x, R1
#2A plus 47B	100x, R1
47B	100x, R1
#2A plus 48	10x, R1
48	10x, R1

Analysis of the data indicated that the best overall combination was the following: primary filter - #7-60, secondary filter - #2A plus 48, instrument settings of 10x, R1. The 47B and 48 filters were Kodak Wratten filters.

Four plates were spotted, in triplicate, with a single standard scopolin solution on each plate and analyzed as previously indicated. The results were again very variable. A Hamilton 10 µl syringe, with a Chaney adapter, was used for spotting since great difficulty had been incurred when spotting with a micropipet.

Two plates were spotted, in duplicate, and analyzed. A new standard scopolin solution was made and diluted to two different concentrations in an attempt to eliminate the continued variability of the data. A scopoletin standard was also used.

Six plates were analyzed in the next series of experiments. The data still had variability, although not as great as previously detected and there was still some photodecomposition occurring. The best straight line still did not pass through the origin.

A 1 mm slit, constructed of black construction paper, was taped over the regular slit of the instrument in an attempt to

reduce the photodecomposition. Nine plates were analyzed with this modification. In addition, different response settings were tested. The results were poorer than those obtained with the regular slit and the photodecomposition was not decreased. The smaller slit was thus abandoned in favor of the regular slit.

Eight plates were analyzed using the regular slit and different response settings (R1, R2, R3, R5, R7). The area was determined by triangulation as a check on the integrator's results. Scopoletin appeared to be less subject to photodecomposition than scopolin and as a result the plots were more nearly linear and closer to the origin. There was still wide variability of the scopolin data, however. The data were also plotted on semilogarithmic graph paper but the linearity of the data was not improved.

Two plates were analyzed using very low concentrations of scopoletin (0.005 to 0.025 μg) and scopolin (0.026 to 0.109 μg). The results were very erratic indicating that these concentrations were apparently too low to be easily determined.

Eight plates were run in two-dimensions using BzMF-1, KFW. Three samples were spotted on the plate: two samples of a treated tobacco leaf sample and a standard solution of scopolin and scopoletin. KFW was only allowed to migrate in the second dimension to a point that was below where the other samples had been spotted. In this way a sample could be checked in one as well as two dimensions on one plate. The standard solutions exhibited small variations, but the treated leaf samples were more erratic.

This prompted another look at the overall procedure. It was noted that in spotting the leaf sample some of the solution appeared to be left on the needle of the Hamilton syringe. The variation might have been due to a partial loss of sample. Six plates were spotted with a standard scopoletin solution. Two were spotted with a Hamilton syringe; two with a micropipet, and two with a 0.2 ml RGI micrometer syringe (Kontes Glass Co., Vineland, N. J.), equipped with a 26 gauge needle. The micrometer syringe had the least amount of variability, although none of the plots passed through the origin.

Several plates were tried in BzMF-4 using improvements in the overall procedure as described in the next section. This data yielded equally poor results.

T. L. Innerarity in this laboratory attempted some later work on scopolin using Avicel-SF (250 microns thick) with BAW and EPW as the developing solvents. A similar lack of success was noted. The direct readout procedure was abandoned for the following reasons. Although much of the variability in the data, itself, was removed by improvements in the overall procedure the plots of the data obtained were not linear and did not pass through the origin. The instrument was not too stable and had to be continually adjusted. The integrater yielded areas with very low counts. The count rate could not be increased even by adjustment of the parameters. In addition, the area obtained seemed to be directly correlated to the spot size. The necessity of placing the plate face down frequently caused a loss of material from the plate. Photo-decomposition was very pronounced, especially with scopolin.

As a result great care had to be exercised not to expose any of the spots to the light source for any length of time before the actual determination. Very small amounts of scopolin and scopoletin could not be determined with this approach.

Development of a One-Dimensional Elution Method. The lack of success in the direct readout method prompted the development of an elution method. The basic approach was similar in design to that used for the direct readout procedure. Woelm polyamide plates (250 microns thick), spotted in triplicate, were developed in one-dimension with BzMF-1, in the first series of experiments. BzMF-4 was substituted in the later series of experiments due to the problems with BzMF-1 discussed in a previous section. They were placed in a drying chamber with exhaust fans. After the last traces of formic acid had been removed, the scopolin and scopoletin spots were located under a long-wavelength ultraviolet light. The spots were outlined with a dissecting needle. A blank area of approximately the same size was also outlined. The outlined spots were removed with a microvacuum cleaner apparatus (38, Fig. 2). A one cm diameter circle of glass fiber (cut from a Cambridge filter, Phipps and Bird, Inc., Richmond, Va.) was pushed into one end of the vacuum cleaner until it was firmly seated. The glass fiber was washed with five portions of approximately two ml of methanol to insure removal of all possible impurities. A vacuum line from a water aspirator was attached to the filter end of the cleaner, and the adsorbent containing the desired sample was swept from the plate

into the cleaner chamber. The vacuum line was then disconnected and a small-diameter polyethylene tube was inserted into the filter end of the cleaner. The cleaner was then fitted, with the filter end down, into a one-hole rubber stopper which seals the top of a specially designed glass vacuum cylinder (37, Fig. 5). The polyethylene tube was inserted into the neck of a 5 ml volumetric flask contained in the vacuum cylinder. Vacuum from a water aspirator was then applied and approximately 5 ml of hot methanol was added, in small increments, to elute the scopolin and scopoletin from the adsorbent. The volumetric flask was then allowed to reach room temperature and then was diluted to the mark with methanol and thoroughly mixed. The fluorescence determinations were made with a model 110 Turner fluorometer (G. K. Turner Associates, Palo Alto, Calif.), using Pyrex cuvets and a high sensitivity attachment. A study of the four ranges available on the fluorometer (1x, 3x, 10x, 30x) indicated that 3x and 10x gave the best results. The primary and secondary filters used for the direct readout procedure were also used in these studies.

Nine plates were analyzed as described in the preceding section to check the one- versus two-dimension results on scopolin and scopoletin. The values obtained indicated that the one-dimensional method was as efficient as the two-dimensional method. The early studies were done with a micrometer syringe equipped with a 26 or 30 gauge needle. Since some of the early data were quite variable the entire procedure was reviewed in an attempt to improve

the overall results. It was first discovered that a larger needle (24 gauge) gave less run-back than the smaller gauge needles. It was also learned by experimentation with different proportions of 2-propanol/water mixtures that a minimum of 70% water was necessary to avoid any run-back on the needle. The solvent system was changed to BzMF-4. New standards were made up and kept in the freezer to avoid any breakdown of the sample in solution. It was also discovered that 2 µl increments of standard solutions and 3 µl increments of fresh tissue could be readily spotted with no loss of the sample.

Standard scopolin and scopoletin solutions were each added directly to a 5 ml volumetric flask and diluted to the mark and their fluorescence determined. Scopolin and scopoletin were determined at 3x with a #2A plus #48 plus 50% neutral density filter for the secondary filter arrangement. Figures 2 and 3 indicated that scopolin was linear in the range of 0 to 5.0 µg and scopoletin in the range of 0 to 0.5 µg.

Six plates were analyzed using the modified procedure. All of the plots yielded linear lines passing through the origin.

Later studies indicated that the small amounts of tissue sample that were spotted on the plate would yield less than 1 µg of scopolin and 0.1 µg of scopoletin. Therefore, the linearity was re-examined in this lower range. Standard scopolin and scopoletin samples were spotted and analyzed as described previously. Scopolin was determined at 3x and the scopoletin at 10x. At these lower

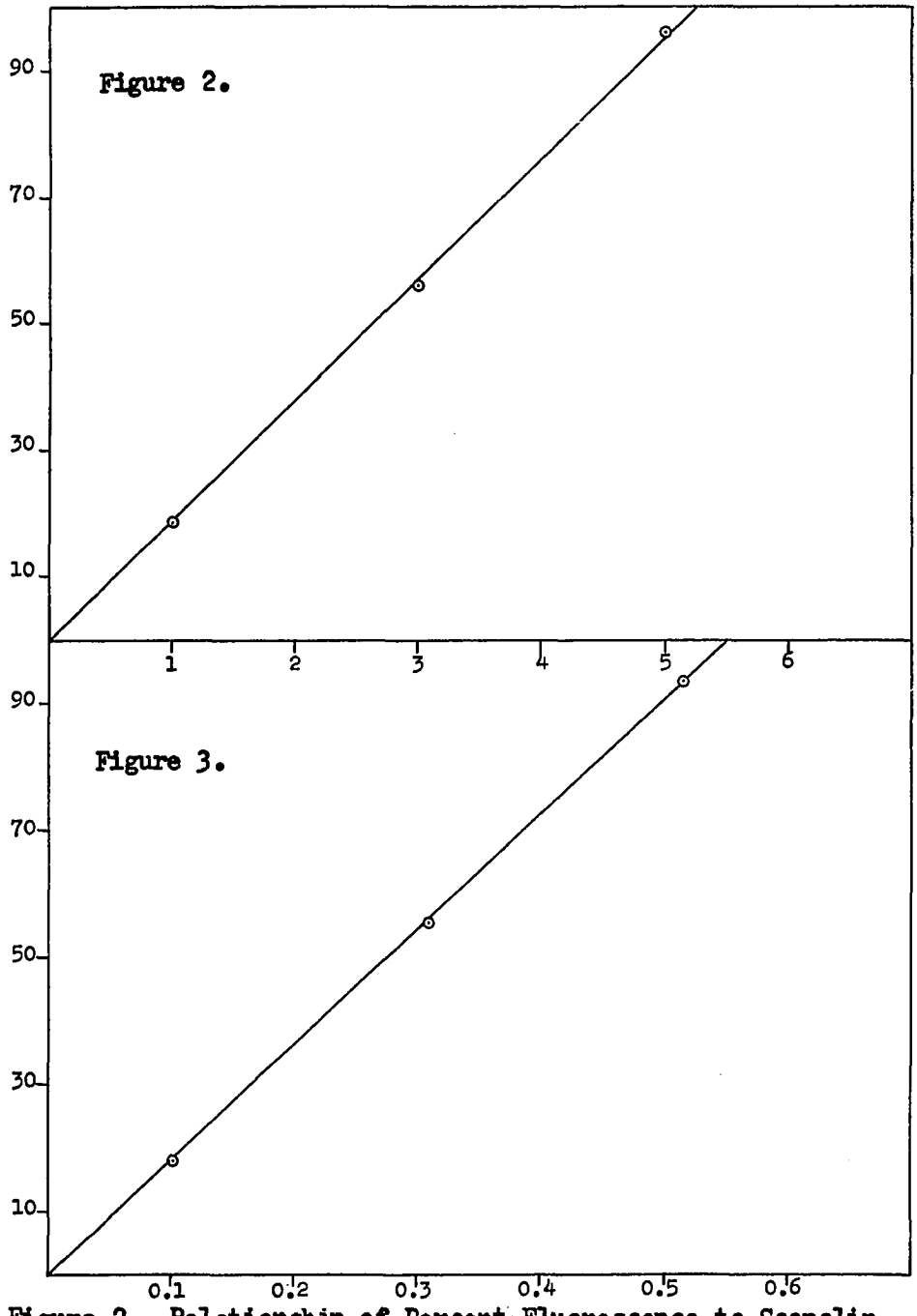


Figure 2. Relationship of Percent Fluorescence to Scopolin Concentration.
Figure 3. Relationship of Percent Fluorescence to Scopoletin Concentration.
Instrument, Turner Fluorometer. Sensitivity Setting, 3x. Primary Filter, 7-60. Secondary Filter, 2A+48+50% Neutral Density Filter. Abscissa: µg Material/5 ml. Ordinate: Percent Fluorescence.

