SEPARATION OF PLANT GROWTH REGULATING SUBSTANCES ON SILICA GEL COLUMNS [1, 2]

LOYD E. POWELL

DEPARTMENT OF POMOLOGY, NEW YORK STATE AGRICULTURAL EXPERIMENT STATION, CORNELL UNIVERSITY, GENEVA

Silica gel columns have been successfully employed to separate a number of synthetic indole derivatives[3] and naturally occurring plant growth regulators. The method is rapid and simple, and appears to be superior to paper chromatography in many respects for this type of work.

MATERIALS AND METHODS

The preparation of the silica gel is similar to the method of Bulen, et al (2) for organic acid chromatography. Silicic acid (Mallinckrodt's 100 mesh analytical reagent) is prepared, generally in one pound batches, by repeatedly washing it in distilled water, and decanting the fine particles that do not settle out of suspension after an arbitrarily chosen period of 15 minutes. After the silicic acid (silica gel) is free of very fine particles that greatly impede solvent flow through the column, it is dried to constant weight at 100° C.

A glass column 1.4 cm inside diameter and 30 cm long, with a 250 ml solvent reservoir on one end and stopcock on the other, is used to support the silica gel bed. A small plug of fine glass wool at the bottom of the column prevents the silica gel from entering the stopcock.

An 8.0 g sample of the dried silica gel is hydrated with 5.0 ml of 0.5 M formic acid. This hydrated sample is suspended in a small amount of the 0.2 % butyl alcohol eluting solvent (petroleum ether: *n*-butyl alcohol saturated with 0.5 M formic acid--99.8: 0.2), poured into the column, and packed with air pressure applied to the top of the column. This results in a column of silica gel approximately 13 cm long.

The sample to be analyzed is placed on top of the column in one of three ways: I. The finely ground tissue sample is mixed with 0.5 g of silica gel which has previously been hydrated with 0.4 ml of 0.5 M formic acid, and transferred directly to the top of the column. This is the method generally used. If the tissue sample has previously been extracted, the liquid extract may be reduced to a small volume and applied to the top of the column, either by II. pipetting it directly on to the column, or III. first mixing it with a small sample of silica gel which has previously been hydrated with formic acid. Method No. II is acceptable if the extracting solvent does not undesirably alter the elution pattern of the regular eluting solvents. If it does, it may be necessary to mix the extract with a small sample of silica gel which has previously been hydrated with formic acid, then carefully evaporate the extracting solvent from the sample with as little loss of the hydrating liquid as possible. As explained in a later section, dehydrated silica gel may account for a substantial loss of certain substances.

The prepared column is placed on a fraction collector adjusted to collect 10 ml fractions. One hundred fifty ml of the 0.2 % butyl alcohol eluting solvent is added to the solvent reservoir, and the air pressure on top of the column regulated to an elution rate of 2 to 3 ml per minute. The first eluting solvent is followed by similar solvents made increasingly polar with butyl alcohol. For routine survey work a suggested solvent schedule is shown in table I. After it has been determined which of these solvents are important for any particular material the others may be eliminated from the solvent schedule, thus shortening the chromatographic process.

The eluate is evaporated at a reduced pressure from the test tubes in which the various fractions have been collected. The residues in the test tubes are bioassayed for plant growth regulating substances using the Avena first-internode test as introduced by Nitsch and Nitsch (8), or by some other satisfactory testing procedure. For the colorimetric detection of indole derivatives the Gordon-Weber modification of the Salkowski reagent (4), and Ehrlich's reagent (3) are employed. The Salkowski reagent is made by dissolving 5.40 g of ferric chloride in 21 of 35 % perchloric acid. Two milliliters of this solution are used for each milliliter of aqueous solution containing the indole derivative. Ehrlich's reagent consists of 10.0 g of *p*-dimethylaminobenzaldehyde dissolved in 100 ml of concentrated hydrochloric acid, sp. gr. 1.188. Two milliliters of this reagent are used for each milliliter of aqueous solution containing the indole derivative, except in the case of indole where the reverse ratio is better.

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[3] The following abbreviations are used in the figures: IAE, ethyl indoleacetate; IAN, indoleacetonitrile; IBA, indolebutyric acid; IPA, indolepropionic acid; IAA, indoleacetic acid; IAM, indoleacetamide.

