[Chem. Pharm. Bull. 18(9)1815—1821(1970)]

UDC 547.751.08.09 : 547.476.8.08.09 : 577.17.581.14.07

Quantitative Estimation of Auxins and Antiauxin in Etiolated Seedlings of Phaseolus Bean by Gas Chromatography and Colorimetry. I

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(Received February 20, 1970)

The quantitative estimation methods were established for Me-IAA, IAN, PAM, IAM, IAA, and PHCA by gas chromatography and colorimetry.

As has been reported in previous papers[2] the presence of indole-3-acetic acid (IAA), methyl indoleacetate (Me-IAA), indole-3-acetonitrile (IAN), phenylacetamide, and *p*-coumaric acid (PCA) in the extract of Moyashi (etiolated seedlings of Phaseolus mungo) was established by the present authors. The method of identification and estimated yields are summarized in Table I.

Table I. Active Substances in Moyashi

Substance	Method of identification	Estimated yield
IAM	isolation	600 mg/900 kg Moyashi
PAM	isolation	90 mg/900 kg Moyashi
IAN	identification by TLC and GC	——
Me-IAA	identification by TLC and GC	——
IAA	isolation as Me-IAA	80—90 mg/1000 kg Moyashi
PCA	isolation as methylated products	——
PHCA	isolation as methylated products	

PAM: phenylacetamide PCA: *p*-coumaric acid PHCA: *p*-hydrocoumaric acid

It would be of interest to know the quantity of these active substances in Moyashi and correlate these data with the growth phenomenon.

For the estimation of auxins the bioassay technique has been generally used. Although bioassay is convenient in terms of the qualititative estimation of a very minute amount of active substances, it has some weak points that there is no evidence what the active substance is and there may be the effect of an impurity that promotes or inhibits the active substance.

To avoid such a weakness, the combination of paper chromatography and bioassay is usually employed. This however is not applicable in the case of Moyashi which contains many auxins and an antiauxin. As is known, separation of neutral auxins by paper chromatography is unsatisfactory. For example, the *Rf* values of IAN and Me-IAA on paper chromatogram are similar and it is very difficult to separate and to estimate Me-IAA and IAN by the combination of paper chromatography and bioassay. Furthermore, identical *Rf* values for IAA and its antagonist PCA make results of bioassay unreliable for the estimation of IAA.

Gas chromatography is a very useful tool for the separation of neutral substances, and hence we applied this technique for the estimation of the isolated active substances.

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2) This paper constitutes Part V of a series entitled "Studies on Plant-growth Regulators" by Toshihiko Okamoto and Yo Isogai. Part IV: T. Okamoto Y. Isogai T. Koizumi, T. Nishino and Y. Satoh, *Chem. Pharm. Bull.* (Tokyo), **18**, 243 (1970).

This paper describes the gas chromatographic estimation of the pure samples of the active substances contained in Moyashi.

Gas Chromatography of Pure Samples and Preparation of Calibration Curves

According to Stowe's report[3] that the Versamide 900 column was the best for the separation of the esters of indolecarboxylic acid, we employed gas chromatograph with hydrogen flame ionization detector and the column packed with 10% Versamide 900 on silanized Chromosorb-W. Since IAA, PCA, and PHCA can not be gas chromatographed directly, their methyl esters were used. Me-IAA, IAN, PAM, and Me-PHCA were separated successfully in sharp peaks by Versamide 900 column, but IAN and Me-PCA gave broad peaks, which made other quantitative estimation method necessary for these two substances. As for IAM, colorimetry was investigated, which will be described in the next section. Though estimation of Me-PCA has not been established yet, one could apply gas chromatography to Me-PHCA resultant from easy reduction of Me-PCA.

For the preparation of calibration curves, internal standard method was employed. An internal standard substance should be selected from substances having a retention time close to the samples. Among those tested, carbazole was selected as the internal standard for Me-IAA and IAN, and *p*-acetophenetidine as that for PAM and Me-PHCA. The schematic gas chromatograms of these substances are shown in Fig. 1.