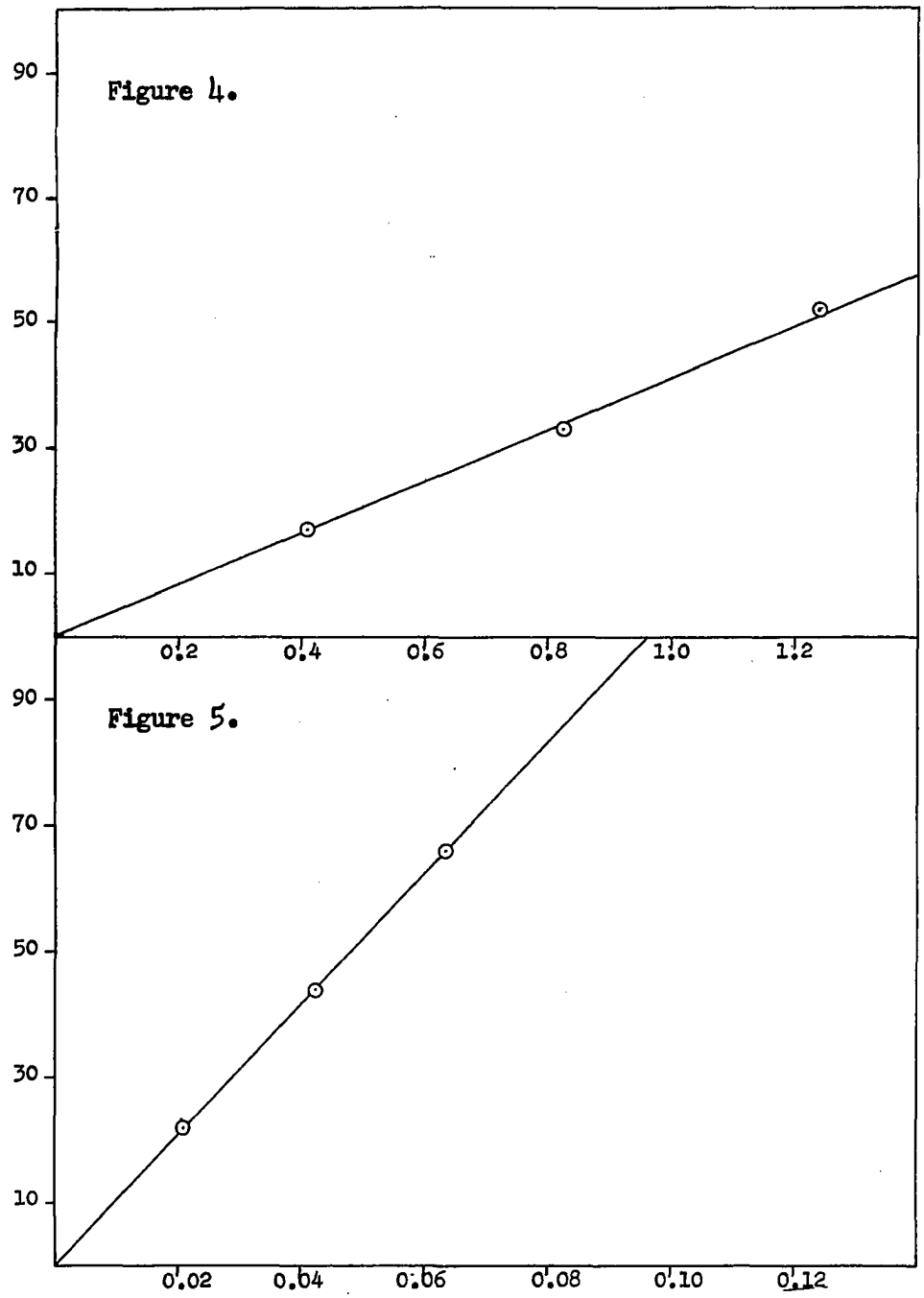


Figure 4. Relationship of Percent Fluorescence to Scopolin Concentration.
Figure 5. Relationship of Percent Fluorescence to Scopoletin Concentration.
Instrument, Turner Fluorometer. Sensitivity Setting, 3x (Fig. 3), 10x (Fig. 4). Primary Filter, 7-60. Secondary Filter, 2A+48.
Abscissa: µg Material/5 ml. Ordinate: Percent Fluorescence.

values of scopolin and scopoletin, no neutral density filter was needed. Figures 4 and 5 indicated that the data were linear with the line passing through the origin.

The reproducibility was checked by spotting eight plates with a standard scopolin, standard scopoletin and a tobacco control root solution. The amount of scopolin and scopoletin present in the control root sample was calculated, and the data obtained indicated that this method was reproducible.

Even though the chromatographic studies indicated no interfering substances the possibility of some was rechecked. Approximately 400 µl of a tobacco root control solution was spotted on a Woelm plate and developed in BzMF-4. The scopolin and scopoletin zones were removed by use of the microvacuum cleaner and eluted with ten ml of hot methanol. The solution was concentrated, in vacuo, to a small volume and 10, 20 and 30 µl of the scopoletin and scopolin samples were spotted on two Woelm and two Avicel-SF (250 microns thick) plates. The Woelm plates were developed with KFW and MW, and the Avicel-SF plates were developed with BAW and IFW (2-propanol: formic acid (98%):water; 5:0.1:95; v/v/v). The plates were then examined under long-wavelength ultraviolet light. The results indicated that there was no significant contamination of either the scopolin or scopoletin zones.

As a final check, the tobacco control root solution was spotted on two Woelm plates along with standard scopolin and developed in BzMF-4. One plate was subjected to the previously described analysis. The standard and unknown scopolin zones were eluted from

the other plate. The solvent was removed, *in vacuo*, and 200 µl of methanol was added. The standard and unknown scopolin solutions were banded, in triplicate, on an Avicel-SF (250 microns thick) plate and developed in IFW (2-propanol:formic acid (98%):water; 5:2:95; v/v/v). These samples were eluted and analyzed in the Turner fluorometer as previously described. The calculated results were within 5% of the average.

The essentials of the procedure finally adopted were:

(1) Woelm plates (containing 0.1 g soluble potato starch/plate, 250 microns thick) were spotted, in triplicate, with two unknown samples and a standard scopolin and scopoletin solution. A 0.2 ml micrometer syringe, equipped with a 24 gauge needle, was used to spot the samples.

(2) The plates were developed in BzMF-4 for approximately 40 minutes and dried for approximately one hour.

(3) The scopolin and scopoletin zones plus a blank were removed with the microvacuum cleaner and eluted with hot methanol.

(4) The fluorescence was determined and a line was drawn through the origin. Curves were thus obtained for the standard scopolin and scopoletin. The amount of scopolin and scopoletin in the unknown samples was then determined graphically. This yielded the concentration of scopolin and scopoletin in the unknown solution in terms of µg/number of µl originally spotted.

(5) The data were then calculated in terms of the number of µg of scopolin and scopoletin present/g of fresh tissue.

This method had the major advantage of speed when compared to the other published methods of determination. A complete analysis of the scopolin and scopoletin present in two unknown samples could be accomplished in less than eight hours. Preliminary purification or separation of the fresh tissue extract was not necessary. The method could be used to detect extremely small amounts of scopolin and scopoletin. This is an advantage accruing to fluorescence analysis methods in general. In addition, a standard solution of scopolin and scopoletin was spotted on every plate. Therefore, the standards were subjected to the same amount of variation as the unknown samples. The method is very specific since the primary and secondary filters chosen very closely approximated the excitation and emission wavelengths of scopolin and scopoletin. Thus, the only compounds which could have interfered would have to have excitation and emission wavelengths very similar to those of scopolin and scopoletin. Very small tissue samples could be determined since less than 30 µl was needed. There appeared to be two disadvantages. The polyamide layer was very fragile and so extreme care had to be exercised in spotting and handling the plate. The polyamide material, itself, seemed to fluctuate in quality somewhat from batch to batch.

Analysis of Maleic Hydrazide-Treated Tobacco and 2,4-D-Treated Tobacco

Maleic hydrazide is used by tobacco growers to eliminate sucker formation chemically. The tobacco produced by this treatment,

however, does not have the same quality or taste as that produced by manually removing the suckers. It was thought that maleic hydrazide might affect the phenolic content of tobacco, particularly the coumarins scopolin and scopoletin. In order to follow quantitatively the change in the scopolin and scopoletin content as a function of time in such treated tobacco, maleic hydrazide was sprayed on tobacco plants, and the quantitative procedure for scopolin and scopoletin as described in the preceding section was used.

The leaves, stems and roots of the maleic hydrazide-treated tobacco plants and the flowers of the 2,4-D-treated tobacco plants were analyzed for their scopolin and scopoletin content. The results are listed in Tables IX and X and Figures 6-15. The standard deviation was also calculated for each result (105). The results listed in Tables IX and X are the average of 2-6 analyses.

Figures 6 and 7 indicate the change in scopolin and scopoletin in leaves as a function of time. Both the treated and control leaves underwent only minor changes in scopolin and scopoletin content for the first 15 days after treatment. The scopolin content then rose rapidly in the treated leaves to the 47th day after treatment.

The scopolin content increased almost 5-fold from the 15th to the 29th day in control leaves and then decreased to a level very similar to the level before the 15th day.