Results

Most of the early work involved in the development of this method employed water to hydrate the silica gel. The method was eventually modified to include 0.5 M formic acid as the hydrating agent in place of the water. Some of the data to be presented are from the early experiments, while tables I and II, and figures 1 and 4 include data from experiments in which formic acid was employed.

It was discovered early in this work that eluting solvents with a nonpolar base, such as petroleum ether, are well suited to chromatography of indole derivatives on silica gel columns. By adding varying amounts of a more polar solvent, such as *n*-butyl alcohol, it is possible to make solvent mixtures which will separate a considerable number of indole derivatives of widely varying characteristics. These solvents must be saturated with an aqueous solution to prevent them from removing the stationary aqueous phase from the column itself.

Care must be exercised that the column is not exposed to temperatures of greater than 30° C. when solvents containing petroleum ether are used, otherwise the boiling point will be exceeded, with resultant cracking of the column. Limited experiments with *n*-hexane indicate that it can be substituted for the petroleum ether in the various solvents, the substitution possibly requiring minor changes in the *n*-butyl alcohol concentration in the 0.2 % butyl alcohol solvent to effect better separation of ethyl indoleacetate and indoleacetonitrile. Hexane, B.P. 68.7° C., overcomes the column-cracking problem associated with elevated laboratory temperatures, yet is relatively easy to evaporate.

A solvent schedule was derived, primarily by empirical methods, which would permit the separation of several indole derivatives. In table I are listed these solvents, the suggested volumes to use, and some indole derivatives eluted by each. Figure 1 shows the elution pattern of these indole compounds when such a solvent schedule is used. The 0.2 % solvent separates and elutes certain neutral indole derivatives such as ethyl indoleacetate and indoleacetonitrile. Indole and skatole are very readily eluted, being nearly on the solvent front. They are not separated from each other by this solvent, however, nor are they separated by completely eliminating the *n*-butyl alcohol from the solvent. The 3 % solvent elutes such acidic substances as indolebutyric acid, indolepropionic acid, and indoleacetic acid. Indolebutyric acid and indolepropionic acid are not separated, but can be eluted separately by using a 2 % solvent. However, the 2 % solvent does not elute indoleacetic acid in as sharp a zone as the 3 % solvent. The 10 % solvent elutes indoleacetamide, and the 75 % solvent trypotophan and tryptamine. The last two substances are

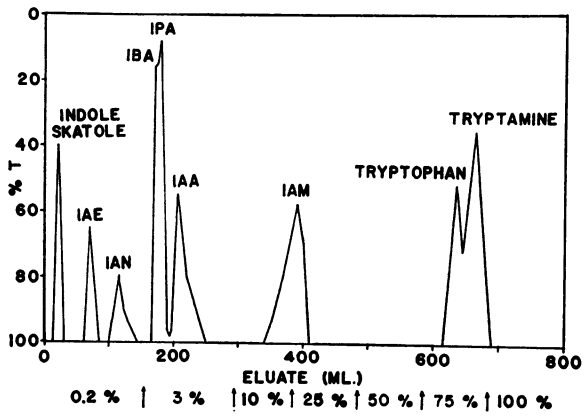
Table I

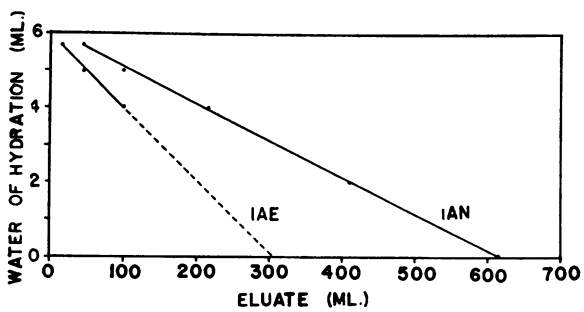
Solvent Schedule for Chromatography of Indole Derivatives on Silica Gel Column