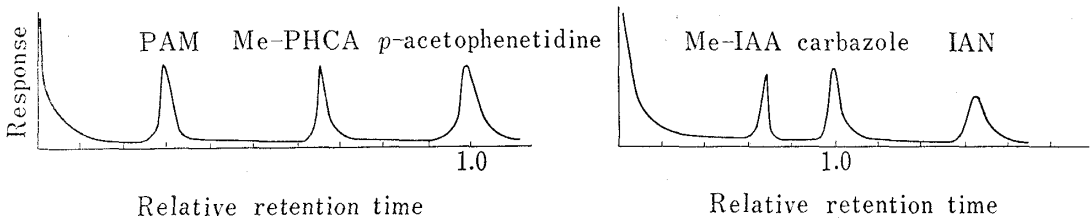

Fig. 1. Schematic Gas Chromatograms of PAM, Me-PHCA, Me-IAA and IAN

column: 10% Versamid 900 on Chromosorb W. 1 m
Hitachi Gaschromatograph Model F 6
(FID detector)
0.5—1.5 μl injection

Both carbazole and *p*-acetophenetidine have no activity in the Avena Section test as shown in Table II, thus a mixture used for gas chromatography can be tested by bioassay using the Avena section test.

Table II. Avena Section Test of Carbazole and *p*-Acetophenetidine

	Concn. (ppm)	Example 1 Av. length (mm)	Example 2 Av. length (mm)
Carbazole	100	7.94	8.47
	10	8.52	8.40
	1	7.85	8.12
p-Acetophenetidine	100	8.47	8.11
	10	7.55	8.26
	1	7.57	7.70
Control		7.85	8.10

With these two internal standard substances, the calibration curves for Me-IAA, IAN, PAM, and Me-PHCA were obtained under the conditions shown in Table III.

3) B.B. Stowe and J.F. Schilke, *Colloq. Intern. Centre Nate. Rech. Sci.* (Paris), **123**, 409 (1963).

Table III. Conditions for the Preparation of Calibration Curves for Me-IAA, IAN, PAM, and Me-PHCA

Substance (μg/μl)	Column temp. (°C)	Evaporation heater temp. (°C)	Carrier gas flow (ml/min)
IAN (>*ca.* 0.2)	220	350	103
Me-IAA (>*ca.* 0.1)	210	350	103
PAM (>*ca.* 0.2)	190	370	103
Me-PHCA (>*ca.* 0.2)	200	325	103

The injection was adjusted in 0.5—1.5 μl range with a microsyringe with methanol as a solvent. The calibration curves are shown in Fig. 2—5.

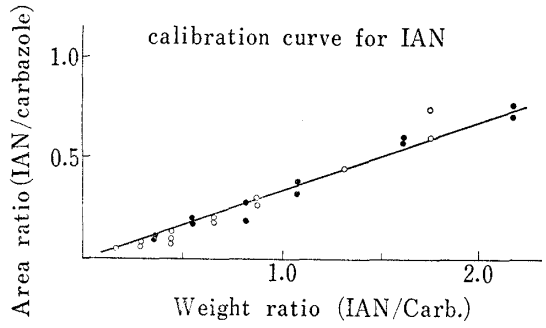

Fig. 2. Calibration Curve for IAN

●: carbazole 0.59 mg/ml
○: carbazole 0.72 mg/ml
Column 10% Versamid 900
column temp. 220° sample heater temp. 350°
H_2 18.9 ml/min N_2 103 ml/min
IAN: Rt. 20.6 min carbazole: Rt. 12.6 min

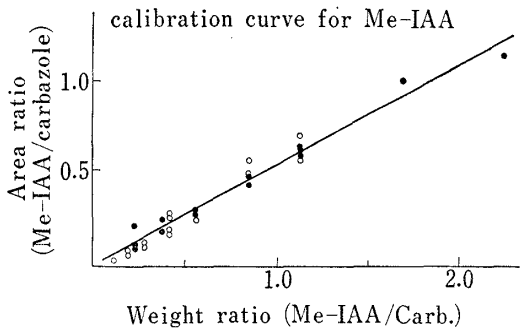

Fig. 3. Calibration Curve for Me-IAA

●: carbazole 1.0 mg/ml
○: carbazole 0.52 mg/ml
Column 10% Versamid 900
column temp. 210° sample heater temp. 350°
H_2 17.3 ml/min N_2 103 ml/min
Me-IAA: Rt. 12.8 min carbazole: Rt. 19.8 min