TABLE IX

SCOPOLIN AND SCOPOLETIN IN MALEIC HYDRAZIDE-TREATED TOBACCO

	TNL				CNL			
D	I	II	III	IV	I	II	III	IV
3	5.3	2.0	0.8	0.1	5.6	0.0	1.1	0.2
7	7.8	0.2	1.0	0.1	4.0	1.3	0.8	0.1
15	8.9	0.3	0.8	0.2	3.3	0.0	0.8	0.0
29	54	3.5	5.2	0.3	16	2.5	2.7	0.4
47	139	1.7	3.3	0.2	6.8	0.8	1.2	0.3
	TNS				CNS			
D	I	II	III	IV	I	II	III	IV
3	31	0.0	0.7	0.1	15	3.2	0.5	0.1
7	63	6.2	1.5	0.1	15	0.0	0.9	0.2
15	223	11	3.8	0.4	23	3.6	0.9	0.4
29	255	3.2	6.3	0.0	20	3.5	1.4	0.1
47	400	6.4	9.6	0.8	42	5.7	0.9	0.1
	TNR				CNR			
D	I	II	III	IV	I	II	III	IV
3	38	0.0	0.6	0.0	47	9.1	0.6	0.1
7	22	1.6	0.6	0.0	63	4.1	1.3	0.1
15	144	1.7	1.9	0.1	38	0.0	0.8	0.1
29	177	4.5	3.0	0.4	79	2.1	2.0	0.1
47	180	4.3	7.7	0.4	128	1.6	3.1	0.3

I: µg of scopolin/g of fresh tissue
II: standard deviation of I
III: µg of scopoletin/g of fresh tissue
IV: standard deviation of III
T: treated sample
C: control sample
N: Nicotiana tabacum
L: leaves
S: stems
R: roots
D: days after treatment with maleic hydrazide

TABLE X

SCOPOLIN AND SCOPOLETIN IN 2,4-D-TREATED TOBACCO FLOWERS

	aTF				aCF			
D	I	II	III	IV	I	II	III	IV
8	163	12	4.0	0.0	55	4.1	1.28	0.1
14	170	3.5	3.7	0.1	53	0.0	1.74	0.0
21	325	1.4	7.1	0.0	69	5.2	1.97	0.2
28	144	8.1	3.2	0.2	52	6.7	2.5	0.0
35	180	5.3	6.4	0.6	107	2.1	4.9	0.0
	bTF				bCF			
D	I	II	III	IV	I	II	III	IV
8	142	2.9	4.6	0.1	44	1.7	1.4	0.1
14	100	3.7	3.1	0.1	37	2.4	2.1	0.1
21	275	0.0	7.4	0.0	60	3.6	1.9	0.2
28	175	9.0	3.4	0.0	56	3.5	2.6	0.3
35	67	12	5.9	0.8	86	2.4	4.6	0.1

T: treated parts
C: control parts
F: flowers
a and B: duplicate sets
I: µg of scopolin/g fresh tissue
II: standard deviation of I
III: µg of scopoletin/g fresh tissue
IV: standard deviation of III
D: days after treatment with 2,4-D

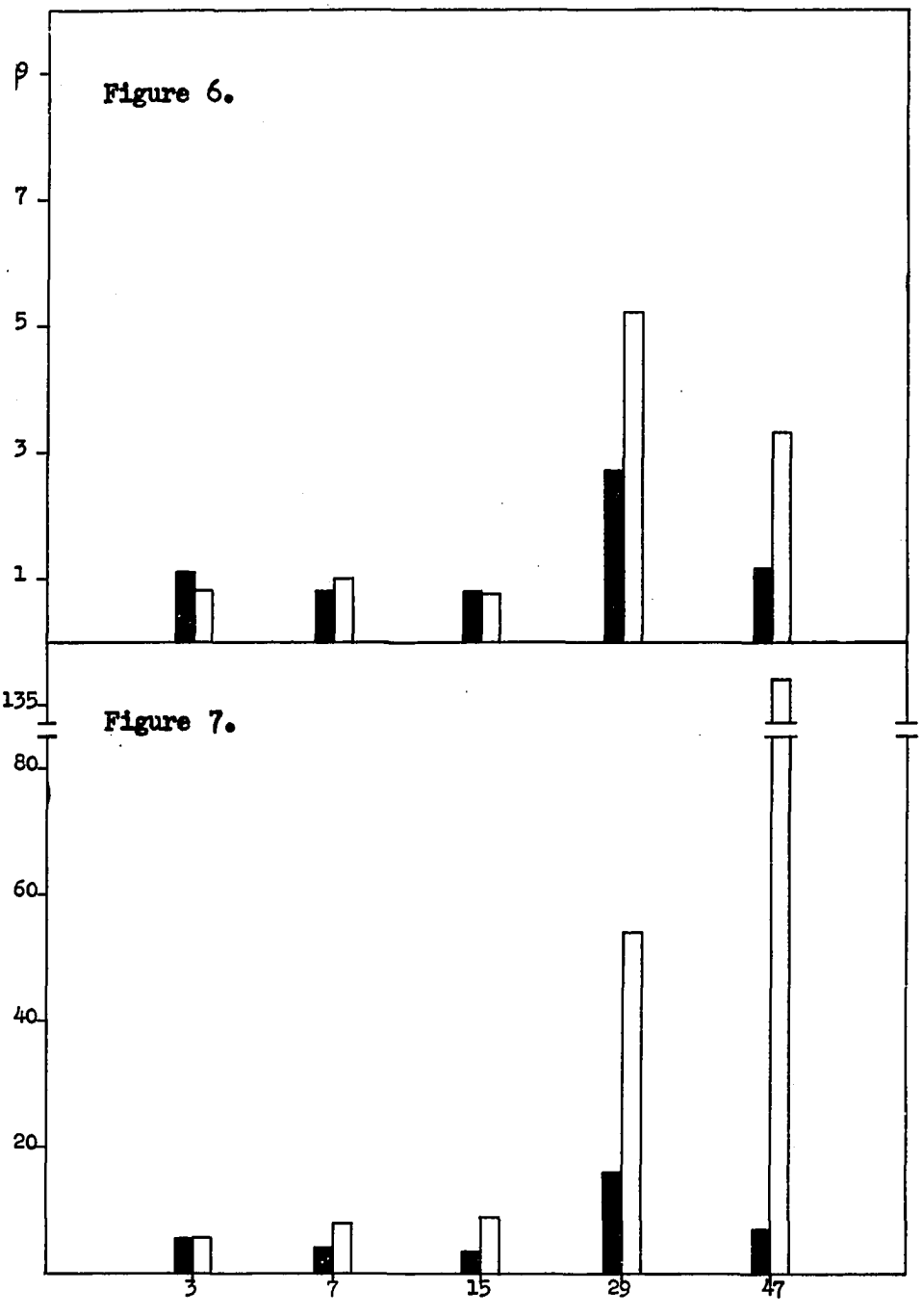


Figure 6. Variation of Scopoletin Content in Leaves of Tobacco Plants Treated with Maleic Hydrazide.
Figure 7. Variation of Scopolin Content in Leaves of Tobacco Plants Treated with Maleic Hydrazide.
Abscissa: Days after Treatment with Maleic Hydrazide. Ordinate: µg Material/g Fresh Tissue. Black = Control Leaves, White = Treated Leaves.

The scopoletin content in treated leaves increased approximately 5-fold from the 15th to the 29th day and then gradually decreased by the 47th day. The control leaves followed a similar pattern to that described for the scopolin levels in the control leaves.

Figures 8 and 9 show the changes in scopolin and scopoletin content of stem tissue. The scopoletin content of treated stems increased continually to the 47th day reaching a level approximately 10-fold higher than the first sample. The scopoletin content of the control stems very slowly increased to the 29th day and then decreased slightly by the 47th day.

The scopolin content of treated stem tissue showed a continued increase to the 47th day reaching a level approximately 20 times that of the first sample. The scopolin content of the control stem tissue showed only minor fluctuations until the 29th day and then gradually increased to a level approximately twice that of the first sample by the 47th day.

Figures 10 and 11 show the changes in scopolin and scopoletin content in root tissue. Both the treated and control root tissues showed a continued increase in scopoletin until the 47th day. The control root tissue showed a 6-fold increase while the treated root tissue gave a 10-fold increase.

The scopolin content of treated root tissue increased rapidly to the 29th day and then appeared to level off at a level approximately 5 times that of the first sample. The control root

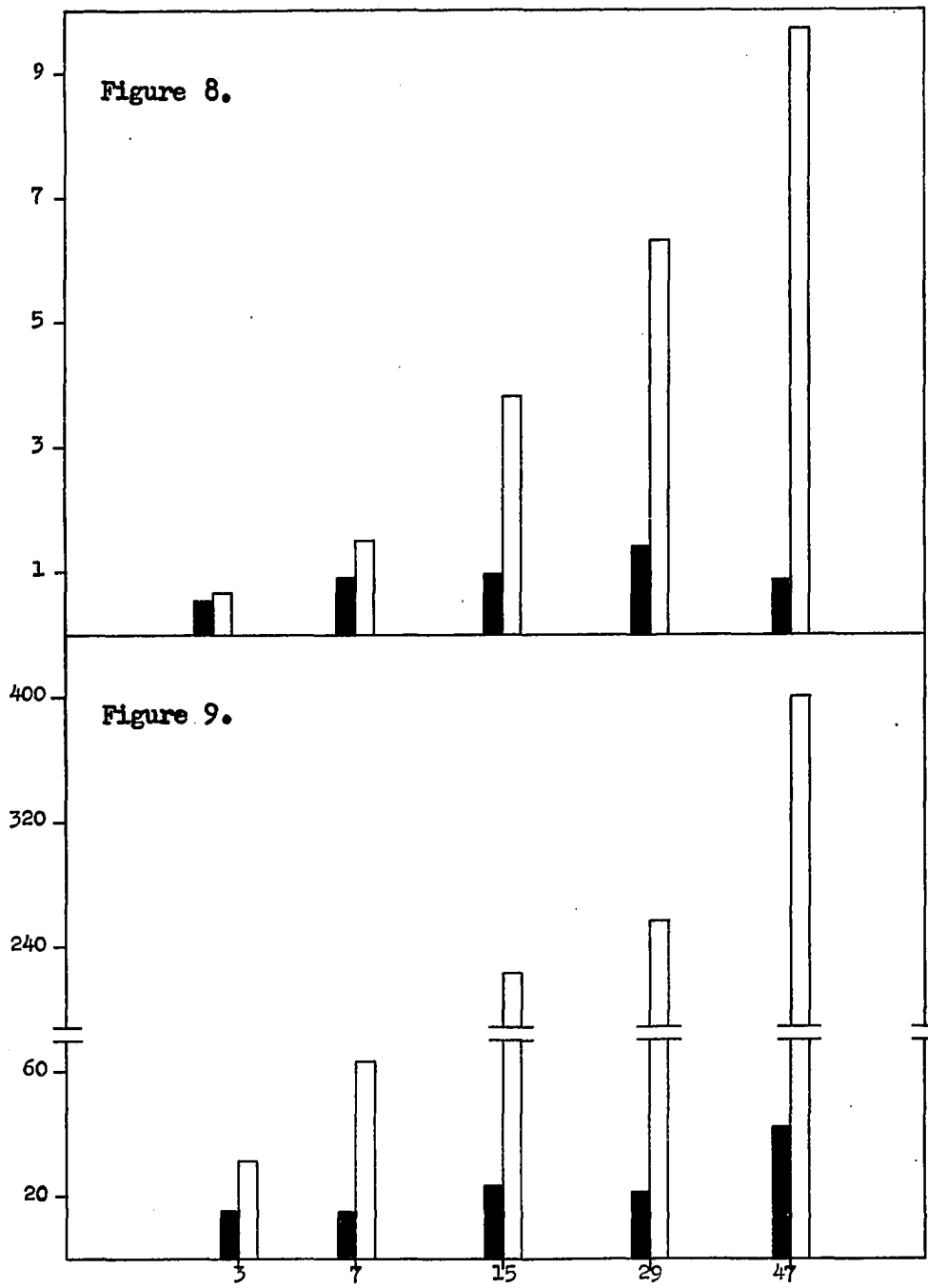


Figure 8. Variation of Scopoletin Content in Stems of Tobacco Plants Treated with Maleic Hydrazide.
Figure 9. Variation of Scopolin Content in Stems of Tobacco Plants Treated with Maleic Hydrazide.
Abscissa: Days after Treatment with Maleic Hydrazide. Ordinate: µg Material/g Fresh Tissue. Black = Control Stems, White = Treated Stems.