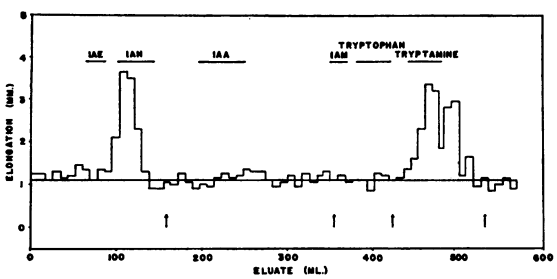
Solvent	Composition	Amount to use (ml)	Indole derivatives eluted
0.2 %	Petroleum ether*: *n*-butyl Alcohol saturated with 0.5 M Formic acid—99.8: 0.2	150	Indole Skatole Ethyl indoleacetate Indoleacetonitrile
3 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—97: 3	150	Indolebutyric acid Indoleopropionic acid Indoleacetic acid
10 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—90: 10	100	Indoleacetamide
25 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—75: 25	100	...
50 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—50: 50	100	...
75 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—25: 75	100	Tryptophan Tryptamine
100 %	*n*-Butyl alcohol saturated with 0.5 M formic acid	100	...

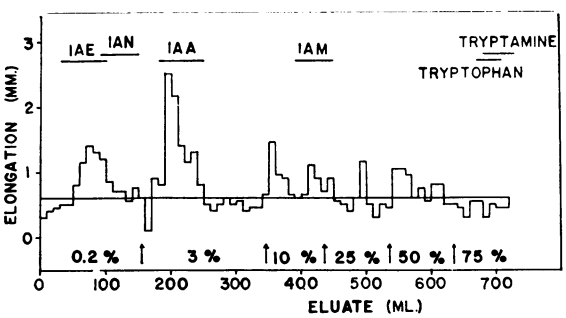
* Mallinckrodt's A.R. petroleum ether, B.P. 30 to 60° C, was used throughout this work without further purification.

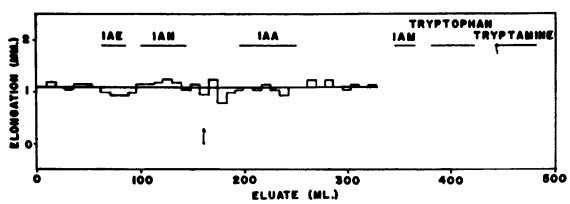
Excess water which separates from solution when the *n*-butyl alcohol and petroleum ether are mixed is removed by centrifugation.











not completely separated with this solvent when an acid-hydrated column is employed, but are well separated by a water-hydrated column. The elution pattern for several indole derivatives on a water-hydrated column may be seen in figure 3. Of the indole derivatives chromatographed the position of only tryptamine is changed by the acid hydration.

First attempts to obtain good recovery of indoleacetic acid after chromatography were not successful. The method used was to pipette 0.5×10^{-6} moles of indoleacetic acid, dissolved in 0.1 ml of absolute ethyl alcohol, into 0.5 g of dry silica gel. This sample was then placed in a vacuum dessicator and held at a pressure of approximately 3 mm mercury for about 30 minutes to remove any traces of alcohol. The dried sample of silica gel plus indoleacetic acid was then hydrated with 0.3 ml of water and placed on top of a silica gel column for chromatography. Under these conditions the recovery rate was never appreciably better than two-thirds. This difficulty was finally overcome by hydrating the 0.5 g sample of silica gel with water or formic acid before adding the indoleacetic acid. Apparently the dehydrated, partially-activated silica gel either adsorbs some of the indole-

FIG. 1 (*top*). Elution pattern of indole derivatives chromatographed on a silica gel column. 0.5×10^{-6} moles of each substance was chromatographed using the following solvent schedule: 0.2 %-150 ml; 3 %-150 ml; 10 %-100 ml; 25 %-100 ml; 50 %-100 ml; 75 %-100 ml; 100 %-100 ml. The Salkowski reagent was used to detect IAE, IAN, IAA, and IAM. Other compounds were detected with Ehrlich's reagent. Silica gel hydrated with 0.5 M formic acid.

FIG. 2 (*second from top*). Volume of 0.2 % solvent required to elute first fraction of IAE and IAN from a silica gel column hydrated with different amounts of water.