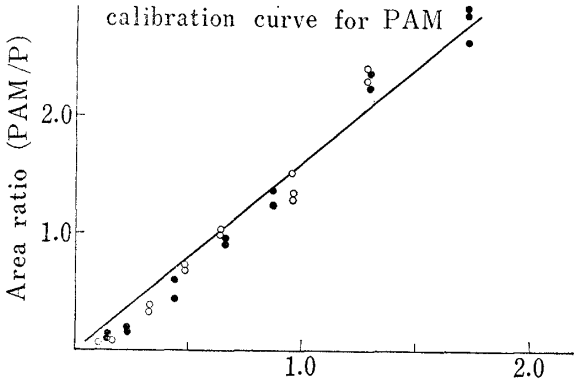

Fig. 4. Calibration Curve for PAM

●: *p*-acetophenetidine 1.1 mg/ml
○: *p*-acetophenetidine 1.5 mg/ml
Column 10% Versamid 900
column temp. 190° sample heater temp. 370°
H_2 18.9 ml/min N_2 103 ml/min
PAM: Rt. 9.2 min *p*-acetophenetidine: Rt. 30.2 min

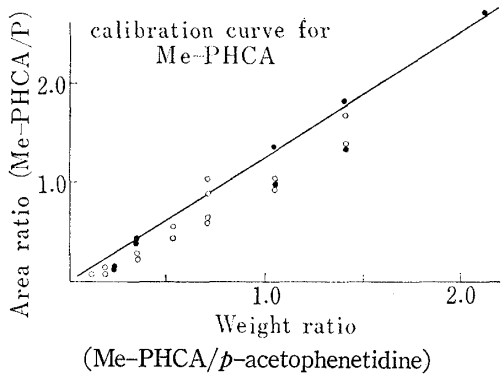

Fig. 5. Calibration Curve for Me-PHCA

○: *p*-acetophenetidine 1.1 mg/ml
●: *p*-acetophenetidine 0.56 mg/ml
Column 10% Versamid 900
column temp. 200° sample heater temp. 325°
H_2 18.9 ml/min N_2 103 ml/min
Me-PHCA: 11.0 min
p-acetophenetididine: 16.8 min

These calibration curves showed a good linearity, establishing quantitative estimation technique for IAA, Me-IAA, IAN, PAM, and PHCA by gas chromatography. Approximately 0.1—1.0 μg/μl of sample solution can be analyzed.

Colorimetry of Auxins isolated from Moyashi

As mentioned previously, the gas chromatographic estimation of IAM was difficult and hence the colorimetry with the Salper reagent ($FeCl_3$–$HClO_4$) was carried out. The quantitative estimation of IAN and Me-IAA by colorimetry was also established. In this experiment, 2 ml of the Salper reagent was added to 1 ml of methanol solution of the sample and, after a certain period, the absorption was measured at a wave length where the color is most stable. The conditions established are shown in Table IV.

Table IV. The Colorimetry of IAM, Me-IAA, and IAN

	Solvent	Wave length (mμ)	Time for color development
IAM	methanol	530	1.5 hr
IAN	methanol	540	4 hr
Me-IAA	methanol	560	10 min

salper reagent (1 ml of 0.5M $FeCl_3$ in 50 ml of 35% $HClO_4$)

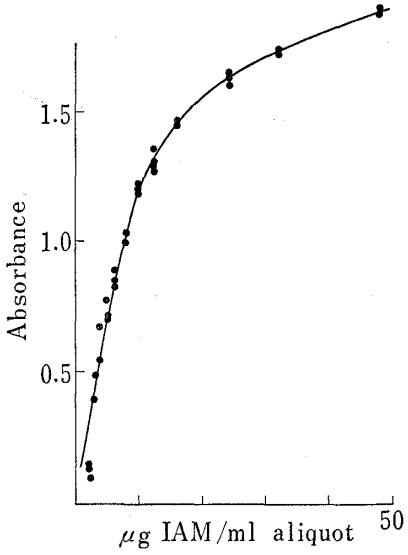

Fig. 6. Concentration Absorbancy Curve for IAM

2 ml Salper reagent added to IAM in 1 ml MeOH. Absorbance measured after 1.5 hr at 530 mμ against blank of reagent plus MeOH.