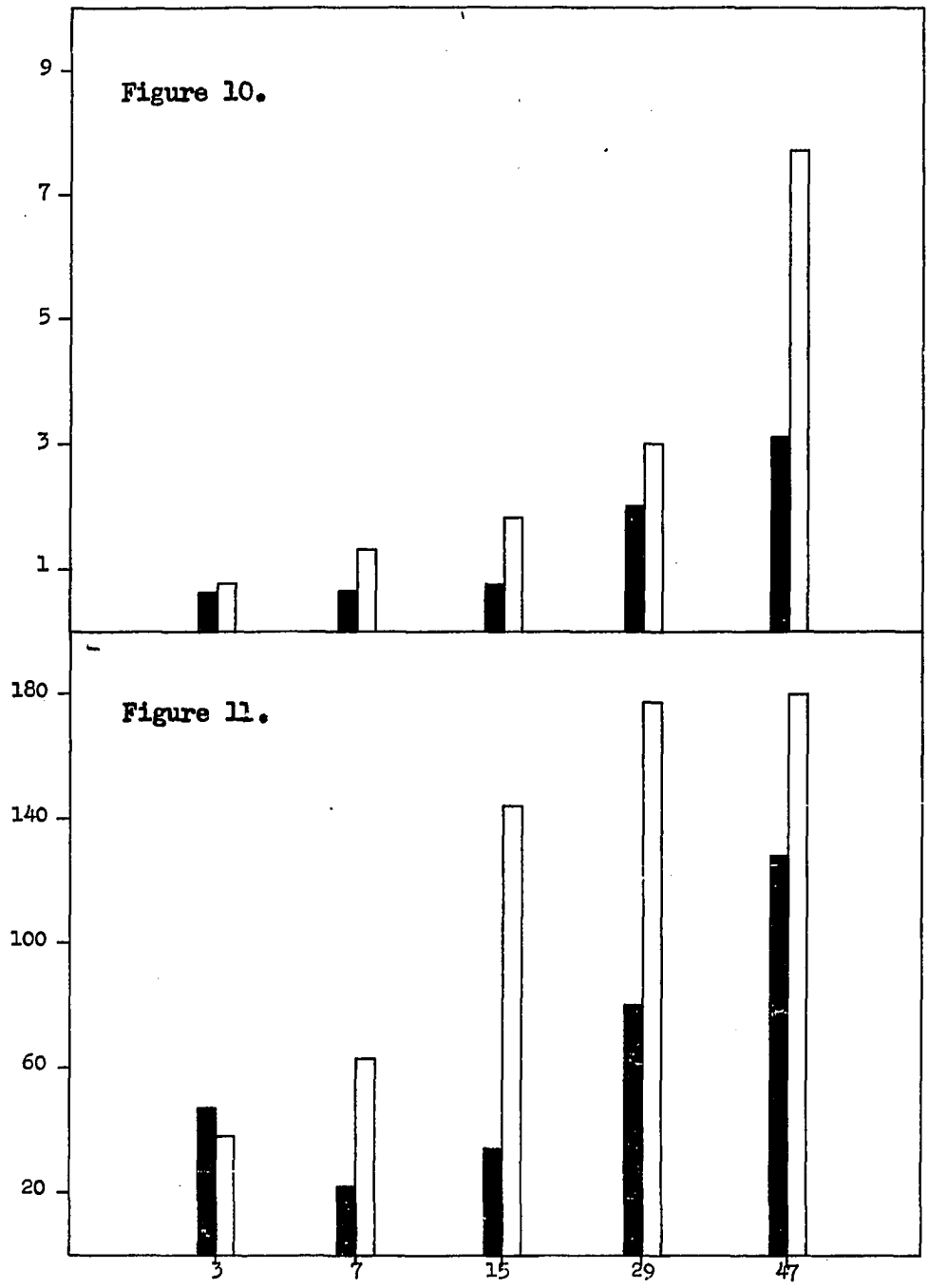


Figure 10. Variation of Scopoletin Content in Roots of Tobacco Plants Treated with Maleic Hydrazide.
Figure 11. Variation of Scopolin Content in Roots of Tobacco Plants Treated with Maleic Hydrazide.
Abscissa: Days after Treatment with Maleic Hydrazide. Ordinate: µg Material/g Fresh Tissue. Black = Control Roots, White = Treated Roots.

tissue, on the other hand, showed a decrease from the third to the seventh day. After the seventh day the scopolin content increased continually to the 47th day reaching a level approximately double that of the first sample. Control root tissue was the only tissue which showed a decrease during the first week after treatment with the control solution.

In the case of tobacco plants treated with maleic hydrazide, the highest levels of scopolin and scopoletin were reached in stem tissue. In the case of treatment with 2,4-D, as reported by Dieterman et al. (20), the treated root tissue had the highest levels of scopolin and scopoletin. The variation of scopolin and scopoletin in the controls indicated the desirability of harvesting a control with each treated sample rather than taking a sample at the beginning of the treatment and at the end of the treatment as reported by Dieterman et al. (20).

Figures 12-15 show the changes in scopolin and scopoletin content of flowers treated with 2,4-D. As indicated by the data in Table X, the absolute values obtained for set a and set b were not the same. Since only single plants were harvested, however, the variation could quite easily be that observed between one plant and another.

Table XI shows the ratio of the amount of scopolin and scopoletin in treated flowers when compared to the amount of scopolin and scopoletin in control flowers. Although the absolute values obtained were not the same, the calculated ratios showed a close agreement in most cases.

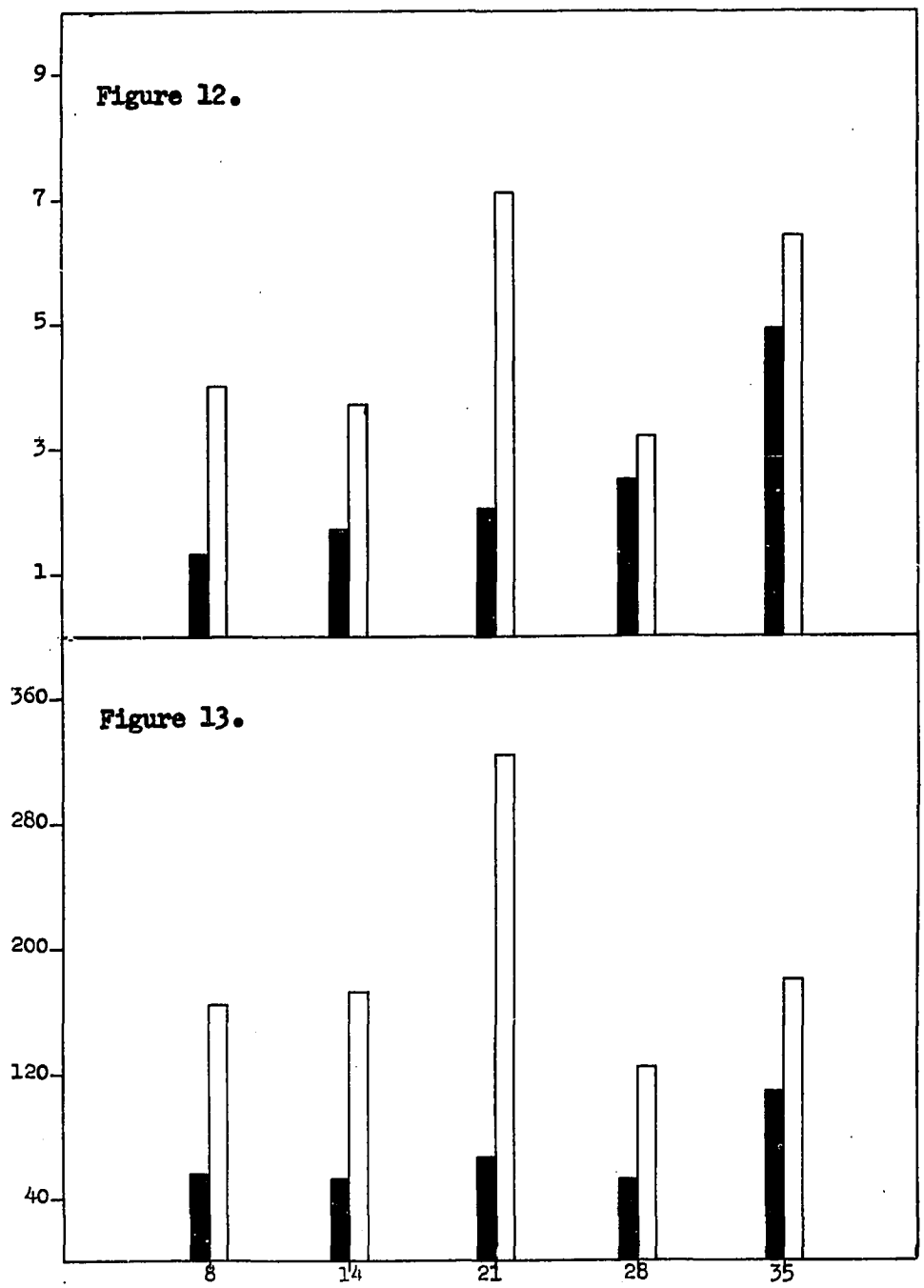


Figure 12. Variation of Scopoletin Content in Flowers of Tobacco Plants Treated with 2,4-D (Set a).
Figure 13. Variation of Scopolin Content in Flowers of Tobacco Plants Treated with 2,4-D (Set a).
Abscissa: Days after Treatment with 2,4-D. Ordinate: µg Material/g Fresh Tissue. Black = Control Flowers, White = Treated Flowers.