FIG. 3 (*third from top*). Silica gel column chromatography of growth substances in cabbage. Solvent schedule: 0.2 %-150 ml; 3%-200 ml; 75 %-75 ml; 100 %-100 ml; water-50 ml. Arrows indicate where solvent changes occurred. Silica gel hydrated with water. Horizontal line running length of histogram indicates growth made by control Avena first-internode sections.

FIG. 4 (*fourth from top*). Silica gel column chromatography of growth substances in peach ovules. Solvent schedule: 0.2%-150 ml; 3 %-200 ml; 10 %-100 ml; 25 %-100 ml; 50 %-100 ml; 75 %-75 ml. Silica gel hydrated with 0.5 M formic acid. Horizontal line running the length of the histogram represents growth made by control Avena first-internode sections.

FIG. 5 (*bottom*). Silica gel column chromatography of a methyl alcohol extract of peach ovules which had previously been extracted on the column by regular eluting solvents. Solvents used for second chromatogram: 0.2 %-150 ml; 3 %-200 ml. Silica gel hydrated with water. Arrow indicates where solvent change occurred. Horizontal line running length of histogram represents growth made by control Avena first-internode sections.

acetic acid so tightly that it cannot be eluted, or inactivates or destroys a part of the indoleacetic acid in some manner. The improved technique permits the recovery of about 90 % of the indoleacetic acid, ethyl indoleacetate, indoleacetonitrile, and indoleacetamide. These results are summarized in table II. The somewhat variable recovery data concerning ethyl indoleacetate is believed due to the fairly rapid fading of the colored compound which develops when the Salkowski reagent is used for detection of this substance.

The separation of certain indole derivatives is affected by the degree of hydration of the silica gel in the column. From figure 2 it is evident that by decreasing the water of hydration greater separation of ethyl indoleacetate and indoleacetonitrile may be obtained. As the amount of hydrating water is decreased the elution fronts for the two compounds become less sharp. That the degree of hydration can be used to alter the elution volumes of indole derivatives which are eluted by solvents other than the 0.2 % solvent has not been investigated, but the method would appear to have its limitations. The solvents containing greater quantities of butyl alcohol also contain larger amounts of acidified water. The silica gel removes the hydrating liquid from the solvent until equilibrium is reached. Therefore, while the degree of hydration of the silica gel remains essentially constant when the 0.2 % solvent, which contains only a trace of water, is used, the water content of the silica gel rapidly tends towards a saturation level when the more polar solvents are employed.

TABLE II

RECOVERY OF SOME INDOLE DERIVATIVES AFTER SILICA GEL COLUMN CHROMATOGRAPHY*

COMPOUND	% RECOVERY
Silica gel hydrated with water after adding indole derivative	
Indoleacetic acid	52.2
" "	66.3
" "	72.8
Silica gel hydrated with water before adding indole derivatives	
Indoleacetic acid	90.2
" "	94.2
Ethyl indoleacetate	101.5
Indoleacetonitrile	91.0
"	88.3
Indoleacetamide	96.8
Silica gel hydrated with 0.5 M formic acid before adding indole derivatives	
Indoleacetic acid	80.7
" "	91.4
" "	92.6
" "	92.1
" "	95.5
" "	96.5
" "	80.6
Ethyl indoleacetate	102.8
" "	126.9
" "	81.8
" "	97.0
Indoleacetonitrile	80.8
"	91.6
"	86.0

* The Salkowski reagent was used to detect the indole compounds.

TABLE III

RECOVERY OF SOME INDOLE DERIVATIVES AFTER SUBJECTING THEM TO MERCURY PRESSURE 3–5 MM FOR 24 HOURS *

COMPOUND	% RECOVERY
Indole	0
Ethyl indoleacetate	95.9
Indoleacetonitrile	92.9
Indoleacetic acid	98.5

* Indole—one test. Ethyl indoleacetate and indoleacetonitrile—mean of four tests. Indoleacetic acid—mean of three tests.

Ehrlich's reagent was used for indole determination. Ethyl indoleacetate, indoleacetonitrile, and indoleacetic acid were detected with the Salkowski reagent.