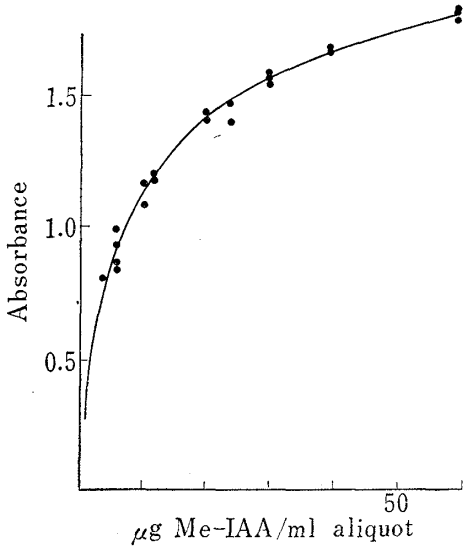

Fig. 7. Concentration Absorbance Curve for Me-IAA

2 ml Salper reagent added to Me-IAA in 1 ml MeOH. Absorbance measured after 10 min at 560 mμ against blank of reagent plus MeOH.

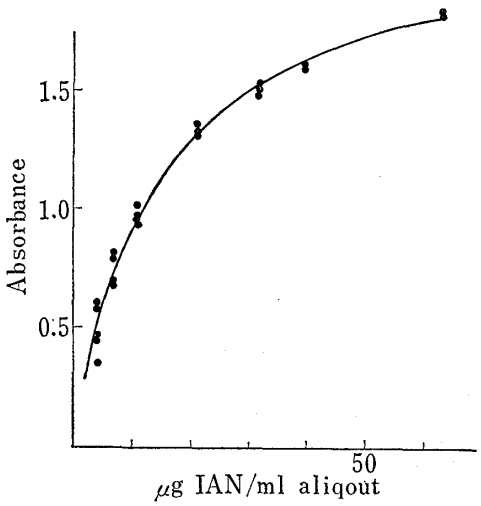

Fig. 8. Concentration Absorbance Curve for IAN

2 ml Salper reagent added to IAN in 1 ml methanol. Absorbance measured after 4 hr at 540 mμ against blank of reagent plus methanol.

The calibration curves for IAM, Me-IAA, and IAN were obtained under these conditions and are shown in Fig. 6—8.

These calibration curves allow estimation of 10—50 μg/ml. Thus the quantitative estimation method of those active substances in Moyashi is now established. The application of these methods to the extract of Moyashi will be reported shortly.

Experimental

Gas Chromatography of the Plant-Growth Regulators——Apparatus: A Hitachi Gas Chromatograph Model F-6 with hydrogen flame detector was used. The U-shape stainless steel column (100 cm × 3 mm, int. diam.) was used. The column packing was made with 10% Versamid-900 on Chromosorb-w (60—90 mesh), acid washed and silanized, and prepared by the evaporation technique. After packing, the column was conditioned for 48 hr at 240° with N_2 flow at the rate of 100 ml/min.

Material: The purity of Me-IAA, IAN, PAM, Me-PHCA, and Me-PCA was checked by gas chromatography (GC) and thin-layer chromatography (TLC). IAM (mp 151°) was checked by TLC.

Determination of Internal Standard Substances——More than 100 substances were tested by GC with the Versamid-900 column and their retention times were compared with the plant-growth regulators. Carbazole was selected as a good internal standard substance for IAN and Me-IAA, and *p*-acetophenetidine for PAM and Me-PHCA. The relative retention times of the plant-growth regulators to the internal standard substances were as follows: Me-IAA 0.65 (to carbazole), IAN 1.63 (to carbzole), Me-PHCA 0.66 (to *p*-acetophenetidine), PAM 0.30 (to *p*-acetophenetidine). The conditions used are shown in Fig. 2—5.