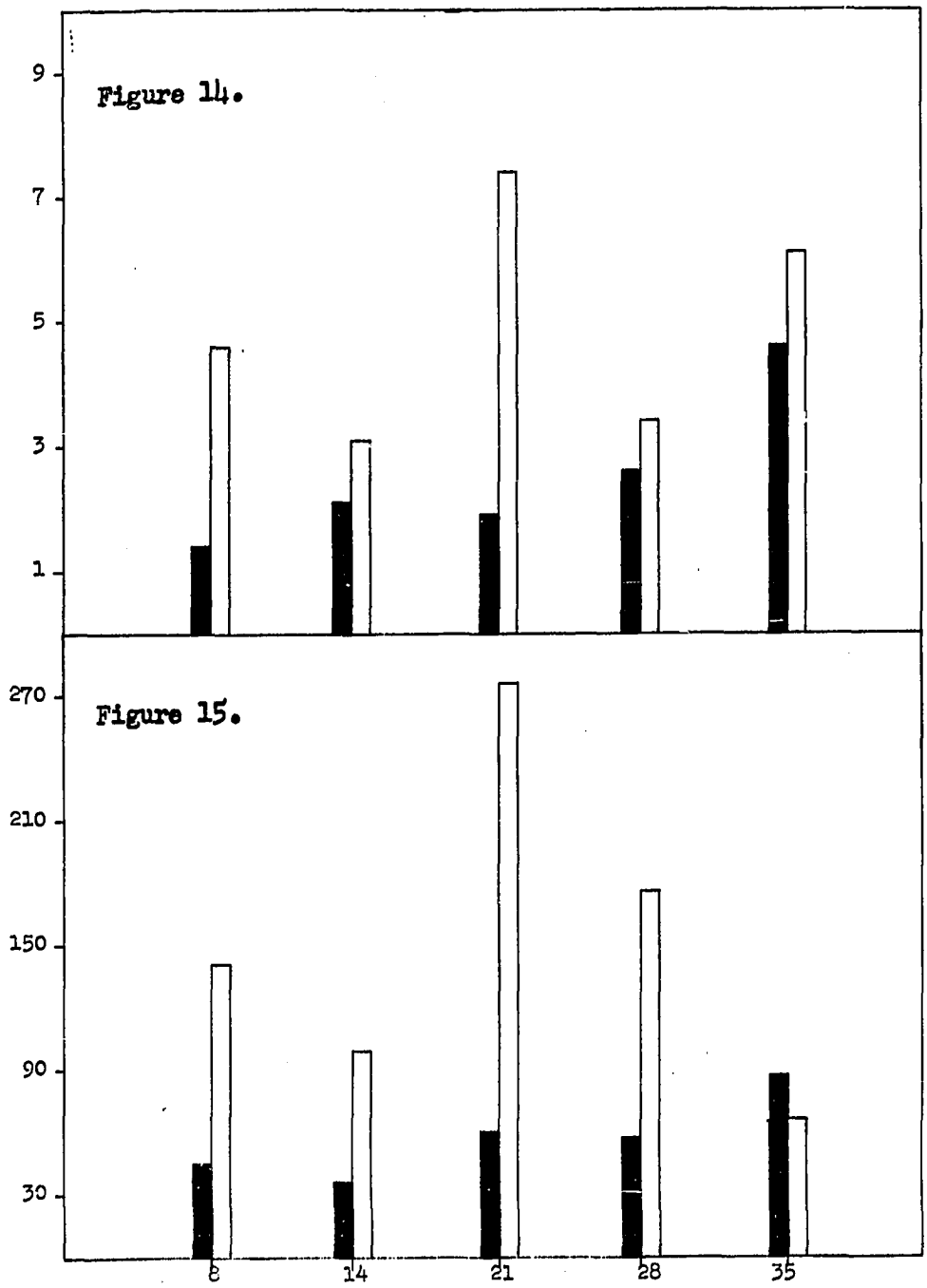


Figure 14. Variation of Scopoletin Content in Flowers of Tobacco Plants Treated with 2,4-D (Set b).
Figure 15. Variation of Scopolin Content in Flowers of Tobacco Plants Treated with 2,4-D (Set b).
Abscissa: Days after Treatment with 2,4-D. Ordinate: µg Material/g Fresh Tissue. Black = Control Flowers, White = Treated Flowers.

TABLE XI

THE RATIOS OF THE AMOUNT OF SCOPOLIN AND SCOPOLETIN IN 2,4-D TREATED TOBACCO FLOWERS TO THE AMOUNTS OF SCOPOLIN AND SCOPOLETIN IN CONTROL FLOWERS

D	Scopolin Ratios aTF/aCF	Scopoletin Ratios aTF/aCF
8	3.0	3.1
14	3.2	2.1
21	4.7	3.6
28	2.8	1.3
35	1.7	1.3
D	**bTF/bCF**	**bTF/bCF**
8	3.2	3.3
14	2.7	1.5
21	4.6	4.0
28	3.1	1.3
35	0.8	1.3

D: days after treatment with 2,4-D
aTF: flowers of tobacco plants treated with 2,4-D (set a)
aCF: flowers of tobacco plants treated with a control solution (set a)
bTF: flowers of tobacco plants treated with 2,4-D (set b)
bCF: flowers of tobacco plants treated with a control solution (set b)

The levels of scopolin and scopoletin in the case of flowers did not show a continued increase or decrease but rather fluctuated. Examination of the figures in the case of scopoletin in the treated flower tissue indicated that the pattern in both set a and set b appeared to be a decrease from the eighth to the fourteenth day, followed by a very sharp increase and reaching a maximum on the 21st day. This was followed by a rapid decrease to the 28th day and then an increase again to the 35th day. The scopoletin in the control flowers appeared to increase gradually to the 35th day reaching a level approximately 3-4 times that of the first sample.

The scopolin content of the treated flower tissue appeared to follow a pattern somewhat similar to that of scopoletin in the treated flower tissue. A maximum level was also reached by the 21st day. The treated flower of set b on the 35th day showed a continued decrease from the 28th day and not an increase as indicated by the treated flower of set a. However, the solution of the sample used for analysis in set b on the 35th day was a very dilute one and the data obtained were quite variable as indicated by the large standard deviation in Table X. The data obtained for the treated flower tissue of set a on the 35th day were from a much larger sample and were much more consistent.

The variation of the scopolin in the control flower tissue of both sets showed essentially the same pattern. The controls appeared to mimic the treated samples but on a much smaller scale. There appeared to be a slight decrease from the eighth to the

fourteenth day, followed by a 15-20 µg/g fresh tissue increase by the 21st day. This was followed by a slight decrease by the 28th day and then a large increase by the 35th day, reaching a level approximately twice as large as the first sample. In the case of the control flowers, the scopolin level at the 35th day was the highest one in contrast to the 21st day for the treated flowers.

Isolation and Partial Identification of a Glycoside from Tobacco Tissue Culture

Thirty-five µl of the fresh tissue extract of the 2,4-D treated and control tobacco leaves, stems and roots, harvested 35 days after treatment, was banded on Avicel-SF (375 microns thick) plates and run in four solvent systems: BzEFW (benzene:ethyl acetate: formic acid:(98%):water; 9:21:6:4; v/v/v/v), BAW, IFW-2, and KFW. The developed plates were examined under ultraviolet light with and without exposure to ammonia fumes. Thirty-five µl of the fresh tissue extract of the maleic hydrazide treated and control tobacco leaves, stems and roots harvested 47 days after treatment, was also examined as described above. Ten µl of an extract of tobacco tissue culture described on the following page was also spotted on each Avicel-SF plate. Examination of the plates indicated the presence of an unknown compound or compounds (two zones which were labeled BG1 and BG2) in the treated stem and control and treated root of the above samples as well as in the tobacco tissue culture extract. The BG1 zone and BG2 zone had very little fluorescence without exposure to ammonia fumes, but turned a bright blue-green when exposed to

ammonia fumes. The BG1 and BG2 zones appeared to be present in much larger amounts in the tissue culture extract. Therefore, the tissue culture extracts were used for the further isolation of this compound.

Nicotiana tabacum, One-Sucker variety or W-38 variety, tissue culture was used. These cultures were grown on RM-1964 medium (63), at room temperature, with approximately 6-8 hours of diffuse fluorescent light/day. The cultures were grown 4-5 weeks and then harvested and extracted under the section entitled Growth, Treatment and Harvesting of Tobacco Plants. Carroll Smith grew the tobacco tissue culture used in this work.

A preliminary separation of the material was done in order to get a spotting sample for future use. Twelve Avicel-SF (500 microns thick) plates were streaked with the tobacco tissue culture extract. These 12 plates were then stacked together (two stacks of 6 plates each), with small polyethylene spacers between the plates, taped to hold the stacks together, and developed with KFW. The BG1 and BG2 bands were located, and the Avicel area containing these zones was scraped off the plates with a large spatula. The Avicel was then packed into a large glass tube which had been drawn to a small opening at one end. This small opening had been previously packed with a 1 cm diameter pad of glass fiber (cut from a Cambridge Filter, Phipps and Bird, Richmond, Va.) and washed with methanol. The fluorescent material on the Avicel was then eluted, under vacuum, directly into a round-bottom flask with the aid of methanol or

methanol:water (1:1; v/v). The solution was then concentrated to dryness, in vacuo, and redissolved in a small volume of methanol. Chromatographic examination of this solution indicated that the combined BG1 and BG2 zones were still contaminated with scopolin, scopoletin and chlorogenic acid. In an attempt to get some preliminary idea of the identity of the zones labeled BG1 and BG2, the above solution was spotted along with quinyl-p-coumarate, ferulic acid glucoside, p-coumaric acid glucoside, scopolin, scopoletin, 5-feruloyl-D-quinic acid, 1-gentisoyl-β-D-glucose and 1-feruloyl-β-D-glucose, on an Avicel-SF (375 microns thick) plate and developed with KFW. The BG1 and BG2 zones had R_f values which were not similar to any of the above compounds. The solution containing the BG1 and BG2 zones was further purified by streaking it on four Avicel-SF (500 microns thick) plates and development in KFW. The plates were stacked together as previously described. The BG1 and BG2 zones were eluted as described above. Chromatographic analysis indicated that the solution contained a BG1 zone, a BG2 zone and a small amount of chlorogenic acid. This spotting solution was kept in the freezer to avoid decomposition as much as possible.

Since the chromatographic studies indicated the presence of two zones, some further preliminary thin layer studies were conducted in order to see if a satisfactory separation could be achieved and the results transferred to a column procedure. Therefore, Woelm plates (250 microns thick) and Silicar-4 (Mallinckrodt, St. Louis, Mo.) plates (250 microns thick) were spotted with the solution

containing the BG1 and BG2 zones which had been isolated as described above. The Woelm plates were made as previously described. The Silicar-4 plates were made as follows:

(1) 8-10 g/plate of Silicar-4 was added to distilled water (ratio: 1 g of Silicar-4 to 2 ml water).

(2) The mixture was shaken vigorously for one minute and spread immediately with a Desaga spreader to a thickness of 250 microns.

(3) The plates were air-dried and activated at approximately 110°C for two hours immediately before use.

The Woelm and Silicar-4 plates were then developed in the following solvents: 1) benzene, 2) benzene:ethyl acetate (1:1; v/v), 3) ethyl acetate, 4) benzene:acetone (1:1; v/v), 5) ethyl acetate: acetone (1:1; v/v), 6) benzene:methanol (1:1; v/v), 7) acetone, 8) acetone:methanol (1:1; v/v), 9) methanol, 10) methanol:water (1:1; v/v) and 11) water.

The results indicated that a good separation of the BG1 zone from the BG2 zone was achieved on the Woelm plate developed in water. This indicated that a separation might possibly be achieved on a Polyclar AT column since both Woelm polyamide and Polyclar AT are similar materials. Polyclar AT is a polyvinylpyrrolidone distributed by General Aniline and Film Corporation, Grasselli, N. J.

Some Polyclar AT was purified according to the directions given by Mizelle et al. (71). The procedure was modified slightly in that all the preliminary washes were done in a batch process. This was less time consuming and larger amounts could be purified.

The material was suspended in water (approximately 2 volumes of water to one volume of Polyclar AT) after the final washing and stored until used.

A Polyclar AT column (3 x 20 cm; Code: 1510-BWII'-76,77-PS-1,2-tiss. cult.-PCAT#-,) was prepared and washed thoroughly with water. Approximately 5 g of clean, dry Polyclar AT was placed in a round-bottom flask and approximately 100 ml of a tobacco tissue culture extract was added. The solvent was removed, *in vacuo*; 2-propanol was added and again removed, *in vacuo*. The dry Polyclar AT containing the adsorbed tobacco tissue culture extract was slurried with water and carefully poured on the Polyclar AT column. The top layer of the column was stirred, and the column was eluted with water. A small amount of pressure was applied to all of the columns described. Nineteen fractions of approximately 125 ml each were taken. Methanol was then added, and two more fractions were taken. An ultraviolet light was used to follow the separations of the major fluorescent bands in all the column work described.

The fractions were concentrated to dryness, *in vacuo*, and redissolved in a small amount of methanol or methanol:water (1:1; v/v). All of the concentrated column fractions were analyzed by spotting 10-20 µl of the concentrated solution on Avicel-SF (250 or 375 microns thick) plates and then developing the plates in KFW or IFW-2. Analysis of the fractions indicated that fraction 7-10 contained most of the BG2 and fractions 11-13 most of the BG1.