Evaporating the solvents from the test tubes prior to bioassay is easily accomplished for tubes containing only trace amounts of butyl alcohol by use of a water aspirator connected to a vacuum dessicator in which the tubes have been placed. Of the several methods investigated for the evaporation of appreciable quantities of butyl alcohol, two have proven satisfactory in routine work. Both require a mechanical high-vacuum pump and dry ice trap. The first one employs a rotating vacuum-type evaporator, thus requiring that individual fractions be transferred to the rotating flask, and the residues transferred back to the test tubes with a lower boiling solvent such as methyl alcohol. A more satisfactory method consists of using a vibrating vacuum evaporator (Evapo-Mix, Laboratory Glass and Instrument Corp., N. Y.) designed to evaporate the solvents from ten test tubes simultaneously. This last method is rapid, and eliminates any transferring of residues.

The effect of reduced pressure over an extended period on recovery of certain indole derivatives is summarized in table III. Indole has a rather high vapor pressure, it being impossible to detect any at all after subjecting 1×10^{-6} moles of this substance to a pressure of 3 to 5 mm mercury for 1 hour. It was found that 1×10^{-6} moles of indole had completely disappeared from unstoppered test tubes at atmospheric pressure after 5 days in the dark at −26° C. Experiments with ethyl indoleacetate, indoleacetonitrile, and indoleacetic acid indicated there was little, and perhaps no, significant loss of these materials after 24 hours at a pressure of 3 to 5 mm mercury.

It has occasionally been advantageous to hasten solvent evaporation by use of an infra-red lamp, but caution must be exercised since it has been shown that this method of applying heat may lead to the disappearance, presumably through evaporation, of some of the more volatile substances such as the ethyl ester and nitrile of indoleacetic acid. Indoleacetic acid, itself, does not seem to be affected.

That the silica gel column method is applicable to naturally occurring growth substances in plant material is demonstrated by experiments with cabbage heads and immature peach seeds. Part of a freshly chopped cabbage head was extracted with several portions of ice cold methyl alcohol in a homogenizer, until most of the chlorophyll had been removed. Figure 3 depicts the bioassay pattern of the extract from 21 mg dry weight of the cabbage head after silica gel chromatography, employing Avena first-internode sections (8) to detect the growth substances. Indoleacetonitrile was clearly evident, as were at least two other growth substances eluted in the 100 % solvent. Possibly one of these was the same as referred to by Bentley, et al (1) and Housley and Bentley (5) as a precursor of indoleacetonitrile in cabbage. The precursor referred to by these workers was water soluble, but not ether soluble, and therefore polar in nature. Such a substance might be expected to be eluted by the 100 % solvent. There was no trace of indoleacetic acid; this was further confirmed by analyzing a second cabbage head, using the extract from as much as 2 g fresh weight. These data concerning the absence of indoleacetic acid are in agreement with those of some investigators (5), and in disagreement with the findings of others (6, 7). Possibly varietal or environmental differences exist among cabbages which explain these results. At least two other possibilities need to be considered in explaining its absence. This experiment was performed before formic acid had been adopted as the hydrating agent for the silica gel. Is it possible that indoleacetic acid was present as a salt and was therefore not eluted from the column, or was never extracted by the methyl alcohol in the first place? Secondly, there is a possibility that an inhibitor was present in the test tubes containing the indoleacetic acid, thus masking its activity. This seems unlikely, however, since cabbage extracts of several concentrations were chromatographed; at no time was there any indication of an inhibitor or an auxin in those test tubes in which indoleacetic acid is normally eluted. To postulate that an inhibitor was responsible for the failure to find indoleacetic acid, then, would require an inhibitor whose dosage-response curve in the bioassay was equal to (but opposite in effect) to that of indoleacetic acid.

The growth promoting substances in immature peach seeds, figure 4, showed an entirely different pattern from that of cabbage growth promoters when the same bioassay was employed. Twenty-five milligrams of ground lyophillized peach ovules, of variety Halehaven, representing 0.87 ovules, collected 50 days after bloom shortly after the endosperm had changed from the free-nuclear to the cellular condition, were subjected to column chromatography. At least six growth promoting substances appear to have been eluted. Three of these were eluted in positions similar to ethyl indoleacetate, indoleacetic acid, and indoleacetamide, but their identities have not been established with certainty. Examination of several similar tests of immature peach seeds collected at different stages of development confirm the presence of these substances in certain other samples as well. Their concentrations appear to fluctuate markedly, being correlated, at least in some instances, with the stage of development of the seed.