Preparation of Calibration Curves——IAN: MeOH solution of IAN (known concentration) was diluted with MeOH solution of carbazole (known concentration) to make standard solutions with a known weight ratio of IAN to carbazole. An aliquot of 0.5—1.5 μl of these standard solutions was injected into the gas chromatograph with a microsyringe (10 μl Jintan Co.) under the condition adjusted as below (This condition was found to be the best for the estimation of IAN as each IAN and carbazole showed a sharp peak without overlapping).

column temp. 220°, sample heater temp. 350°
flow rate: N_2 103 ml/min, H_2 18.9 ml/min
retention time: 20.6 min, carbazole 12.6

The ratio of peak areas of IAN and carbazole was obtained by weighing the peaks on the chromatogram. And the average value was obtained by several experiments of the same solution. The calibration curve was drawn by plotting the ratio of peak areas versus weight ratio as shown in Fig. 2.

Carbazole	IAN/Carbazole		Carbazole	IAN/Carbazole	
	Weight ratio	Area ratio		Weight ratio	Area ratio
0.72 μg/μl	0.17	0.056	0.59 μg/μl	0.36	0.096
	0.29	0.076		0.36	0.10
	0.29	0.077		0.54	0.21
	0.45	0.099		0.54	0.17
	0.45	0.14		0.82	0.27
	0.45	0.061		0.82	0.18
	0.67	0.18		1.07	0.32
	0.67	0.20		1.07	0.38
	0.88	0.27		1.61	0.57
	0.88	0.31		1.61	0.59
	1.32	0.44		2.16	0.74
	1.32	0.44		2.16	0.69
	1.76	0.60			
	1.76	0.75			

Me-IAA: The same procedure as IAN was applied to a MeOH solution of Me-IAA and carbazole. The calibration curve is shown in Fig. 3. The condition for gas chromatography and results are shown below.

column temp. 210°, sample heater temp. 350°
flow rate: N_2 103 ml/min, H_2 17.3 ml/min
retention time: Me-IAA 12.8 min, carbazole 19.8 min

Carbazole	(Me-IAA/carbazole)		Carbazole	(Me-IAA/carbazole)	
	Weight ratio	Area ratio		Weight ratio	Area ratio
0.52 μg/μl	0.23	0.10		0.85	0.49
	0.23	0.11		1.14	0.59
	0.23	0.22		1.14	0.64
	0.38	0.25		1.71	1.00
	0.38	0.20		1.71	0.99
	0.57	0.29		2.27	1.1
	0.57	0.31		2.27	1.5
	0.85	0.45			

Carbazole	(Me-IAA/carbazole)		Carbazole	(Me-IAA/carbazole)	
	Weight ratio	Area ratio		Weight ratio	Area ratio
1.03 μg/μl	0.11	0.04		0.57	0.25
	0.19	0.08		0.57	0.31
	0.19	0.07		0.57	0.28
	0.28	0.11		0.85	0.45
	0.28	0.14		0.85	0.58
	0.43	0.19		0.85	0.51
	0.43	0.27		1.14	0.57
	0.43	0.29		1.14	0.62
	0.43	0.17		1.14	0.61
	0.57	0.28			

PAM: MeOH solution of PAM (known concentration) was diluted with MeOH solution of *p*-acetophenetidine (known concentration) to make standard solutions of known weight ratio of PAM to *p*-acetophenetidine. The standard solution (0.5—1.5 μl) was injected into the gas chromatograph preadjusted as shown below (This condition was found as the best condition for the estimation of PAM, because PAM and *p*-acetophenetidine showed a sharp peak respectively and did not overlap).

column temp. 190°, sample heater temp. 370°
flow rate: N_2 103 ml/min, H_2 18.9 ml/min
retention time: PAM 9.2 min, *p*-acetophenetidine 30.2 min

The ratio of peak areas of PAM and *p*-acetophenetidine was obtained by measuring the weight ratio of peaks corresponding to both substances and the average value was found by the several experiments. The calibration curve was obtained by plotting the ratio of peak areas against the weight ratio. This is shown in Fig. 4.

p-Aceto-phenetidine	(PAM/*p*-acetophenetidine)		*p*-Aceto-phenetidine	(PAM/*p*-acetophenetidine)	
	Weight ratio	Area ratio		Weight ratio	Area ratio
1.526 μg/μl	0.11	0.048	1.126 μg/μl	0.15	0.12
	0.16	0.050		0.15	0.11
	0.32	0.32		0.23	0.11
	0.48	0.72		0.23	0.16
	0.48	0.69		0.44	0.43
	0.64	1.04		0.44	0.59
	0.64	1.02		0.65	0.92
	0.96	1.30		0.65	0.92
	0.96	1.53		0.87	1.25
	0.96	1.34		0.87	1.35
	1.29	2.40		1.30	2.38
	1.29	2.29		1.30	2.24
				1.74	2.86
				1.74	2.91
				1.74	2.62