All of these fractions were still heavily contaminated with other compounds.

Fractions 11-13 were combined, the solvent removed, in vacuo, and the residue suspended in water. This water suspension was applied to a Woelm polyamide column (2.5 x 28 cm; Code: 1510-BWII'-78-PS-1, 2-F11,12,13-W#-,) packed in water. The Woelm polyamide had been previously purified by placing it in a column with methanol followed by elution with two liters of methanol, 4 liters of water and 2 liters of methanol. The material was then removed from the column and dried. The column was eluted with water (fractions 1-16), methanol:water (1:1; v/v; fractions 17-19), methanol (fractions 20, 21), and dimethylformamide (fraction 22). Chromatographic analysis indicated that the BG1 and BG2 zones were contained in fractions 4-11.

Since these fractions were still contaminated with compounds other than BG1 and BG2, they were combined, concentrated to dryness, in vacuo, and redissolved in a small amount of water. The water solution was applied to a Polyclar AT column (2.4 x 16.5 cm; Code: 1510-BWII'-79-PS-1,2-W-F-4-11-PCAT#-,) packed in water and eluted with water (fractions 1-4) and methanol (fractions 5-8). Chromatographic analysis indicated that fractions 1-6 contained most of the BG1 and BG2 zones. There were small amounts of impurities still present. Fractions 1-6 were combined, concentrated to dryness, in vacuo, and redissolved in methanol:water (1:1; v/v).

Fractions 7-10 (Code: 1510-BWII'-76,77-PS-1,2-tiss. cult.-PCAT#-F-&-10) were combined and placed on a Polyclar AT column (2.5 x 12 cm) packed in water and eluted with

water, methanol:water (1:3; v/v), methanol:water (1:1; v/v), and methanol. Chromatographic analysis indicated the BG1 and BG2 zones were located in fractions 4 and 5. These fractions were combined, concentrated to dryness, in vacuo, and redissolved in a small amount of methanol:water (1:1; v/v).

The initial column chromatography experiments indicated that the BG1 and BG2 zones could be separated from most of the major impurities by chromatography on Polyclar AT and Woelm polyamide. However, the BG1 zone was not separated from the BG2 zone and many minor impurities still remained.

Since there did not appear to be a great deal of material isolated from the initial columns, another 150 ml of tobacco tissue culture extract was taken and chromatographed on a column of Woelm polyamide (2.5 x 18 cm; Code: 1510-BWII'-80,81-PS-1,2-tiss. cult.-W#-,) and eluted with water, methanol:water (1:e; v/v), methanol: water (1:1; v/v), and methanol. The fractions containing the BG1 and BG2 zones were rechromatographed on a Polyclar AT column (2.5 x cm; Code: 1510-BWII'-84-Wp.81-F1,2-PCAT#-,) packed in water and eluted with the same solvents as the previous column. Fraction 2 containing the BG1 and BG2 zones plus minor impurities was concentrated to dryness, in vacuo, and the residue redissolved in a small amount of methanol:water (1:1; v/v). The total amount of material isolated was very small (less than 1 mg).

A number of other column procedures was tested in an attempt to separate the BG1 zone from the BG2 zone. A Polyclar AT

column (2.5 x 12.5 cm; Code: 1510-BWII'-85-PCATp.79-F-1-6-PCAT#-,) was packed in 20% methanol/benzene (v/v). A partially purified sample containing the BG1 and BG2 zones was placed in 20% methanol/benzene (v/v) and added to the column. The column was eluted with 20%, 30%, 50% and 75% methanol/benzene (v/v) followed by methanol. Chromatographic analysis indicated no separation of the BG1 zone from the BG2 zone.

Since the BG1 zone could be separated from the BG2 zone on a thin layer plate of Avicel, several columns were tried using Avicel-Technical Grade (distributed by FMC Corporation, American Viscose Division, Marcus Hook, Pa.) cellulose.

A column of Avicel-Technical Grade (2.5 x 13 cm; Code: 1510-BWII'-90-AvTG-#-,) was packed in 5% 2-propanol/water (v/v). A partially purified sample of the BG1-BG2 mixture (1510-BWII'-84-Wp.81-F1,2-PCAT#2) dissolved in 5% 2-propanol/water (v/v) was added to the column and eluted with 5% and 10% 2-propanol/water (v/v) followed by IFW-2. Chromatographic analysis indicated that all of the BG1-BG2 mixture was contained in fraction 4 eluted with IFW-2. No separation of the BG1 zone from the BG2 zone had occurred although some minor impurities had been removed.

Therefore, a column of Avicel-Technical Grade (2.5 x 18 cm; Code: 1510-BWII'-94-AvTG-90-F4-AvTG#-,) was packed in water. Fraction 4 from the preceding column was concentrated to dryness, <u>in vacuo</u>, and the residue redissolved in a minimal amount of water and placed on the column. The column was eluted in water (fractions

1-3), methanol:water (1:3; v/v; fractions 4-6), methanol:water (1:1; v/v; fractions 7-9), methanol:water (3:1; v/v; fractions 10-12), methanol (fractions 13, 14), methanol containing 0.1% formic acid (fraction 15), methanol containing 0.5% formic acid (fraction 16), methanol containing 2% formic acid (fraction 17) and IFW-2 (fractions 18, 19). Chromatographic analysis indicated that the BG1 and BG2 zones were located in fractions 14-17, with fraction 14 containing the major portion. Although some purification had taken place, no separation of the BG1 zone from the BG2 zone had occurred. The results also indicated that the BG1-BG2 mixture remained on the column until methanol was applied.

A further attempt at separating the BG1 zone from the BG2 zone was made by placing fraction 14 of the preceding column on another column of Avicel-Technical Grade (2.5 x 18 cm; Code: 1510-BWII'-95-AvTG-94-F14-AvTG#-,) packed in 80% methanol/water (v/v). The column was eluted with 80% (fractions 1-4), 85% (fractions 5-8), 90% (fractions 9-12), and 95% (fractions 13-16) methanol/water (v/v) followed by methanol (fractions 17-20). Each fraction contained approximately 25 ml. Chromatographic analysis indicated that fractions 3-20 contained the BG1 and BG2 zones with fractions 3-6 containing the major portion of this material.

A column of silica gel (2.5 x 10.5 cm; Mallinckrodt 100 mesh, washed twice in acetone and dried at approximately 105°C for 1 hour; Code: 1510-BWIII-29-AvTGp.95-F3-6-S.G.#-,) was packed in 25% methanol/benzene (v/v). Fraction 3 of the preceding column was

placed on the column. The column was then eluted with 25% (fractions 1-4), 50% (fractions 5-8), and 75% (fractions 9-12) methanol/benzene (v/v), followed by methanol (fractions 13-16). Each fraction was approximately 25 ml. An additional 300 ml of methanol (fractions 17-19) was added, followed by 100 ml of methanol containing 0.5% formic acid. Chromatographic analysis indicated that most of the BG1-BG2 mixture was concentrated in fraction 20 with small amounts of BG1 and BG2 scattered among the other fractions.

The following conclusions were drawn from the above data:

1) The BG1 zone could not be separated from the BG2 zone by any column chromatographic procedure tried.

2) The major impurities (scopolin, scopoletin, chlorogenic acid isomers) could be removed from the tobacco tissue culture extract by column chromatography on Polyclar AT and Woelm polyamide packed in water and eluted with water to which increasing amounts of methanol have been added.

3) Preparative thin layer chromatography probably would have to be employed for the final purification of the BG1-BG2 mixture and hopefully to separate the BG1 zone from the BG2 zone.

Since only very small amounts of the BG1-BG2 mixture had been isolated, 1800 ml of a tobacco tissue culture extract was concentrated to dryness, <u>in vacuo</u>. The residue was suspended in 180 ml of water and placed onto a column of Polyclar AT (6 x 40 cm; Code: 1510-BWII'-91,92-PCAT#-,) packed in water. The column was eluted with water (fractions 1-7), methanol:water (1:1; v/v; fractions

7 and 8), and methanol (fractions 9-12). Chromatographic analysis indicated that the BG1-BG2 zones were located in fractions 3-8.

These fractions were combined, concentrated to dryness, in vacuo, and the residue was suspended in 15 ml of water and added to a column of Woelm polyamide (2.5 x 25 cm; Code: 1510-BWII'-93-PCAT-91,92-F-3-8-W#-,) packed in water. The column was eluted with water (fractions 1-7), methanol:water (1:1; v/v; fractions 8 and 9), and methanol (fraction 10). Chromatographic analysis indicated that fractions 3, 4, and 5 contained the BG1-BG2 mixture plus 4-12 fluorescent impurities in each fraction.

Fraction 3 was further purified by chromatography on a column of Avicel-Technical Grade (2.5 x 18 cm; Code: 1510-BWIII-32-WF3-VolII8p.93-AvTG#-,) packed in methanol:water (1:1; v/v). The column was eluted with methanol:water (1:1; v/v; fractions 1 and 2), and methanol (fraction 3).

Fractions 4 and 5 from the above Woelm polyamide column were further purified by chromatography on columns of Avicel-Technical Grade packed in water and eluted with water, methanol:water (1:1; v/v), methanol, and methanol containing 0.5% formic acid. These partially purified fractions were further purified by preparative thin layer chromatography on Avicel-SF (500 microns thick) plates.

The BG1-BG2 mixture which had been partially purified by column chromatography was streaked on 56 plates of Avicel-SF (500 microns thick) and developed in IFW-2. This solvent was chosen since it gave the widest separation of the zones labeled

BG1 and BG2. The plates were stacked together (6-8/stack) using the polyethylene spacers as previously described. The plates had been scored approximately 1/4 of an inch from either edge with a dissecting needle. The Avicel containing the BG1 and BG2 zones were each scraped off the developed plates. Each zone was then eluted with MW (methanol:water; 1:1; v/v). Chromatographic analysis (Avicel-SF; 375 microns thick; plates developed in IFW-2) indicated that although all of the minor impurities had been removed, each zone yielded both BG1 and BG2. The purified extracts were combined and the solvent removed, in vacuo. The material was then redissolved in methanol.

The BG1-BG2 mixture which was later isolated from the 1800 ml of tissue culture extract by column chromatography was streaked on 182 Avicel-SF (500 microns thick) plates and purified as described above. The BG1 zone and BG2 zone were isolated separately and appropriate zones recombined for further preparative thin layer chromatography. In this case the large amount of impurities present necessitated the use of KFW and BzEFW in addition to IFW-2 to eliminate all of the impurities. Chromatographic analysis of the isolated BG1 zone and BG2 zone indicated that both zones always contained BG1 and BG2. Therefore, all the purified fractions were recombined into 3 fractions and filtered through several small Polyclar AT columns in order to eliminate any impurities which had been extracted from the Avicel-SF itself. These purified fractions were then subjected to hydrolysis and ultraviolet analysis.

Table XII lists the R_f values obtained for the purified BG1-BG2 mixture in various solvents. The R_f's were determined on Avicel-SF (375 microns thick) plates developed in tanks which had been saturated with solvent before development. The solutions were banded rather than spotted and the R_f values were measured to the top of the band.