It may be questioned as to whether extraction is complete when a sample of powdered plant material is placed on top of the silica gel column and extracted during the chromatographic process. To test this a 100 mg sample of peach ovules was first extracted on the column in the usual manner. Previously a similar sample had been shown to have a growth promoter pattern corresponding with the sample described in figure 4 as regards the substances in the ethyl indoleacetate and indoleacetic acid positions. The extracted plant sample was then recovered from the column and ground in a mortar with methyl alcohol, a standard method of extracting auxins for paper chromatography. This methyl alcohol extract was reduced to a small volume and transferred to the top of a silica gel column and chromatographed using the 0.2 % and 3 % solvents only. As figure 5 shows, there were no traces of growth promoting substances. The first extraction apparently completely eluted the growth promoting substances. There are, however, some indications that the column extraction method may not be satisfactory for all plant tissues. Inconclusive, but suggestive evidence from experiments with powdered apple leaves containing rather large amounts of inhibitors indicates that complete extraction may not be obtained with this method.

Discussion

The foregoing results show that certain synthetic indole derivatives (neutral, acidic, and basic) and some naturally occurring growth substances, may be separated on silica gel columns. The method is relatively rapid, and, so far as the author is aware, permits better separation of a number of indole compounds of rather different chemical characteristics than any single-dimension paper chromatographic process now in use. Using the technique described a sample may be extracted and fractionated into neutral, acidic, and basic indole derivatives, and the solvents evaporated from the test tubes preparatory to bioassay, within 10 to 12 hours, under rather mild conditions. Since little or no preliminary purification of the extract is needed before chromatography, the method minimizes transfer of the extract or eluate from one container to another, thus reducing losses and saving time.

The column method can be used with small samples. With the column size described here, cabbage samples of 21 mg dry weight, and peach ovule samples of 25 mg dry weight have been used in conjunction with the Avena first-internode bioassay (8), although less material could have been used satisfactorily. Larger samples can be used but may present certain problems such as overlapping of closely-running substances, and supra-optimal concentrations of biological materials. For much of the work reported

here, either 0.5×10^{-6} or 1×10^{-6} moles of synthetic indole derivatives were used for colorimetric determination, although 1×10^{-7} moles or less is adequate, at least for some substances. When bioassay with the Avena first-internode test was used, 1×10^{-9} moles of ethyl indoleacetate, indoleacetonitrile, and indoleacetic acid were satisfactory.

When a particular solvent does not give satisfactory resolution, better results may often be obtained by changing the amount of *n*-butyl alcohol in the solvent. Changing the fraction size collected is generally less helpful. Employing a column of different length, or changing the degree of hydration of the silica gel may be a solution to some problems.

Experience with this method to date indicates that, contrasted with paper chromatography, the chief disadvantages are minor. Controls cannot be run simultaneously with the chromatography of the extract, such as can be done with paper chromatography, making it necessary to rely on the chromatographic pattern of control substances from another experiment as a guide to where various substances will be eluted. Internal standards that may exist or be placed in a sample, such as pigments, however, aid in relating the behavior of the chromatographic process from sample to sample. Generally the threshold volumes of elution for a particular substance does not vary more than a few milliliters between tests. The fact that reduced pressure is employed to hasten evaporation of solvents from the test tubes causes some loss of volatile constituents, but such losses have been shown to be negligible for ethyl indoleacetate, indoleacetonitrile, and indoleacetic acid.

Summary

Silica gel column chromatography has been successfully employed to separate a number of indole derivatives and naturally occurring plant growth regulating compounds. By placing the tissue sample to be analyzed on top of the column, it is possible to extract and separate several neutral, acidic, and basic indole derivatives, and naturally occurring growth regulating substances, in one operation by a suitable choice of solvents. A bioassay is carried out in the test tubes used to collect the various fractions, thus eliminating the transfer of plant extract from one container to another. The method is simpler and faster than a comparable fractionation employing paper chromatography. It is quantitative, at least for certain substances.

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