Me-PHCA: The same procedure as for PAM was used for a MeOH solution of Me-PHCA and *p*-acetophenetidine, and the calibration curve (Fig. 5) was obtained. The condition for gas chromatography and the results are shown.

column temp. 200°, sample temp. 325°
flow rate: N_2 103 ml/min, H_2 18.9 ml/min
retention time: Me-PHCA 11.0 min, *p*-acetophenetidine 16.8 min

p-Aceto-phenetidine	Me-PHCA/*p*-acetophenetidine		*p*-Aceto-phenetidine	Me-PHCA/*p*-acetophenetidine	
	Weight ratio	Area ratio		Weight ratio	Area ratio
1.126 μg/μl	0.12	0.068		0.71	0.60
	0.18	0.089		0.71	0.60
	0.18	0.072		0.71	0.90
	0.18	0.15		1.07	0.90
	0.36	0.30		1.07	0.96
	0.36	0.22		1.07	0.94
	0.53	0.47		1.42	1.68
	0.53	0.56		1.42	1.37

p-Acetophenetidine	Me-PHCA/*p*-acetophenetidine		*p*-Acetophenetidine	Me-PHCA/*p*-acetophenetidine	
	Weight ratio	Area ratio		Weight ratio	Area ratio
0.563 μg/μl	0.24	0.15		1.06	1.36
	0.24	0.11		1.42	1.82
	0.36	0.41		1.42	1.89
	0.36	0.42		2.13	3.63
	0.71	0.61		2.13	2.70
	0.71	1.03		2.84	2.62
	1.06	0.95		2.84	4.44

Avena Section Test for Carbazole and *p*-Acetophenetidine——The following procedure was employed for the bioassay of carbazole and *p*-acetophenetidine. Dehusked oat seeds (Avena sativa Victory) were soaked in H_2O for 2 hr, and grown for 72 hr at 25° in darkness, except for occasional exposure to red light. Section of 5 mm in length were cut from coleoptiles at 3 mm below the tip, and 15—20 sections per dish were floated in the dark on 1 ml of aqueous solution containing a test substance. After 24 hr, the length was measured and compared with that of the control. The example of the results are shown in Table II.

Colorimetry of IAM, IAN, and Me-IAA——Material: The samples were the same as used for gas chromatographic estimation and 70% $HClO_4$ and $FeCl_3 \cdot 6H_2O$ (both reagent grade). were purchased from Wako Pure Chemical Co.

Procedures: The Salper reagent prepared from 500 ml of 35% $HClO_4$ aq. solution and 10 ml of 0.5M $FeCl_3$ aq. solution was added to MeOH solution of a sample and after a certain period, the absorption was measured.

Determination of the Conditions——IAM: To 1 ml of MeOH solution of IAM was added to 2 ml of the Salper reagent and the absorption at 440—600 mμ region was measured after 10 min to 4 hr. The result is shown in Fig. 9. The absorption at 530 mμ was the most stable after 1.5 hr.

IAN and Me-IAA: Similar procedure as above was carried out and results shown in Fig. 10, and 11 were obtained. The absorption is the most stable after 4 hr at 540 mμ in the case of IAN and after 10 min at 560 mμ in the case of Me-IAA.

Preparation of Calibration Curves——IAM: To 1 ml of a series of MeOH solution of IAM of known concentration was added 2 ml of the Salper reagent and after 1.5 hr the absorption was measured at 530 mμ. A mixture of 1 ml of MeOH and 2 ml of the Salper reagent was used as a blank solution. The calibration curve shown in Fig. 6 was obtained by plotting the absorbance against the concentration.

IAN and Me-IAA: Similar procedures were carried out and the calibration curves were obtained at 540 mμ after 4 hr for IAN and at 560 mμ after 10 min for Me-IAA (Fig. 7 and 8).