TABLE XII

R_f VALUES OF BG1 AND BG2

Compounds	BAW	KFW	KFW-2	Solvent I, Table VII
BG1	61	51	54	63
BG2	54	45	79	63
	Solvent II, Table VII			Solvent III, Table VII
BG1	72			74
BG2	72			74
	Solvent IV, Table VII			
BG1	43			
BG2	43			

BzEFW was also tried but the R_f values were quite variable. BzEFW appeared to be a solvent mixture which was extremely temperature dependent.

An ultraviolet absorption spectrum (in methanol) of the combined BG1-BG2 mixture purified in the first series of experiments was determined. The ultraviolet spectrum obtained was very similar

to that of scopolin. Therefore, 15 µl of the combined BG1-BG2 mixture was banded on an Avicel-SF (375 microns thick) plate and developed with IFW-2. Examination under ultraviolet light indicated that there was only a small BG1 zone and BG2 zone. The major zone was a blue-fluorescing band with an R_f similar to scopolin. Another plate banded more heavily indicated the presence of scopoletin.

Apparently the high temperature used to remove the water on the rotary evaporator had caused a hydrolysis or pyrolysis.

Therefore the material was spotted on Avicel-SF (375 microns thick) plates along with scopolin and developed with the seven solvents listed in Table XII and BzEFW. The R_f of the blue-fluorescent compound from the BG1-BG2 mixture corresponded to that of scopolin in all of the solvent mixtures studied.

Approximately one ml of the BG1-BG2 mixture, containing the suspected scopolin, was subjected to hydrolysis with 3% hydrochloric acid. The water solution was extracted with ethyl acetate to remove the aglycone and the water phase was neutralized by filtration through a small column (1.2 x 5.5 cm) of Amberlite IR-45 resin (OH form). The eluate from the column was concentrated to dryness, in vacuo, and redissolved in a small amount of 10% 2-propanol/water, v/v. This solution was banded on Avicel-SF (375 microns thick) plates along with a standard solution of glucose and galactose and developed in solvents I, II, III, and IV of Table VII, used to separate sugars. The plates were sprayed with aniline hydrogen oxalate (115).

Examination of the plates indicated that glucose was the only sugar present in the aqueous phase.

The ethyl acetate extract was concentrated to dryness, *in vacuo*, and redissolved in a small amount of methanol. An ultraviolet absorption spectrum of this material was determined. Comparison of the spectrum with the values listed by Sargent and Skoog (87) indicated that the aglycone isolated was possibly scopoletin although all the peaks did not correspond exactly. Therefore, the aglycone was further purified by chromatography on a small Polyclar AT column eluted with methanol:water (1:1; v/v). A minor contaminant remained on the column. The ultraviolet absorption spectrum (Table XIII) of the purified aglycone (in methanol) indicated that the aglycone was scopoletin. The purified aglycone was also chromatographed on Avicel-SF (375 microns thick) plates using the seven solvents listed in Table XII and BzEFW, along with a known sample of scopoletin. The R_f values of the purified aglycone corresponded to those of scopoletin in all of the solvent systems studied.

The rest of the combined BG1-BG2 mixture thought to contain scopolin (as indicated by the R_f values) was further purified by preparative thin layer chromatography using six Avicel-SF (500 microns thick) plates developed in IFW-2. The blue-fluorescent zone thought to be scopolin was eluted from the plates as described previously with methanol and an ultraviolet absorption spectrum was determined. Although the spectrum was similar to that of scopolin (87), the results indicated that there were still impurities present. Therefore

the material thought to be scopolin was chromatographed on a Polyclar AT column (1.5 x 10 cm); and eluted with water. A minor impurity was retained by the column. The eluate was concentrated to dryness, *in vacuo*, and redissolved in methanol. An ultraviolet absorption spectrum of this solution (Table XIII) corresponded very closely to that reported by Sargent and Skoog (87).

The above results indicated that the BG1-BG2 mixture contained a derivative of a scopoletin glycoside, and more specifically, a derivative of scopolin. The two zones which appeared during thin layer chromatography (labeled BG1 and BG2) in some solvent systems were thus suspected of being isomeric in nature.

TABLE XIII

ULTRAVIOLET ABSORPTION SPECTRA DATA

Level	Scopoletin (86)	Isolated Aglycone
max	230 mμ	228 mμ
min	246	245
max	255	258
min	271	270
max	299	294
min	307	308
max	346	342
Level	**Scopolin (86)**	**Suspected Scopolin**
min	-	214 mμ
max	228 mμ	227
min	265	265
max	291	288
min	306	305
max	340	338

A solution of the BG1-BG2 mixture was then subjected to gas chromatography. The mixture was silyated by dissolving it in dimethylformamide and treatment with hexamethydisilizane. The silyated mixture was injected into a stainless steel column (1/4 inch by 6 ft.) which had been packed with Gas Chrom Q and coated with UCW-98 (3%). The temperature was programmed from 200-300°C at a rate of approximately 8°C/minute. The carrier gas was helium at a flow rate of approximately 60 ml/minute. Since no peak was observed, the material was silyated again using the above conditions except that trimethylchlorosilane was added to promote silyation. The material was again injected under the above conditions. No peak was observed under these conditions. This indicated that the BG1-BG2 mixture was a high molecular weight compound. This work was conducted by J. L. Wilson.

Since the material was a derivative of scopolin, a sample of scopolin was silyated and subjected to nuclear magnetic resonance analysis. The procedure reported by Mabry, Kagan and Rösler was used (64). C. A. Peters performed the nuclear magnetic analysis. The following delta values were obtained:

(1) 3.10-4.09 (multiplet which integrated for 9 protons)

(2) 3.82 (singlet which integrated for 3 protons)

(3) 4.76-5.12 (multiplet which integrated for 1 proton)

(4) 6.08 and 6.22 (doublet which integrated for 1 proton)

(5) 6.83 (singlet which integrated for 1 proton)

(6) 6.97 (singlet which integrated for 1 proton)

(7) 7.43 and 7.59 (doublet which integrated for 1 proton)

By analogy to the nuclear magnetic resonance spectrum of esculin (Fig. 1) reported by Mabry, Kagan and Rösler (64) the delta values were assigned to the following protons of scopolin (Fig. 1). The multiplet at 3.10-4.09 contained the glucose protons. The singlet at 3.82 was the $-OCH_3$ group located at carbon 7. The multiplet at 4.76-5.12 was the glucose-H-1 proton. The 6.08 and 6.22 doublet was the proton at carbon 3 and the 7.43 and 7.59 doublet was the proton at carbon 4. The singlets at 6.83 and 6.97 were the protons on carbons 5 and 8.

It was hoped that enough of the BG1-BG2 mixture could be obtained so that it could be subjected to nuclear magnetic analysis. Unfortunately the total amount of the BG1-BG2 mixture isolated was less than 4 mg. Therefore, a nuclear magnetic resonance analysis could not be performed.

An ultraviolet analysis of the isolated mixture was then undertaken. The material was dissolved in methanol and its ultraviolet absorption spectrum determined. Then 2 drops of 1% sodium hydroxide was added and its ultraviolet absorption spectrum determined. A fresh sample was saturated with solid, fused sodium acetate and its ultraviolet absorption spectrum determined. The results are indicated in Table XIV.

The BG1-BG2 mixture has an ultraviolet spectrum (Table XIV) which is very similar to that of scopolin (Table XIII). All of the major peaks appeared to have been shifted hypsochromically approximately 10 to 20 mμ. The large shift of

TABLE XIV

ULTRAVIOLET ABSORPTION SPECTRA OF THE BG1-BG2 MIXTURE

Level	BG1-BG2 Mixture	plus 1% NaOH	plus $NaC_2H_3O_2$
min	210 mμ	-	-
max	216	-	-
min	229	240 mμ	-
min	264	280	263 mμ
max	293	306	293
min	300	316	300
max	318	362	320

the long wavelength band when 1% NaOH was added indicated that the BG1-BG2 mixture was not a simple derivative of scopolin. The molecule or molecules attached to the scopolin nucleus must contain a highly ionizable group to cause the large spectral shift observed or they may undergo a possible oxidation in the alkaline medium breaking down or rearranging to a functional group which would show a large spectral shift in the presence of base.

The BG1-BG2 mixture was subjected to a number of spray reagents after chromatography on Avicel-SF (375 microns thick) plates in several of the sugar solvents listed in Table VII. The BG1-BG2 mixture reacted immediately with a $KMnO_4$ spray reagent (34), turning a bright yellow. A scopolin solution on the same plate did not react nearly as rapidly. When the BG1-BG2 mixture was sprayed with a $AgNO_3$ solution (97) a positive test was observed. A negative test was observed with aniline hydrogen oxalate (115). The BG1-BG2 mixture turned a light yellow when sprayed with a periodate/benzidine spray reagent (34). A slight positive test was also observed when

the BG1-BG2 mixture was sprayed with the Cartwright-Roberts reagent (15). This reagent is used to detect quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid) and is fairly specific.

Shikimic acid (3,4,5-trihydroxy-1-cyclohexenecarboxylic acid) and quinic acid were subjected to the above spray reagents. Shikimic acid reacted immediately with the $KMnO_4$ solution (34) producing a bright yellow spot while quinic acid reacted very slowly. Both quinic acid and shikimic acid gave a negative test with $AgNO_3$ (97) and only a faint positive test with aniline hydrogen oxalate (115). Both quinic acid and shikimic acid reacted rapidly with the periodate/benzidine reagent (34). Shikimic acid gave a positive test with the Cartwright-Roberts test (15) in addition to the reported quinic acid.

This information suggested that the BG1-BG2 mixture might contain a shikimic acid molecule. The last of the purified BG1-BG2 mixture was subjected to acid hydrolysis as described previously. A solution of shikimic acid was also subjected to acid hydrolysis under similar conditions.

The water phase was banded on Avicel-SF (375 microns thick) plates and developed in several of the solvents listed in Table VII. The plates were then examined under an ultraviolet light. This examination indicated that some of the BG1-BG2 mixture remained unhydrolyzed. In addition, upon exposure to ammonia fumes, a bright yellow band and an orange band appeared, which were not present in

a blank. The shikimic acid solution which had been heated with hydrochloric acid also contained a spot with a similar R_f value which turned bright yellow with ammonia.

Cartwright and Roberts (15) reported the appearance of three trace spots when quinic acid had been heated with hydrochloric acid. They suggested that this might have been due to lactonization or a process similar to lactonization. Since shikimic acid is very closely related to quinic acid the same process might have occurred. There did not appear to be any other fluorescent areas or bands present.

When a plate banded with the above solutions was sprayed with the $KMnO_4$ reagent (34), a bright yellow spot with an R_f similar to that of a standard shikimic acid solution and the shikimic acid solution heated with hydrochloric acid was noted.

Another plate was sprayed with the Cartwright-Roberts reagent (15) and a faint yellow orange spot with an R_f similar to that of the standard shikimic acid solution and the shikimic acid solution heated with hydrochloric acid was observed. This information suggested that shikimic acid might possibly be present in the BG1-BG2 mixture. This was supported somewhat by the fact that the BG1-BG2 mixture was much more soluble in water and methanol than scopolin and the addition of an acid function could greatly increase the solubility. Also the BG1-BG2 mixture had a faint blue-purple fluorescence (when heavily spotted) when viewed under an ultra-violet light and turned a bright blue-green upon exposure to ammonia

fumes. Materials with quinic acid attached frequently have similar color reactions (ex: the feruloylquinic acids). The attachment of a shikimic acid could have an effect similar to that of quinic acid. Also the presence of shikimic acid with its α,β-unsaturated carboxyl group would be expected to produce a shift in the ultraviolet spectrum when NaOH is added.

Although the above information suggests the possible presence of shikimic acid, nothing can be said definitely about its point of attachment to the scopolin nucleus. The most likely point of attachment would be the glucose moiety since scopoletin is the aglycone isolated upon acid hydrolysis.

The positive $AgNO_3$ test observed by the BG1-BG2 mixture and the negative $AgNO_3$ test for shikimic acid indicated that the material may be more complex. The positive $AgNO_3$ test indicated the presence of an oxidizable function like a carbonyl group. The lack of any other $AgNO_3$ positive spot, other than glucose, in the hydrolysate could indicate the presence of a glucose molecule attached to the shikimic acid or the presence of some molecule which is degraded or destroyed by the acid hydrolysis.

Chromatographic Studies on the Tissue Extracts

The bean tissue and tomato tissue extracts were subjected to thin layer chromatography on Avicel-SF (375 microns thick) plates. Twenty to thirty µl of tissue extract was banded on the plate and developed in IFW-2. The plate was examined under an ultraviolet light. Then the plate was sprayed with a ninhydrin solution to

detect the amino acids present. The beans and tomatoes which had been subjected to 50 ppm NO_2 showed no significant differences between the control and treated plant extract in either the polyphenol or amino acid content. The bean and tomato extracts did not contain scopolin, scopoletin or a BG1 or BG2 zone. All of the R_f values listed in the following section were determined on Avicel-SF (375 microns thick) plates which had been developed in IFW-2.

The bean leaves appeared to have only chlorogenic acid of the phenolic depsides and coumarins of interest to us, while the tomato leaves contained chlorogenic acid and band 510. The bean leaves also had four other major bands:

(1) a faint red band which turned a very bright deep yellow brown with ammonia (R_f = 0.15-0.18);

(2) a band which turned very bright blue-purple with ammonia (R_f = 0.62-0.65);

(3) a band which turned very bright yellow-blue with ammonia (R_f = 0.72-0.75); and

(4) a band which turned bright blue with ammonia (R_f = 0.75-0.77).

The tomato leaves did not contain these three bands just listed but had four other major bands:

(1) a band which turned brown-yellow with ammonia (R_f = 0.28-0.31);

(2) a band which turned deep yellow with ammonia (R_f = 0.66-0.71);

(3) a band which turned bright blue with ammonia (R_f = 0.77-0.80); and

(4) a band which turned very bright blue-pink with ammonia (R_f = 0.83-0.85). The band which turned brown-yellow with ammonia was probably rutin.

In addition, two bands appeared in both the treated and control tomato leaves harvested 10 days after treatment and later: (1) a band which turned very bright sky blue with ammonia (R_f = 0.03-0.05); and (2) a blue-purple band which turned bright blue with ammonia (R_f = 0.06-0.07). These bands were also present in the treated and control extracts of tomato stem and root tissue harvested 10 days after treatment and later.

The bean stem tissue appeared to contain no chlorogenic acid isomers. Three major bands appeared: (1) a band which turned yellow-brown with ammonia (R_f = 0.14-0.15); (2) a band which turned yellow-brown with ammonia (R_f = 0.30-0.32); and (3) a band which turned bright purple with ammonia (R_f = 0.75-0.92).

The tomato stem tissue contained neochlorogenic acid and chlorogenic acid but no band 510. Two other major bands appeared: (1) a brown-yellow band which turned bright brown-yellow with ammonia (R_f = 0.30-0.35); and (2) a band which was faint yellow and turned yellow with ammonia (R_f = 0.75-0.77). The brown-yellow band with an R_f of approximately 0.30-0.35 was probably rutin.

The bean root tissue contained no chlorogenic acid isomers. Five major bands appeared to be present.
(1) a band which turned very bright purple with ammonia (R_f = 0.03); (2) a band which was bright blue-violet (R_f = 0.05-0.06); (3) a band which was faint white and turned red with ammonia (R_f = 0.08-0.09); (4) a band which was deep purple and turned bright purple with ammonia (R_f = 0.29-0.31); and (5) a band which turned yellow with ammonia (R_f = 0.35-0.36).

The tomato root tissue contained chlorogenic acid and three other major bands: (1) a band which turned blue-green with ammonia (R_f = 0.43-0.45); (2) a band which turned white with ammonia (R_f = 0.70-0.75); and (3) another band which turned white with ammonia (R_f = 0.78-0.82).

The bean and tomato plant extracts which had been treated with 10 ppm SO_2 also exhibited no significant differences in the polyphenol or amino acid content. They contained the same major bands which were discussed above.

The bean and tomato plants which had been treated with larger doses of SO_2 and NO_2 were then examined. Again, no major changes were noted in the polyphenol or amino acid content. The only changes that had occurred at all were found in the two low R_f bands which were present in tomato leaf, stem and root tissue. Only the 500 ppm NO_2 and 1000 ppm NO_2 treated leaves of tomatoes contained these two bands.

In tomato roots there appeared a faint green-blue band (with ammonia) with an R_f = 0.04 instead of the very bright sky blue with ammonia bands (R_f = 0.05-0.07 and 0.06-0.07). The one exception was the roots and stems of tomatoes treated with 100 ppm SO_2 which did contain these latter bands.

The bean roots treated with SO_2 and 750 and 1000 ppm NO_2 contained an additional band which the bean roots treated with 50 ppm NO_2 did not contain (the band turned yellow with ammonia and had an R_f = 0.12).

All of the bean and tomato tissues which had been subjected to the larger doses of NO_2 and SO_2 were banded on Avicel-SF (375 microns thick) plates and developed in both BAW and BzEFW. These plates were examined under an ultraviolet light and then sprayed with a solution of ninhydrin to detect the amino acids present. In addition, p-coumaryl-glucose, quinyl-p-coumarate and 3-feruloyl-D-quinic acid were banded on these plates. Chromatographic analysis of the plates developed with BAW and BzEFW indicated (as had those developed in IFW-2) that there were no significant changes in any of the samples spotted. None of the bands in the tomato or bean extracts matched the p-coumaryl-glucose, quinyl-p-coumarate or 3-feruloyl-D-quinic acid in both R_f and color reactions under ultraviolet light with and without exposure to ammonia fumes.

Several investigators (68, 75, 90, 103) have reported that the content of the free proline increased dramatically under various stress conditions. Since ninhydrin gave only a light yellow spot with proline, all of the tissue extracts were re-examined for any difference in proline.

Fifteen µl of the tissue extract was spotted on an Avicel-SF (375 microns thick) plate and developed in solvent VI of Table VIII. This solvent was chosen since proline had a relatively high R_f value. A standard solution of proline was also spotted on the plates at a level of approximately 5 µg. The plates were examined under an ultraviolet light and then sprayed with an isatin solution. Isatin produces a deep blue color when proline is present. The following

results were obtained: (1) All of the NO_2 and SO_2 treated tomatoes contained a fair amount of proline (approximately 1-4 µg/15 µl) but there were no significant differences in the treated samples versus the control samples. There did appear to be a gradual increase of proline with time. (2) All of the NO_2 and SO_2 treated bean extracts contained very small amounts of proline (approximately 0-2 µg/15 µl) and there were no significant differences in the treated versus the control extracts. The amount of proline appeared to remain relatively constant with time. (3) All of the tobacco plants treated with 2,4-D contained very large amounts of proline (greater than 5 µg/15 µl) and there were no significant differences in the treated versus the control extracts. The amount of proline appeared to remain relatively constant with time. (4) All of the tobacco plants treated with maleic hydrazide contained proline. In this case, however, there did appear to be differences in the amount of proline contained in a treated sample versus a control sample. In all of the maleic hydrazide samples tested, the proline content of the treated sample was larger than in the corresponding control.

It appeared from the above studies that beans and tomatoes treated with 50 ppm NO_2 and 10 ppm SO_2 showed no significant changes in the polyphenol or amino acid content. Even the application of these air-pollutants in much larger doses did not essentially alter either the polyphenol or amino acid content even though physical damage was visible.

2,4-D alters the polyphenol content of tobacco but not the amount of free proline present, whereas maleic hydrazide alters both the polyphenol content and the free proline content.

CHAPTER III

SUMMARY

A new, rapid procedure for the quantitative analysis of the coumarins, scopolin and scopoletin was developed using a combination of thin layer chromatography and fluorescence.

An exhaustive extraction procedure was found to be necessary for the quantitative removal of free phenolic material in Nicotiana tabacum plants. The macerated material was successively washed with hot solutions of 2-propanol:water (1:1, v/v), 2-propanol:benzene: methanol:water (2:1:1:1, v/v/v/v) and 2-propanol:water azeotrope. The plant material was then extracted for 24 hours with 2-propanol: water azeotrope and then for another 24 hours with 2-propanol. A slightly modified procedure was used to extract quantitatively the free phenolic material from bean and tomato plants.

After extraction and concentration, the plant extracts were spotted on thin layers of Woelm polyamide (250 microns thick) with the aid of a micrometer syringe. Ten to thirty µl of extract was spotted, in triplicate, along with standard solutions of scopolin and scopoletin.

The plate was then developed in the solvent system benzene:methanol:formic acid (98%) (4:1:0.1, v/v/v) for approximately

forty minutes. The scopolin and scopoletin zones were scraped off the plate, and the scopolin and scopoletin were eluted from the polyamide material with the aid of hot methanol.

The per cent fluorescence of these solutions was then determined and compared to that of the standard solutions spotted on the same plate. The fluorescence response of the standard scopolin and scopoletin solutions was found to be linear between 0-5 µg/5 ml for scopolin and 0-0.5 µg/5 ml for scopoletin.

Tobacco plants sprayed with 2,4-dichlorophenoxyacetic acid and maleic hydrazide were harvested on various days after spraying and the free phenolic material extracted. The flowers of the plants treated with 2,4-dichlorophenoxyacetic acid and the roots, stems and leaves of the plants treated with maleic hydrazide were then analyzed for their scopolin and scopoletin content and the results discussed.

A scopoletin glycoside occurring in very small amount was isolated from a tobacco tissue culture extract, and was partially identified. The material was found to be a derivative of scopolin. In addition, shikimic acid was tentatively identified as being present.

Bean and tomato extracts from plants subjected to varying concentrations of two air-pollutants (nitrogen dioxide and sulfur dioxide) were examined by thin layer chromatography on layers of a microcrystalline cellulose called Avicel-SF. No significant differences in the free polyphenol and free amino acid content (particularly proline) were detected. Tobacco plants sprayed with

2,4-dichlorophenoxyacetic acid exhibited no significant differences in the free proline content between the treated and control extracts. The free proline content of tobacco plants sprayed with maleic hydrazide was significantly larger than in the corresponding control plants.

In addition, a number of chromatographic studies were made to explore the usefulness of thin layer chromatography in relation to phenolic materials. The R_f values of a number of depsides, phenolic acids, and coumarins in several new solvent systems, tested on thin layers of Woelm and Macherey and Nagel polyamide as well as on Avicel-SF were reported.

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